Current approaches to vaccine preparation

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Abstract

Numerous conventional vaccines for animal use are currently available, and many of these vaccines have been instrumental in the control of infectious diseases of major economic importance. A vaccine has even been instrumental in global eradication of smallpox, an important human disease. However, many of the current vaccines are deficient in efficiency, potency, or safety. It has been recognized that the conventional methodologies are a limitation to further vaccine development. Introduction of monoclonal antibodies, recombinant DNA, and protein engineering techniques has facilitated a rather rapid increase in the knowledge of pathogenetic mechanisms, as well as of protective antigens at the molecular level. This knowledge provides the basis for development of a new generation of vaccines. As a rule, these vaccines contain purified immunogens, or even isolated epitopes, identified and prepared by molecular biological techniques. The efforts to find better delivery systems and better adjuvants accompany the research on vaccines.

Résumé

Méthodes actuelles de préparation des vaccins

De nombreux vaccins pour utilisation animale sont couramment disponibles et plusieurs de ceux-ci ont été indispensables dans le contrôle et la prévention de maladies infectieuses d'importance économique majeure. Un vaccin a même été utile dans l'éradication mondiale d'une maladie humaine importante, la variole. Toutefois, plusieurs des vaccins courants sont déficients dans leur efficacité, puissance et sécurité. Les méthodologies de production conventionnelles sont maintenant reconnues comme des facteurs limitants dans l'amélioration et le développement de vaccins. L'introduction des anticorps monoclonaux, la recombinaison d'ADN et les techniques d'ingénierie de protéines ont facilité une compréhension et une accumulation de connaissances rapides sur la pathogénie des maladies de même que des antigènes protecteurs au niveau moléculaire. Ces connaissances forment la base du développement d'une nouvelle génération de vaccins. Généralement, ces vaccins peuvent contenir des immunogènes purifiés, ou même des épitopes isolés, identifiés et préparés par des techniques de biologie moléculaire. Les efforts effectués pour produire de meilleurs vaccins et de meilleurs adjuvants accompagnent la recherche sur les vaccins et sont aussi décrits dans le texte.

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Current vaccines

Although it is not our purpose in this article to review all of the major historical milestones in microbiology and immunology that have led to development of current vaccines, the extraordinary intellectual contributions of Jenner and Pasteur will be mentioned. In 1798, the first report of a safe, live vaccine was published by the English physician Edward Jenner who used cowpox virus to successfully protect against smallpox and thus initiated the new era of prophylactic vaccination (1). However, not much progress was made until the great French chemist Louis Pasteur reported in 1880 that *Pasteurella multocida*, grown *in vitro*, immunized chickens against challenge with cultures of virulent *Pasteurella multocida*. This has been referred to as the groundstone of immunology (2). Following these initial experiments with what was at that time known as fowl cholera, Pasteur further developed vaccines against anthrax and rabies by culturing the pathogenic agents under unfavorable growth conditions (2), and thus introduced a technique for purposeful production of vaccines. A decade later, chemically inactivated whole organisms began to be used to prevent human diseases; the first killed bacterial vaccines (bacterins) against typhoid fever and cholera, were produced in 1896 (2).

After the encouraging invention of several vaccines, using both attenuated and killed organisms, exciting achievements continued to be made in the field of vaccine preparation. In the early part of this century, some success was also achieved in production of vaccines against other pathogens and bioactive agents, such as rickettsiae (3) and tetanus toxin (2). Nowadays, there are numerous vaccines for veterinary use and approximately 20 vaccines for human use (4). Many of these vaccines play important roles in limiting serious animal and human diseases. Smallpox, one of the most devastating diseases in human beings, has even been eradicated through vaccination, while many other diseases have been brought under control.

The classic vaccines can be divided into two groups according to the status of an organism or a bioactive agent included as the antigen: live or killed (inactivated). The term killed is used when bacterial or protozoal vaccines are discussed, whereas the term inactivated is used with vaccines against viruses or toxins. It was recognized very early that killed or inactivated microorganisms prepared under certain inactivation conditions preserved immunogenicity, and these vaccines have been widely used. Bacterial exotoxins collected from culture supernatant can also be used, as in the cases of vaccines for shipping fever of cattle (*Pasteurella haemolytica*), tetanus, and diphtheria (5-7).

Live vaccines can be obtained by selecting the naturally occurring pathogens which may be virulent for some species, but are avirulent for the immunized

Table 1. Disadvantages of conventionally prepared vaccines

Live vaccines

1. Possibility of residual pathogenicity or immunosuppression
2. Possibility of reverting to the virulent state through
   a) mutation
   b) recombination (exchange of a piece of genome) with wild organisms
3. Possibility of perpetuation of the organism in the environment
4. Limited shelf-life
5. Requirement of a defined temperature for storage
6. Demand a biological system for vaccine production
7. Possibility of contamination with adventitious agents

Killed vaccines

1. Possibility of incomplete inactivation or immunosuppression
2. Generally induce short-lived immunity
3. Must be given parenterally. This is disadvantageous where individual handling of animals is not practical
4. Generally high antigenic mass needed, and multiple dose required, resulting in high cost
5. Often associated with an undesirable immune response (hypersensitivity) because of antigenic modification or contamination

species (heterologous vaccines). Marek's disease, one of the most economically important diseases of chickens, has been successfully controlled by vaccination with the turkey herpesvirus (8). Incidentally, this is the first and only successful vaccine against virally induced tumors. Alternatively, the pathogenic organism can be used via an unnatural route of infection, or the disease agent can be artificially attenuated. The former strategy is still used in protection of lambs against contagious pustular dermatitis of sheep caused by a parapoxvirus. Scarification into the skin of a leg at one month of age prevents occurrence of oral lesions; this protects against economic losses due to reduction in food uptake, caused by infection later in life leading mainly to oral lesions. Following the principle of Pasteur's discovery of microbial attenuation due to cultivation of infectious agents *in* vitro (fowl cholera) or due to propagation of an agent in an unnatural host (canine rabies propagated in rabbits), many vaccines were developed. Introduction of embryonated eggs during the 1920s and cell culture during the 1940s for cultivation of viruses provided yet another vehicle for purposeful reduction of pathogenicity.

Both live and inactivated vaccines have disadvantages (Table 1).

The ease with which some pathogens change their genetic make-up (resulting in their antigenic diversity) contributes to ineffectiveness of some current vaccines (7,9). Mutation and recombination are the main sources of antigenic instability of microorganisms. Viruses with an RNA genome are particularly subject to a high frequency of mutations because the host cells do not possess the enzymes necessary to correct misincorporation of RNA nucleotides, whereas such enzymes are available for DNA. Many important veterinary pathogens are in the category of highly mutable viruses, e.g. foot-and-mouth disease virus, bovine viral diarrhea virus, influenza viruses, canine distemper virus, rabies virus, coronaviruses, and rotaviruses. RNA viruses with segmented genomes, e.g. influenza viruses, rotaviruses, and orbiviruses (bluetongue), can, in addition to mutation, exchange individual segments of nucleic acids when two different viruses of the same group replicate concomitantly in the same individual. Whenever these continuously occurring genetic changes involve important antigenic determinants, vaccines based on the old genotype become inefficient. In addition, some of the vaccines manufactured presently exhibit residual pathogenicity and cause undesirable side effects such as local erythema and swelling, fever, irritability, convulsions, seizures, shock, irreparable brain damage, and even death (4,10,11). Moreover, the simplistic view that immune response leads to protection, even if the same microorganism is involved, can no longer be held. This is well demonstrated in the case of dengue hemorrhagic fever virus infection (12). In this case the specific antibody has been implicated in enhancement of pathology by facilitation of virus entry into macrophages, followed by the loss of macrophage function. The same mechanism is suspected in infection with human immunodeficiency virus-1 (13–15). Therefore, it is obvious that the traditional idea of using nonpathogenic whole organisms or crudely purified subunits to stimulate immune responses against pathogenic infectious agents has to be abandoned in favor of more meticulous analysis of the protective immune mechanisms, and the antigenic determinants involved.

Conventional vaccines may also not succeed in situations where immunopathology, such as immunosuppression, hypersensitivity, chronic inflammation, potentiation of infection by antibody, or autoimmunity triggered by antigenic mimicry (immunity against normal tissues elicited by microbial antigens mimicking normal tissue antigens), is prominent. Therefore, the development of vaccines must be based on a solid knowledge of the elementary surface antigenic structures (epitopes or antigenic determinants), as they pertain to protection and immunopathology. Otherwise, vaccines can lead to disease potentiation as is best documented with inactivated measles virus and human respiratory syncytial virus vaccines (16,17). People vaccinated with these inactivated vaccines, when naturally infected, developed more severe clinical symptoms than the unvaccinated controls. The reason for this seems to be antigenic modification of the fusion protein due to the inactivation processes. For all of these reasons, the interest in developing second generation vaccines is greatly increasing.

New generation vaccines

The new vaccines are characterized by the use of techniques involving recombinant DNA, monoclonal antibodies, and protein engineering (chemical synthesis of protein antigens). These techniques are commonly, and perhaps inappropriately, called "biotechnology".

Confusion sometimes arises from two different uses of the word biotechnology. Apart from the narrow meaning of the word, used to indicate recombinant DNA and protein engineering technologies, this term is often used to include all biological technologies. This causes confusion, and we would like to use this opportunity to argue against the ambivalent terminology that

poses far reaching implications, e.g. in distribution of research funds by granting agencies. While recombinant DNA and protein engineering are undoubtedly strategic technologies of extremely high national importance, other biological technologies do not have the same prominence. We believe that Canada is not profiting from recombinant DNA technology to the extent that some other countries are, partly because the resources rightly marked for the strategic recombinant DNA technology have often been directed to other areas due to the loophole of ambivalent terminology.

This section on current efforts towards vaccine production is organized under the following headings:

1.0. Subunit vaccines
1.1 Preparation of protective antigens by recombinant DNA techniques (cloning of the corresponding genes from pathogenic microorganisms into the prokaryotic and eukaryotic expression hosts)
1.2 Synthetic peptides
2.0 Anti-idiotypic vaccines
3.0 Attenuated live and recombinant viral vaccines
3.1 Attenuation by mutation
3.2 Selection of epitope mutants using neutralizing monoclonal antibodies
3.3 Recombinant viruses used as vectors for heterologous protective antigens
4.0 Adjuvants

1.0 Subunit vaccines

Whole pathogens, such as bacteria and viruses, contain many antigens. When these whole agents are in a vaccine, not all of the antigens are involved in the protective response of the host; some can cause hypersensitivity, immunosuppression, or other side-effects (11). However, by using pure individual protective antigens (subunit vaccines), these problems can be avoided. There are two basic ways of preparing subunit vaccines:

1) Components can be purified from infectious agents by the use of conventional physicochemical techniques. Bacterial exotoxins from *Corynebacterium diphtheriae* and *Clostridium tetani* lose toxicity but retain antigenicity after inactivation with formaldehyde. These toxoids are able to elicit formation of neutralizing antibodies against the exotoxins. Cytotoxin from the supernatants of *Pasteurella haemolytica* cultures has been successfully used to vaccinate cattle against pneumonic pasteurellosis (5,6).

2) Secondly, subunits can be prepared by recombinant DNA technology or by chemical synthesis (protein engineering). Research preceding production of such vaccines must first identify immunologically relevant peptides. Before a peptide is used in a vaccine, it should be identified as immunologically relevant. This can be done in several ways (18). Fragments obtained from isolated, chemically or enzymatically cleaved native proteins are screened for their ability to bind specific antibodies or to interfere with the interaction between antibodies and intact antigens. Crystallographic studies of three-dimensional structures of proteins are also used (11). X-ray crystallography (three-dimensional analysis of crystallized pure protein) supplies information about the relative atomic mobility of different regions of the protein. This is important because it has been shown that highly mobile areas in proteins are more likely to bind antibodies to synthetic peptides than less mobile areas (19). The antigenic determinant can be identified in some cases by a hydrophilicity plot (20). The point of the highest local average hydrophilicity in the amino acid sequence of a protein antigen is invariably located in, or immediately adjacent to, an antigenic determinant. This can be rather easily determined by computer programs, once the nucleotide sequence of a determinant in question is known. Another approach for identifying epitopes of a given protein is to systematically synthesize overlapping peptides of previously identified proteins and to measure their reactivity with antibodies against the native protein. The peptides showing the highest reactivity presumably contain epitopes (11). Immunogenic epitopes can be either continuous (i.e. a short linear sequence of amino acids) or discontinous (i.e. linear distant residues brought together by folding to form an epitope) (21). A discontinuous epitope may be mimicked by synthetic linear sequences of only those amino acids that participate in formation of that epitope. The excess amino acid sequence is removed from a discontinuous epitope, and such sequences of amino acids are called mimotopes because they mimic continuous epitopes (11).

We have already given the main reasons for the need to include in a vaccine only epitopes that elicit protective immunity. However, reduction of size of vaccine antigens results in weakened immunogenicity, because of lack of the surrounding structures that normally act as immunological carriers. Therefore, in order to stimulate a high level of protection, the peptides are often coupled with larger carrier molecules such as bovine serum albumin or keyhole limpet hemocyanin. Although this seems contrary to the original effort to reduce the peptide size, this time the excess protein is inert with respect to its pathogenicity. Coupling of the small peptides and carrier protein molecules is achieved by glutaraldehyde, bi-functional reagents such as N-succinimidyl 3-[2-pyridyldithio] propionate, or benzidine dihydrochloride (19). The research into adjuvants as the means of enhancement of immunogenicity of small peptides will be discussed later.

Once the immunogenic peptide has been identified, mass production can be achieved by cloning of the gene into a vector, or by chemical synthesis.

1.1 Preparation of protective antigens by recombinant DNA techniques (cloning of the corresponding genes from pathogenic microorganisms into the procaryotic and eukaryotic expression hosts)

Since 1981, when Kleid and colleagues succeeded in cloning the antigenic polypeptide VP3 of foot-and-mouth disease virus within *Escherichia coli* and preparing it as a vaccine used for cattle and swine (22), many attempts have been made to use recombinant DNA techniques to prepare vaccines. This method is based on the fact that a selected portion of a pathogen's genome (one which codes for antigenic determinants important for induction of protective immunity) can be expressed in, and purified from, bacterial, yeast, or mammalian vector cells (23). Thus far, genes for

components of bacteria, protozoal parasites, viruses, and genes for toxins have been cloned and expressed in *Escherichia coli*, *Salmonella typhimurium*, *Saccharomyces cerevisiae*, and other vectors (23–25). A polypeptide manufactured through this technology can be used as a fusion protein that contains, apart from the antigenic peptide of interest coded for by the inserted DNA sequence, a peptide coded for by the adjacent DNA sequences of the vector. These extra sequences may serve as immunological carriers, thus rendering unnecessary not only cleavage and purification but also conjugation to a carrier molecule.

1.2 Synthetic peptides

In 1971, Arnon *et al* reported that chemically synthesized peptides could induce antibody production to intact protein (26). Since that time, synthetic peptides mimicking components of viruses, bacteria and parasites have been widely studied. These peptides have been shown to elicit antibodies and protection against foot-and-mouth disease virus (27), human influenza virus (28), and herpes simplex virus 2 (29). A difficult problem in preparation of vaccines to some pathogens is caused by their multiple antigenicity and antigenic drift. Induction of an immune response against type-common or conserved sequences of an organism could be mediated by multivalent vaccines constructed to contain the desired sequences. Such products have begun to emerge in the study of human influenza virus, where a synthetic hybrid peptide containing antigenic determinants of more than one strain, and facilitating protection against all these strains, has been described (11). A synthetic peptide covalently binding the copy fragments of antigens of *Streptococcus pyogenes*, diphtheria toxin, hepatitis B virus, and *Plasmodium knowlesi* has been reported to elicit production of high levels of antibodies to each of these antigens (30). A synthetic peptide representing the carboxyl-terminal 37 amino acids of the beta-subunit of human chorionic gonadotropin has also been used as a vaccine. This vaccine reduced the fertility rate of baboons significantly, suggesting the possibility of a novel, nonsurgical route for sterilization of animals (31).

2.0 Anti-idiotypic vaccines

Antibodies are proteins, and as such they are antigenic when taken from an animal and inoculated into an unrelated species. This was first described by Kunkel and co-workers (33) in 1963 whose studies, contrary to the then accepted opinion, demonstrated that individual antibodies could elicit secondary antibodies in another species. The group of epitopes found within the hypervariable antigen-binding regions of antibodies are known as idiotypes (Id). The hypervariable regions of the secondary antibody molecules, complementary to idiotypes, have a configuration of the antigen that gives rise to the idiotype (34).

Idiotypes and anti-Id, like any other pairs of antigens and antibodies, have a lock-and-key complementarity to their three-dimensional surfaces. In 1974, the network theory for regulating the immune response based on the above phenomenon was proposed by Jerne (35). Jerne reasoned that a host's response to an antigen is controlled by a series of idiotype-anti-Id reactions that might enhance or suppress the immune response to an antigen. A given idiotype is under the control of an anti-Id, or Ab2, whereas the anti-Id can be regulated by another set of antibodies referred to as an anti-anti-Id, or Ab-3. This complex set of reactions operates via a feedback mechanism to control the immune response (36). When the equilibrium of the idiotype and anti-Id interaction is disturbed by immunization with an antigen, a particular Id interaction would dominate and an immune response would be induced (37). The dual immunological property of idiotypes, also referred to as Ab1, has been well documented (36–38). The anti-Id induced when the idiotope on Ab1 is used as an antigen is again complementary, and thus mimics the structure of the original antigenic determinant (36). For diagram see Figure 1.

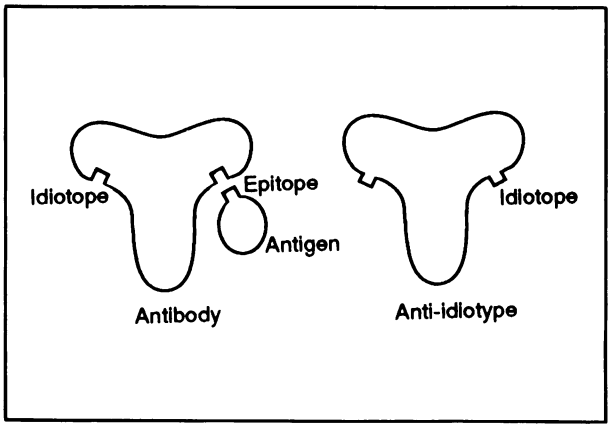

Figure 1. Method of constructing a vaccine virus vector carrying a selected gene from another virus. TK = thymidine kinase gene of vaccinia virus; BudR = bromodeoxyuridine; start-ATG and stop-TAA translation codons denote foreign DNA sequence to be inserted in vaccinia virus. (Courtesy, Dr. B. Moss. In: Fenner F, Bachmann PA, Gibbs EPJ, Murphy FA, Studdert MJ, White DO, eds. Veterinary Virology. Orlando: Academic Press, 1987: 269, with permission.)

A new strategy for vaccination has been extrapolated from this theory, namely employing anti-Id as surrogate antigens in vaccines. Both humoral and cell-mediated immune responses could be induced (40,41). It would be especially useful when immunity to only a single epitope of an infectious agent is adequate for protection. Anti-Id vaccines would have several advantages. First, such a vaccine would not contain nucleic acid or undesirable proteins, and that would increase the safety of the vaccine. It was also reported that anti-Id vaccine was capable of stimulating an immune response in newborns unresponsive at that age to polysaccharide antigens (39). This provides a way to circumvent nonresponsiveness to bacterial capsular antigens in neonates (19,37,38). The anti-Id vaccines are good candidates for the manufacture of vaccines in which the antigens are polysaccharide and carbohydrate because these cannot be produced by recombinant DNA technology. With the use of monoclonal antibody techniques, the anti-idiotypic antibodies can be made from impure antigens, and, once

Table 2. List of infectious agents and their antigens against which Ab was induced by anti-idiotypic antibody

Infectious agents	Antigen	References
Viruses		
Feline leukemia	whole virus	(36)
Newcastle disease	whole virus	(36)
Herpes simplex	glycoprotein	(40)
Hepatitis B	surface antigen	(44,46,47)
Human immunodeficiency	envelope glycoprotein	(48)
Murine leukemia	envelope glycoprotein	(49)
Polio type II	viral protein 2	(50)
Rabies	surface glycoprotein	(51)
Reovirus	hemagglutinin	(39)
Sendai	receptor (specific T helper)	(52)
Tobacco mosaic	whole virus	(53)
Venezuelan equine encephalomyelitis	whole virus	(54)
Bacteria		
Streptococcus pneumoniae	phosphorylcholine	(55)
Escherichia spp.	polysaccharide	(38)
Listeria monocytogenes	T cell specific to L.M.	(56)
Parasites		
Trypanosoma rhodesiense	surface glycoprotein	(41)
Schistosoma mansoni	membrane glycoprotein	(56)
Trypanosoma cruzi	glycoprotein	(58)

the required hybridomas are established, production in large quantities is rather inexpensive. Alternatively, anti-idiotypes used in vaccines might be synthesized chemically (protein engineered) (33).

Inspired by Jerne's theory, Sacks pioneered the anti-Id vaccine against experimental African trypanosomiasis (42). Since then, anti-Id have been used to induce immune responses to antigens associated with a large number of parasites, bacteria, and viruses (Table 2). Anti-Id have been shown to bind to the idiotype displayed on B-cell tumors and to cause growth inhibition or lysis of these tumors without affecting normal tissues (43,44). This immunotherapeutic method provides an exciting new pathway for therapy of bovine leukemia, some forms of feline leukemia, and other B-cell tumors. In spite of the considerable amount of information available, the use of anti-Id vaccines is still in the experimental phase (19).

3.0 Attenuated live and recombinant viral vaccines

In the past, many attenuated vaccines were produced by passing the etiological agent through an unnatural host, and they were successfully used without knowledge of the underlying genetic basis. Recent advances in viral genetics and molecular biology provide a basis for rational construction of stable, attenuated mutants for use in immunoprophylaxis.

3.1 Attenuation by mutation

Attenuation can theoretically be achieved by any mutation that diminishes the capacity of a virus to replicate in a host. It was established that, in the case of rabies virus, the attenuation is associated with a single amino acid substitution (79). An essential property of any attenuated live-virus vaccine is the stability of the attenuation phenotype. Reversion to pathogenicity occurs primarily by suppression, i.e. by development of a second-site mutation that corrects the defect caused by the original mutations. Therefore, the attenuation phenotype should be stabilized by inducing as many mutations as are consistent with viability and satisfactory immunogenicity (79). Deletion mutants, such as those used recently for pseudorabies virus (thymidine kinase gene deleted) (81,82), should be stable because the absence of a gene cannot be substituted by a mutation elsewhere in the genome. It was shown that herpes simplex virus deletion mutants have reduced ability to be reactivated from ganglion cells (83). A deletion of an additional gene in the case of the pseudorabies virus makes the immune response to this vaccine virus distinguishable from that against wild viruses (81). This is a highly desirable feature for eradication efforts because pigs infected with wild pseudorabies viruses harbor the pathogenic virus for life, with the possibility of shedding at any time.

3.2 Selection of epitope mutants using neutralizing monoclonal antibodies

Attenuation can also be achieved by selecting spontaneous mutants of a virus that resist neutralization by neutralizing monoclonal antibodies (mAb). However, only a few of these mutants exhibit attenuation, and they can revert rather easily. The fact that neutralization mAb resistant mutants of neurovirulent reovirus are stable (80) indicates that this approach might be fruitful with at least some viruses.

3.3 Recombinant viruses used as vectors for heterologous protective antigen

Recombinant viruses that express foreign genes of pathogenic organisms are a special case of live viral vaccines. No such vaccines have yet been licensed, but

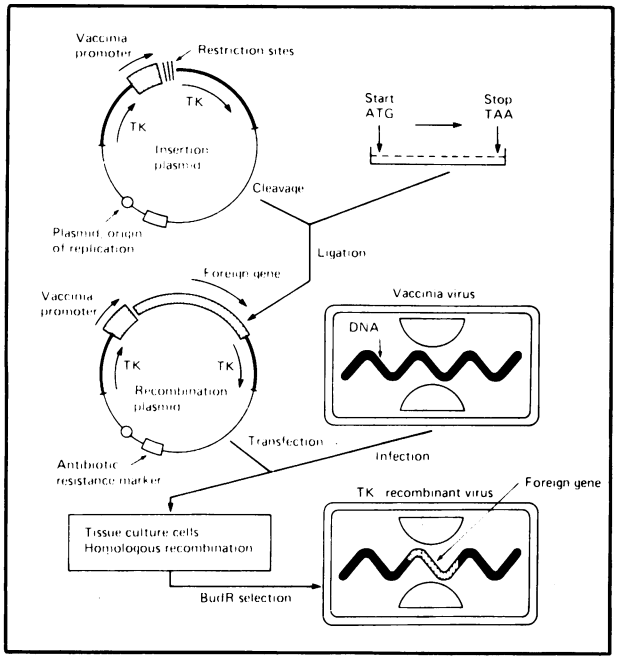

Figure 2. Schematic representation of anti-idiotypic antibody serving as antigen in vaccines. Note that the shape of the binding site (idiotope) of the anti-idiotype antibody is the same as that of the epitope on the antigen. Therefore, the idiotope of an anti-idiotypic antibody mimics the epitope (it has the same antigenic property) that was used in production of the first antibody, which was used subsequently to produce anti-idiotypic antibody.

the high expectations for these vaccines are reflected in the intense research efforts currently underway in many laboratories concerned with vaccines against important veterinary and human pathogens. With the use of recombinant DNA technology, genetically altered viruses can be constructed that carry foreign genes coding for antigenic determinants of other pathogens. The foreign gene(s) should be expressed on the surface of that virus, and the virus should replicate in a vaccinated species without any harmful effect to the host. In the process of the self-limiting infection with the recombinant virus, the vaccinated animal will be immunized concomitantly against the agent from which the foreign gene was acquired.

Much of the research so far has been done with vaccinia virus as the vector. The biological features that influenced the intense interest in vaccinia virus include a DNA genome of nearly 200,000 base pairs that allows replacement of many genes without affecting the ability of the virus to grow in tissue culture (59). Vaccinia virus also has a virus-encoded transcription system with unique regulatory sequences, apparent absence of RNA splicing (exclusion of some nucleotide sequences from mRNA after transcription from DNA), and a cytoplasmic site of replication (60). The procedure for insertion of the immunizing foreign gene into vaccinia virus is schematically depicted in Figure 2.

The protocol for inserting foreign genes into vaccinia virus involves, first, molecular cloning of that gene. The gene previously identified to code for the antigenic determinant of interest is sequenced. Once the sequence is known, specific restriction endonucleases can be used to dissect the gene, and then the gene is inserted into a convenient cloning vehicle (most often plasmid pBR 322) by ligation. At the same time, specific vaccinia virus transcriptional regulatory sequences and sequences from a nonessential vaccinia virus gene (which determines the place of insertion of the recombinant DNA) are cloned into the same vector such that they flank the foreign gene. The plasmid containing the chimeric inserts is then introduced into tissue culture cells via a process called transfection. During transfection the tissue culture cells are treated with calcium chloride to break the permeability barrier of the cell membrane, which in turn facilitates the entrance of the plasmid (61). The cells are concomitantly infected with infectious vaccinia virus that is to incorporate the new hybrid DNA sequences. Vaccinia virus contacts the DNA sequences of the recombinant plasmid during its replication, and through recombination incorporates the plasmid DNA into its own DNA. The vaccinia virus sequence in the plasmid serves as the lead for incorporation at the predetermined site. Once this occurs, replication of the recombinant vaccinia virus continues. After maturation and release, a novel recombinant vaccinia virus is identified among the parental vaccinia virions by an immunological assay such as RIA (62), by nucleic acid hybridization (63), or by other methods based on its phenotypic characteristics (62,63).

The use of recombinant vaccinia viruses has several advantages over other types of vaccination. The most important feature is its ability to stimulate vigorous humoral and cell-mediated immunity (19,62,64). It has been shown that recombinant virus elicits both primary and secondary immune responses (61). Moreover, the products expressed by recombinant vaccinia virus are properly glycosylated, processed, and transported to membranes, and consequently they mimic the native state of antigens. Foreign genes are thus expressed in a manner similar to native synthesis and this presents an "authentic" antigen to the immune system (59). This is not true for the products prepared by gene expression in prokaryotic and eukaryotic expression vectors. Heat stability, economy of manufacture, and ease of administration are additional advantages of these vaccines. The possibility of including genes responsible for immunization and excluding genes that might cause undesirable side effects provides a basis for increased safety of these vaccines. Multiple genes can also be inserted into the vaccinia virus to produce polyvalent vaccines for agents that exhibit a number of immunogenic surface proteins (62).

The most serious obstacle to licensure of these vaccines has been public concern over the safety of possible environmental release of recombinant viruses. There is an additional concern with recombinant vaccinia vaccines, which stems from the pathogenicity of unaltered vaccinia virus itself to humans with impaired cell-mediated immune functions. Vaccinia virus is known to cause rare, severe, generalized infections in individuals with immunodeficiencies of the cell-mediated type. Thus the possibility of spread of these viruses from vaccinated animals to humans, however remote, presents another obstacle to licensure. However, attempts to attenuate vaccinia virus have been

successfully undertaken, hopefully paving the way to successful licensing of these vaccines (63).

During the past few years, other viruses such as adenoviruses and herpesviruses have also been explored as vectors. Fowlpox virus was shown to be a suitable vector for both avian and nonavian species (64). Apathogenic enteric bacteria have some potential as vectors, particularly against enteric pathogens and in situations where the intestinal route of vaccination might be advantageous (65).

Vaccinia virus is still the most widely employed organism for the purpose of producing recombinant vaccines. The main reasons are: the ability to grow in the laboratory; stability in freeze-dried preparations; broad host range; ease of administration (scarification or orally); and, most importantly, a large genome allowing more than 25,000 base pairs to be inserted and expressed relatively readily. Chimeric vaccinia viruses have been constructed which expressed vesicular stomatitis virus glycoprotein (66), influenza virus hemagglutinin (67,68), retrovirus envelope protein (69), rabies virus glycoprotein (70), and HA and F protein of rinderpest virus (71). The list of important human pathogens, the immunizing components of which were expressed by the recombinant vaccinia viruses, includes Lassa virus glycoprotein (72), herpes simplex virus glycoprotein (73,74), HA and F protein of measles virus (75), F protein of human syncytial virus (76), hepatitis B virus surface antigen (77), and HTLV-III/LAV envelope protein (61). All of these recombinant viruses protected experimental animals against challenge with corresponding virulent viruses.

4.0 Adjuvants

The small size of antigens produced by the new technologies results in low immunogenicity, and this has led to increased research activity into old immunological adjuvants, as well as to a search for new, more potent ones. Although the literature on adjuvants is immense, the purpose of this paragraph is to list the areas of high research activity in connection with the new generation vaccines.

Freund's incomplete (FIA) and complete (FCA) adjuvants are the most powerful candidates. FCA contains killed mycobacteria, in addition to the mineral oil and emulsifier present in FIA. The undesirable side effects caused by the mycobacterial component of FCA can be reduced by using derivatives of the active component of the mycobacterial peptidoglycan. Other candidates that are in use or under consideration are metabolized lipid preparation, synthetic liposomes and synthetic muramyl dipeptide, aluminum hydroxide, saponins including Quil A and ISCOMs (immune stimulating complexes), slow-releasing biodegradable capsules, pluronic block polymer surfactants, stearyl tyrosine and related structures, bacterial lipopolysacharides, and lymphokines (84–90).

The mode of action of adjuvants is under intensive investigation. It has been suggested that macrophages are primary targets for adjuvant action and are stimulated to release interleukin 1 which acts on T-helper (Th) cells and is also important in B-cell responses Adjuvants may increase the efficiency of interaction of B cells with accessory cells and may augment Th activity (24).

In addition, various other approaches are being contemplated to increase immunogenicity of antigens. The list of possibilities includes: conjugation of immunogenic peptides (constructed by previously discussed technologies) with peptides possessing affinity for appropriate lymphocyte receptors or with peptides mimicking lymphokines; addition of peptides that increase affinity of antigens for class II MHC (MHC II is a cell membrane structure necessary for cooperation among cells of the immune system); and use of bifunctional antibodies with one specificity to an antigen and the other specificity to T helper of cytotoxic cells (90,91).

Summary and conclusions

Introduction of recombinant DNA and monoclonal antibody technologies in the 1970s caused expansion of the knowledge of molecular structure of the individual protective antigens of pathogenic microorganisms. Furthermore, a considerable amount of knowledge about pathogenic mechanisms has been accumulated through studies that utilized these molecular tools. It became obvious that traditional methods of vaccine preparation, although quite successful in many situations over the past 100 years, will now have to be gradually replaced by products of biotechnology. These molecular techniques will make it possible for vaccines to be constructed which contain epitopes relevant to protection and which lack microbial components that can be harmful. CVJ

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