CHAPTER II

LITERATURE REVIEW

II. LITERATURE REVIEW

2.1 *Entamoeba histolytica*

2.1.1. Importance of the disease amebiasis

Amebic dysentery, also known as amebiasis, is a common disease of the developing world and is caused by *Entamoeba histolytica*, a one-celled parasite (Figure 2.1). The World Health Organization estimates that 50 million cases of colitis and liver abscess and 100,000 deaths result from infection by this organism annually (World Health Organization, 1995). Most of the infections occur in the developing nations of Central and South America, Africa, and Asia. Microbial adhesion is often a first step during infections leading to overt disease. *E. histolytica* adheres to and destroys cells in the human gut, causing severe tissue damage in the colon. In rare cases, the parasite spreads outside the intestine to the liver and forms abscesses. Even less commonly it spreads to other parts of the body, such as the lungs or brain. Therapies are aimed at blocking adhesion and may prevent colonization and subsequent disease. Since humans are the only reservoir for *E. histolytica*, a vaccine that prevents colonization by blocking adherence could eradicate amebiasis (Dodson *et al.*, 1999).

2.1.2. Life cycle of *Entamoeba histolytica*

The life cycle of *Entamoeba histolytica* (Figure 2.2) involves trophozoites that live in the host s large intestine and cysts that are passed in the host s feces. Humans are infected by ingesting cysts, most often via contaminated food or water. In most infections, the parasite lives in the intestine and does not cause illness, or causes mild intestinal symptoms. Symptoms of the mild form of amebiasis include loose or watery stools, abdominal discomfort, and stomach cramps. Some people develop a severe form of amebiasis called amebic dysentery. Symptoms of this form of the disease are stomach pain, bloody stools, and fever. In rare cases, the parasite spreads outside the intestine to the liver and forms abscesses. Even less commonly it spreads to other parts of the body, such as the lungs or brain.

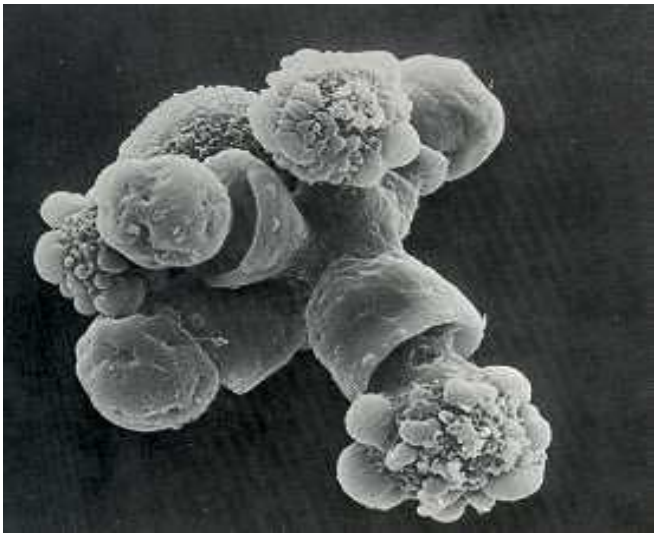

Fig. 2.1. Scanning electromicrograph of a trophozoite of *E. histolytica* ingesting epithelial cells (Martinez-Palomo, 1986). X 2200

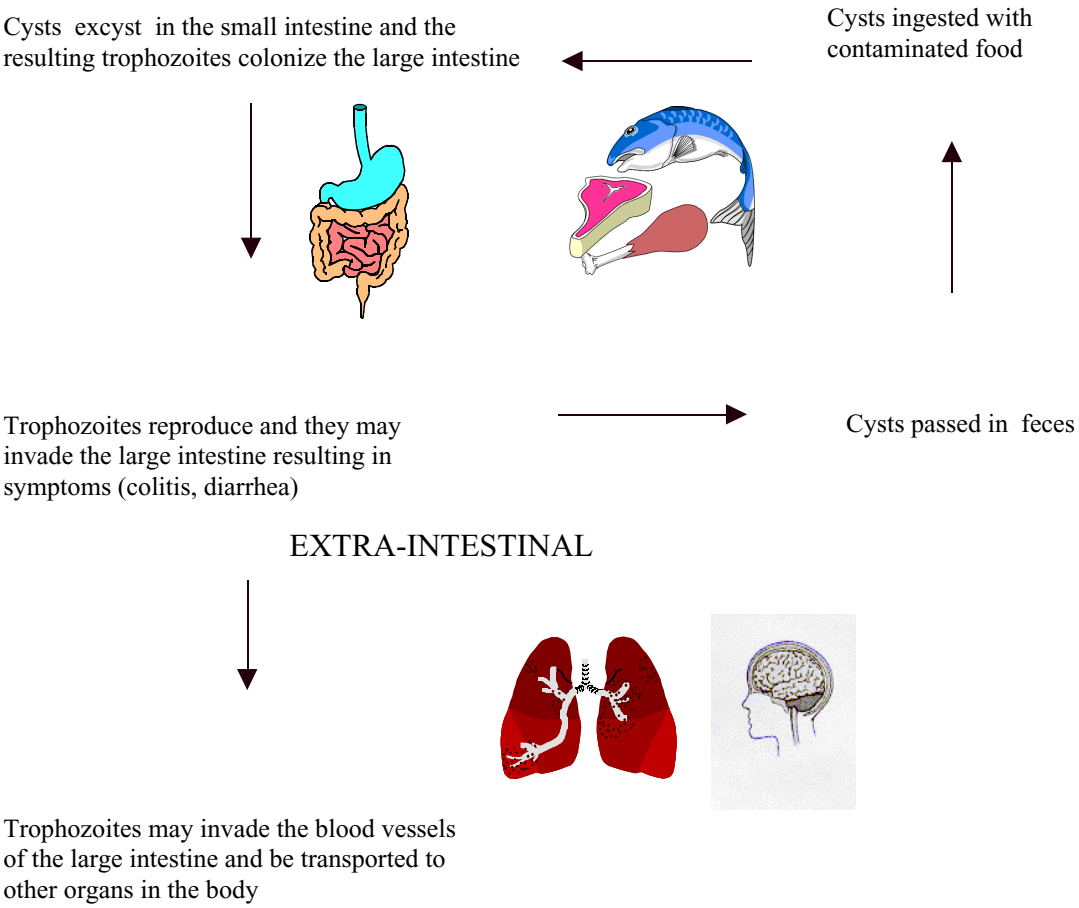

Fig. 2.2. Life cycle of *Entamoeba histolytica*

2.1.3. The amebic cell surface lectin

All strains of *E. histolytica* express a surface lectin that is immunogenic. This lectin is a 260 kDa heterodimer of heavy (170 kDa) and light (35 kDa) subunits linked by disulfide bonds and is required for adherence to human intestinal epithelial cells and contact-dependent killing of immune effector cells (Dodson *et al.*, 1999). It binds exposed terminal galactose (Gal) and N-acetyl-D-galactosamine (GalNAc) residues of target cell glycoproteins (Ravdin *et al.*, 1985; Saffer and Petri, 1991). The lectin is an obvious vaccine candidate. However, animals immunized with the intact lectin produce antibodies against adherence-enhancing and adherence-inhibiting epitopes on the lectin heavy subunit and, in some cases, the disease is exacerbated (Petri and Ravdin, 1991). A carbohydrate recognition domain (CRD) has been identified within the lectin heavy-subunit cysteine-rich region (Dodson *et al.*, 1999). The CRD lacks these adherence-enhancing antibody epitopes and immunization with the CRD does not result in the production of adherence-enhancing antibody responses. Recent studies are investigating the use of CRD in vaccine design to confer protection from invasive amebiasis (Dodson *et al.*, 1999).

2.2 The vaccine concept

2.2.1. Introduction

An ideal vaccine may be considered to be a modified, non-pathogenic form of an infectious agent, not as capable of replication and spread as the wild type or pathogenic agent, but still able to stimulate the immune system (Brown *et al.*, 1993). With the growth of the human population and the prevalence of infectious agents (e.g. HIV), vaccination costs have been increasing and are therefore no longer affordable on a global scale. Subunit vaccines produced using conventional methods such as yeast, bacteria or mammalian cell systems, require extensive purification to remove host proteins and compounds. Packaging, refrigeration during shipping and storage, the use of needles and syringes and the need for administration by trained personnel also increase the cost of such vaccines (Moffat, 1995). In 1990, the World Health Organization launched the Children s Vaccine Initiative to encourage the accelerated development of novel vaccines

for delivery to children worldwide. The initiative stressed the importance of developing oral vaccines that would be affordable, reliable, and heat-stable, and called for the development of multicomponent vaccines that could immunize against several infections at once (Wong *et al.*, 1998).

2.2.2. Oral immunization

At present many vaccines are delivered to by injection. This method has some practical advantages but it also has drawbacks. Intramuscular or subcutaneous administration of vaccines often does not lead to optimal or long-lasting protection against infectious agents. In contrast, the oral route of delivery can stimulate strong protective responses on mucous membranes and in the circulation (Cui *et al.*, 1991). Oral vaccines, which do not require the use of needles and medically qualified personnel for administration, would be of great advantage in developing countries. Also, combining antigens from a variety of pathogens to form effective multicomponent vaccines is one of the challenges of modern vaccine research. A potential problem for multicomponent vaccines is limitations on the volume of material that can be injected, particularly in infants (Mannino and Gould-Fogerite, 1995), which could also be circumbented using oral vaccines.

2.2.3. Transgenic plants — an exciting alternative for cost-effective vaccine production and delivery

Currently, the most common large-scale production systems for proteins are genetically engineered bacteria and yeast, due to the relative ease of manipulation and rapid predictable growth of these organisms. Recombinant proteins overexpressed in these systems are extensively purified to remove host proteins and other compounds. These processes add to the cost of recombinant proteins. Transgenic plants provide an alternative system that can be scaled up to high production capacity. A great practical advantage of producing vaccines in transgenic plants is the ability to directly use edible plant tissues for oral administration without purification (Richter and Kipp, 1999). It has been shown that transgenic plants can assemble foreign proteins in their native immunologically-active configurations because plants and human cells share many

fundamental biosynthetic processes (Wong *et al.*, 1998). *Agrobacterium*-mediated transformation is the most widely used approach for creating transgenic plants and has been used in all examples of transgenic plants expressing antigenic proteins. The protocols for engineering transgenic plants to express immunogenic proteins are summarized in Figure 2.3 (Edelman, 1997). The ultimate desired endpoint is vaccine production in a food source that could be directly used for immunization of children and infants as well as adults. Table 2.1 shows some of these candidate crops (Edelman, 1997).

In 1998 clinical trials were conducted using transgenic potatoes expressing the B subunit of the *E. coli* heat-labile enterotoxin (LT-B) as oral vaccine. Volunteers ingested raw transgenic potato tubers (50-100g) and developed both systemic and mucosal immune responses. They also showed comparable levels of antibody secreting cells as volunteers challenged with 10^9 entero-toxigenic *E. coli* (Tacket *et al.*, 1998). Today, a variety of human disease antigens have been expressed in transgenic plants (Table 2.2, Tian *et al.*, 2000) and results have shown protective antibody production in humans to these antigens. This provides a proof of concept of transgenic plants as edible vaccines.

2.3 The common mucosal immune system

Mucous membranes are the primary routes of entry for a large number and wide variety of human disease-causing agents, including those that are inhaled, ingested, or sexually transmitted. Antigens or invading pathogens that can survive the harsh acid and degradative environments encountered following oral entry may be taken up by the specialized microfold or M cells of the small intestine. These cells can take up antigens, including whole bacteria, and transport them to the underlying follicle known as a Peyer s patch (Figure 2.4). The induction of T- and B-cell responses to antigens occurs here and is followed by migration of the immune cells to the mesenteric lymph nodes. The T and B cells then travel via the efferent lymphatics to the thoracic duct, where they enter the circulation. Later, they migrate to various effector sites in the gastrointestinal, respiratory, and genitourinary tracts. This is known as the common mucosal immune system (Mestecky and McGhee, 1989). Thus, stimulation in the gut can lead to the development of some local immunity in the lungs and urogenital tract. This further increases the attraction of the mucosal route for vaccination (Brown *et al.*, 1993).

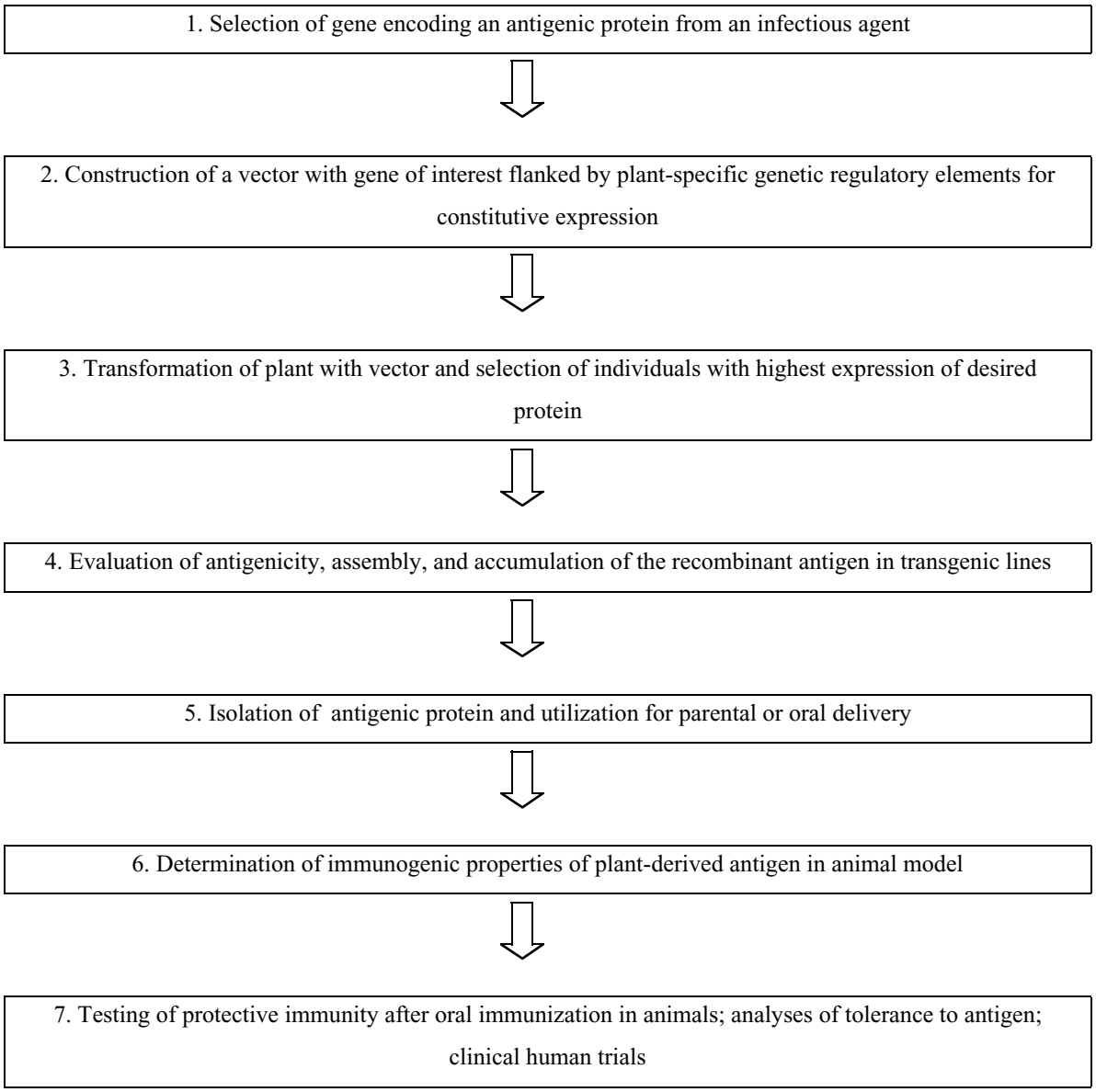

Fig. 2.3. Flowchart describing the experimental steps utilized to create transgenic plants that produce orally immunogenic proteins (adapted from Edelman, 1997)

Table 2.1. List of some plant species that may be considered as expression systems for recombinant vaccines (adapted from Edelman, 1997)

Plant species	Advantages	Disadvantages	Time to obtain transgenic samples
Tobacco	• research tool • well-characterized gene expression system • easily maintained biomass supply • abundant material facilitates protein characterization	• leaf tissue may contain toxic alkaloids • not commonly consumed	• fast: leaf tissues can be analyzed within 5-6 weeks
Potato	• facile transformation • tuber-specific gene expression elements are well characterized • potato tissues accepted as food by many animal models • easily stored	• relatively low protein content in edible tissue causes low recombinant protein levels • food is cooked for human consumption, which may cause loss of secondary structure and immunogenicity in most recombinant proteins	• Microtubers can be assayed for tissue-specific expression in 8-10 weeks to evaluate levels of gene expression and selection of desired transformant line • soil-grown tubers available in 3-4 months for feeding
Legumes (beans, peanuts) or cereal crops (maize, rice, wheat)	• universal agronomic production technology • seeds are relatively rich in protein • seeds can be easily stored • may be an excellent means of delivery for animal vaccines	• heated or cooked before human use, which would very likely denature recombinant proteins	• timing and efficiency of generating transformants varies among species
Banana	• grown in many developing countries where vaccines are needed • eaten by infants and adults • uncooked food • once established, abundant and inexpensive fruit is available on a 10-12 month cycle	• transformation system is relatively inefficient • no data available on gene expression, especially for fruit specific promoters • large space needed —intensive growth conditions needed	• analysis of constitutive gene expression in leaves in 3-4 months • approximately 18 months from transformation to fruit production
Tomato	• genomics well known • uncooked food • transformation system well established	• low biomass • relatively low protein content	• analysis of gene expression in 20-25 weeks

Table 2.2. Human disease antigens being tested for plant-based production of vaccines (Tian *et al.*, 2000) (AIDS=acquired immunodeficiency syndrome; HIV=human immunodeficiency virus)

Disease	Target antigen	Strategies to make plant-based vaccine	Reference
AIDS	HIV-1 capsid glycoprotein gp160	Tobacco mosaic virus (TMV)	Sugiyama *et al.*, 1995
Human influenza	Hemagglutinin epitope	Tobacco mosaic virus (TMV)	Sugiyama *et al.*, 1995
Malaria	Sporozoite B-cell epitope AGDR	Tobacco mosaic virus (TMV)	Turpen *et al.*, 1995
Measles	T-cell and B-cell epitopes	Cowpea mosaic virus (CPMV)	Lomonossoff and Hamilton, 1999
Cholera	Cholera toxin subunit B	Transgenic potato	Arakawa *et al.*, 1997
Hepatitis B	Hepatitis B surface antigen	Transgenic tobacco	Mason *et al.*, 1992
Norwalk viral enteritis	Whole virus capsid	Transgenic tobacco and potato	Mason *et al.*, 1996
Enterotoxigenic *E. coli*	Heat labile enterotoxin subunit B	Transgenic tobacco	Haq *et al.*, 1995
Tooth decay	Streptococcus mutans cells surface-adhesion protein	Transgenic tobacco	Curtiss and Cardineau, 1990

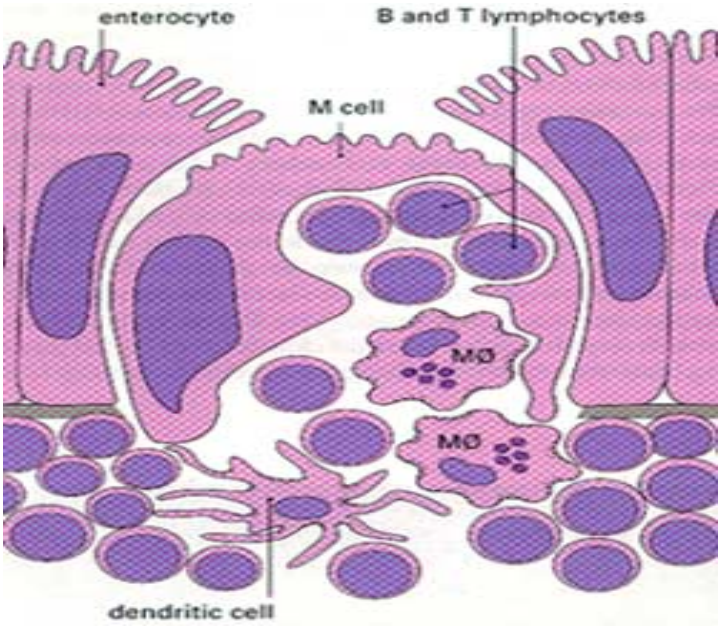

Fig. 2.4. M cells serve as passage ways for virus or antigen to be transported into the Peyer s patches (Roitt, 1993)

IgA-producing plasma cells, initially stimulated by cognate B- and T-cell interactions in gut-associated lymphoid tissue (GALT), secrete antibodies locally that are specifically taken up by epithelial cells, transported to the mucosal surface, and released with a part of the receptor as secretory IgA. Secretory IgAs can protect against pathogens that replicate on or enter via mucosal surfaces (Bergmann and Waldman, 1988; Walker *et al.*, 1992).

There are two major obstacles in developing mucosal vaccines: 1) the requirement for much higher levels of antigen than injected vaccines and 2) the fact that a poor immune response is often seen, inducing tolerance rather than evoking a protective immune response. The mucosal immune system is known to be the site of priming for two paradoxically opposite purposes, i.e., tolerance and mucosal immunity. The usual response of the gastrointestinal tract to antigens is tolerance rather than immunity (Chen *et al.*, 1995). The mechanisms of oral tolerance remain unclear. Oral tolerance has been shown to bias T-cells toward the development of Th2-type (the interleukins IL-4 and IL-10) responses when they are re-exposed to antigen, which can result in predominantly IgG1 rather than IgG2a antibody responses (Ma *et al.*, 1997). To prevent this, the use of an adjuvant is frequently required to potentiate the immune response. At the present time, the development of effective oral vaccines is hampered by an incomplete understanding of the minimal requirements for induction of a positive immune response versus tolerance.

2.4 Adjuvants

2.4.1. Introduction

The term adjuvant was first coined by Ramon in 1926 for a substance used in combination with a specific antigen that produces more immunity than when the antigen is used alone (Ramon, 1926). Ramon demonstrated that it was possible to increase artificially levels of diphtheria or tetanus antitoxin by the addition of bread crumbs, agar, tapioca, starch oil, lecithin, or saponin to vaccines (Ramon, 1925). Today interest in vaccine adjuvants is growing rapidly for several reasons. First, dozens of new vaccine candidates have emerged over the past decade against infectious agents, cancer, fertility,

and allergic and autoimmune diseases. Many of these candidates require adjuvants. Second, the Children s Vaccine Initiative (CVI) initiated in 1990 has helped to energize political and public health interest in vaccine adjuvants by establishing ambitious goals for enhancing present vaccines and for developing new ones (Douglas, 1993). Finally, refinements in the fields of analytical biochemistry, macromolecular purification, recombinant technology, and improved understanding of immunological mechanisms and disease pathogenesis have helped to improve the technical basis for adjuvant development and application (Edelman, 1997). The enormous diversity of compounds that increase specific immune responses to an antigen and thus function as vaccine adjuvants makes any classification system somewhat arbitrary. Table 2.3 (Edelman, 1997) shows examples of immunopotentiators used during the past 25 years. These adjuvants are grouped according to origin rather than mechanism of action, because the mechanism for most adjuvants are incompletely understood.

A carrier is an immunogenic protein bound to a weakly immunogenic antigen (Edelman and Tacket, 1990). A carrier may also be a living organism (or vector) bearing genes for expression of the foreign antigen on its surface. Carriers increase the immune response by providing T-cell help to the antigen. A vehicle also provides the substrate for the adjuvant, the antigen, or the antigen-carrier complex but unlike carriers, vehicles are not themselves immunogenic. Like carriers, most vehicles can alone enhance antigens and so are sometimes considered to be another class of adjuvants, although their immunostimulatory effects are often augmented by the addition of conventional adjuvants to constitute adjuvant formulations. Thus, an adjuvant formulation is composed of an adjuvant in a suitable vehicle. Examples of such adjuvant formulations include (1) monophoshoryl lipid A and cell wall skeleton of *Mycobacterium phlei* adjuvant in a squalene-in-water emulsion vehicle and (2) monophosphoryl lipid A adjuvant in a liposome vehicle (Edelman, 1997).

Most subunit vaccines are not highly antigenic by themselves. Subunit antigens fail for a variety of reasons, such as incorrect processing by the immune system, rapid clearance, stimulation of inappropriate immune response, and lack of critical B- or T-cell epitopes.

Table 2.3. Classes of modern vaccine adjuvants, carriers and vehicles (adapted from Edelman,1997)

I. ADJUVANTS

- A. Aluminium and calcium salts
  - 1. Aluminium phosphate
  - 2. Aluminium hydroxide
  - 3. Calcium phosphate
- B. Bacterial and plant products
  - 1. Mycobacterial products (eg. Complete Freund s adjuvant)
  - 2. Gamma inulin and algammulin
  - 3. Beta-glucan
  - 4. Monophosphoryl lipid A
  - 5. Neuraminidase-galactose oxidase
  - 6. *Klebsiella pneumoniae glycoprotein*
  - 7. *Bordetella pertussis*
  - 8. *Cornynebacterium parvum*
- C. Surface-active agents
  - 1. Saponin
  - 2. Deoxycholic acid/alum complex
  - 3. Dimethyl dioctadecyl ammonium bromide
  - 4. Avridine
  - 5. Nonionic block copolymers
- D. Polyanions
  - 1. Dextran
  - 2. Double-stranded polynucleotides
  - 3. Acetylated polymannose
  - 4. Sulfolipopolysaccharide
- E. Polyacrylics
  - 1. Polymethyl methacrylate
  - 2. Acrylic acid-allyl sucrose
- F. Vitamins
  - 1. Vitamin A
  - 2. Vitamin D3
  - 3. Vitamin E
- G. Cytokines
  - 1. Granulocyte-macrophage colony stimulating factor
  - 2. INF-α
  - 3. INF-γ
  - 4. IL-1
  - 5. IL-2
  - 6. IL-7
  - 7. IL-12
- H. Hormones
  - 1. Human growth hormone
  - 2. Dehydroepiandrosterone
- I. Imidazo-quinolines
- J. Glycolipid bay R1005
- K. Stearyl tyrosine
- L. 7-allyl-8oxoguanosine
- M. Unique synthetic antigen construct
  - 1. Multiantigen peptide
  - 2. Linear polymerization of haptenic peptides
  - 3. Peptide linkage to T-or B-cell epitopes

II. CARRIERS

- A. Bacterial toxoids (tetanus, diphtheria, pertussis, *Pseudomonas*)
- B. Meningococcal outer membrane proteins
- C. Fatty acids
- D. Cholera toxin, LT toxin, B subunit
- E. Ty virus-like particles
- F. Nucleic acid vaccines
- G. Living vectors
  - 1. Vaccinia virus
  - 2. Adenovirus
  - 3. Canary virus
  - 4. Poliovirus
  - 5. BCG
  - 6. Attenuated *Salmonella*
  - 7. Attenuated *Vibrio cholerae*
  - 8. Attenuated *Shigella*

III. VEHICLES

- A. Mineral oil emulsions
  - 1. Incomplete Freund s adjuvant
- B. Vegetable oil emulsions
- C. Squalene and squalene emulsions
- D. Lipid containing vesicles
- E. Biodegradable polymer microspheres
- F. Protein cochleates
- G. Edible plants

Potentially, some of these failures can be overcome by administering subunit antigens with adjuvants. The best adjuvant, however, will never correct the choice of the wrong epitope.

In contrast to subunit vaccines, traditional live vaccines or whole-cell inactivated microbial vaccines are strong immunogens. These vaccines are structurally more complex and contain many redundant epitopes, which offer more opportunity to bypass genetic restriction of the vaccinee. They provide a larger antigen mass and their antigens are larger molecules, portions of which may serve as carrier proteins and thus function as intrinsic adjuvants to enhance immunogenicity by providing T-cell help (Edelman, 1997).

2.4.2. Mechanisms of action

A vaccine adjuvant can influence the immune response in one or more ways (Hunter and Lal, 1994): (1) It introduces antigen into the appropriate *in vivo* microenvironment. (2) It retains and releases antigen slowly from the site of deposition. This is called the depot effect whereby long-term release of antigen results in increased immune response (Freund, 1956; Glenny *et al.*, 1931). (3) The adjuvant recruits and activates antigen-presenting cells and lymphoid cells. (4) It activates complement and induces synthesis, secretion, and binding of cytokines. (5) It delivers T-cell epitopes to the MHC (Major Histocompatibility Complex) class I (cytoplasmic) pathway of antigen-presenting cells for CD8+ cytotoxic T-lymphocyte induction. (6) It delivers T-cell epitopes to the MHC class II (phagolysosome) pathway of antigen-presenting cells for CD4+ T-lymphocyte-mediated responses and antibody induction. The ability of adjuvants to influence so many parameters of the immune response greatly complicates the process of finding an effective adjuvant, because our knowledge of how any one adjuvant operates on a cellular level is insufficient to support a completely rational approach for matching the vaccine antigen with the proper adjuvant (Edelman, 1997).

2.4.3. Safety

As with all drugs and biological products, the absolute safety of adjuvants can never be guaranteed. Inherent toxicities can be ascribed in part to the unintended stimulation of various aspects of the immune response. Consequently, safety and

adjuvanticity must be balanced in order to obtain maximum immune stimulation with minimum side effects (Bussiere *et al.*, 1995). The most important attribute of any adjuvanted vaccine is that it is more efficacious than the aqueous vaccine and that this benefit outweighs its risk. During the past 70 years many adjuvants have been developed that were never accepted for routine vaccination because of their immediate toxicity and the possibility of delayed side effects (Edelman, 1997). Table 2.4 shows the real or theoretical risks of administering vaccine adjuvants (adapted from Edelman, 1997). Recombinant DNA technologies provide the tools for site-specific mutagenesis to modify toxicity and adjuvanticity and the promise of will yield a new generation of protein-based adjuvants.

2.4.4. Characteristics of an ideal adjuvant

A universal ideal adjuvant does not exist, because each immunogen and the targeted antigen will have their unique requirements. Nevertheless, the following generic characteristics would be desirable (Edelman, 1997): (1) The safety of the adjuvant must be assured which includes freedom from immediate and long-term side effects. (2) The adjuvant must be defined chemically and biologically so that there are no lot-to-lot variations in the manufactured product, thereby assuring consistent responses in vaccines over time. (3) The adjuvant combined with antigens should elicit a more robust protective immune response leading to efficacy with fewer doses and/or lower concentration of antigen. (4) The adjuvanted vaccine should be stable on the shelf for at least two years before commercially and clinically useful. (5) The adjuvant should be biodegradable and easily removed from the body after its adjuvant effect is exhausted. (6) The adjuvant should be inexpensive. To date, no adjuvant meets all of these criteria.

When looking for mucosal adjuvants that can be easily expressed in plants, one prefers a plant protein that is known to survive the digestive process and bind to the intestinal mucosa of humans. Among the most promising proteins for this approach to vaccine delivery are multivalent plant lectins. Lectins are carbohydrate binding proteins that usually contain multiple sites for binding to specific carbohydrate moieties. Different lectins have different levels of toxicity, though not all lectins are toxic.

Table 2.4. Real and theoretical risks of vaccine adjuvants (Edelman, 1997)

1. Local acute or chronic inflammation with formation of painful abscess, persistent nodules, ulcers, or draining lymphadenopathy
2. Induction of influenzalike illness
3. Anaphylaxis
4. Systemic clinical toxicity to tissues or organs
5. Induction of hypersensitivity to host tissue
6. Cross-reactions with human antigens
7. Sensitization to tuberculin or to other skin-test antigens
8. Immune suppression
9. Carcinogenesis
10. Teratogenesis
11. Abortogenesis
12. Dissemination of live vector within the host to cause disease; spread of the vector to the environment and other persons

Lectins are involved in the recognition between cells or cells and various carbohydrate-containing molecules and play an important role in the defense mechanisms of plants against the attack of microorganisms, pests, and insects. Other functions of lectins in plants include storage of proteins, transport of carbohydrates and packaging and/or mobilization of storage material. In 1988, lectins were shown to provoke an immune response when given orally (De Aizpurua and Russell-Jones, 1988) which suggests that they would make good carriers for other antigens. For this reason, ricin, a plant lectin from castor bean, could be a good adjuvant.

2.5Ricin

2.5.1. Introduction

Ricin is a protein produced by *Ricinus communis* (castor bean, Figure 2.5) which is highly toxic (the minimal lethal dose is around 1μg/kg body weight). *Ricinus communis* accumulates ricin to more than 5% of the total protein in vacuoles within the seeds (Lord, 1994). Many cytotoxic proteins from a variety of plants have been identified that are related to ricin both in structure and function. They inhibit protein synthesis by specifically and irreversibly inactivating eukaryotic ribosomes. These ribosome inactivating proteins (RIPs) are typically N-glycosylated 30 kDa monomers (type 1 RIPs). However, in order to bind to the cell surface galactosides and enter the cytosol to reach ribosomes, they require a second monomer, a galactose-binding 30 kDa lectin. The monomers are joined by a disulfide bridge to form the toxic heterodimers (type 2 RIPs). Ricin is a heteromeric type 2 RIP consisting of an RIP A (RTA) chain linked by a disulfide bond to the lectin or binding B (RTB) chain. The non-toxic B subunit binds to terminal galactose residues on the cell surface and facilitates internalization of the toxic A chain (Olsnes and Phil, 1982). Once inside the cell, the A chain appears to be routed via the retrograde pathway through the Golgi to the endoplasmic reticulum (ER) where it is transported into the cytosol. In the cytosol, CTA inactivates eukaryotic ribosomal RNA by catalyzing the depurination of an adenine at a specific site in the 28S rRNA (Endo and Tsurugi, 1987; Munishkin and Wool, 1995). A single ricin molecule that enters the cytosol can inactivate over 1500 ribosomes per minute and kill the cell.

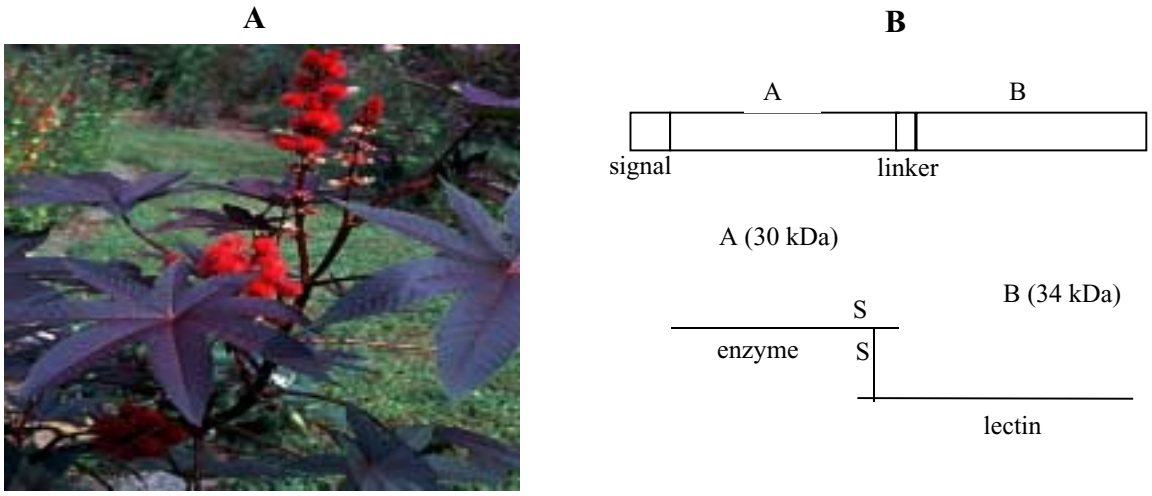

Fig. 2.5. **A**. Castor bean (*Ricinus communis*); **B**. Schematic diagram of ricin gene and mature ricin protein toxin

The production of active ricin is a complex, multistep process. Ricin synthesis begins as a prepropolypeptide that contains both A and B chains. The signal sequence of the NH_2—terminus targets the nascent chain to the ER where it is cleaved to form proricin. Proricin elongates and is N-glycosylated within the lumen of the ER. Protein disulfide isomerases catalyze disulfide bond formation as the proricin molecule folds itself. Proricin undergoes further oligosaccharide modifications within the Golgi complex and is then transported within vesicles to the protein bodies (storage vacuoles) (Wiley and Oeltmann, 1991). Targeting information for routing to the vacuoles requires the 12 amino acid linker between the A and B chain (Frigerio *et al.*, 1998). Several mechanisms permit the production of the cytotoxin ricin in plants without a cytotoxic effect in the plant itself. First, prepro-, pro-, and ricin are compartmented within the endomembrane system and thus do not come in contact with the ribosomes in the cytosol. Second, catalytically active ricin is not produced until proricin is proteolytically cleaved by an endopeptidase within the protein bodies. This splits the polypeptide into the A chain and the B chain, which are still linked by a single disulfide bond. Because ricin is inactive until this occurs, the plant avoids inactivating its own ribosomes in case some proricin accidentally passes into the cytosol during synthesis and transport (Robertus, 1991). Additionally, plant ribosomes are less sensitive to ricin than are animal ribosomes. Bacterial ribosomes are generally not susceptible to inactivation by ricin.

2.5.2. Adjuvant activity of ricin

It has previously been shown that ricin has adjuvant activity. Koga T. *et al.* (1971) reported that the toxic protein ricin from *Ricinus communis* exerts an adjuvant activity expressed in terms of an increase in the number of plaque-forming cells. Mice were injected with various amounts of ricin (from 0.002 to 0.2 μg) and at the same time with sheep red blood cell suspension. Antibody-forming cells of the spleen were estimated four days after injection according to the Jerne s plaque technique (Jerne *et al.*, 1963). A significant increase in the number of plaque-forming cells was observed as compared to a control group, indicating that ricin posseses an adjuvant activity.

Thorpe *et al.* (1989) also found a marked IgE response in rats after injection of 100 μg of castor bean ricin extract. Enhanced IgE responses to other antigens such as

albumin were also observed when ricin extract was administered together with an optimal dose of ovalbumin. There was a substantial increase in ovalbumin-specific IgE but not IgG. In addition, total serum IgE but not IgG increased up to 20-fold. The effect was more sustainable than that of an established IgE-specific adjuvant, *Bordetella pertussis* (Thorpe *et al.*, 1989).

In a recent study at Virginia Tech, the adjuvancy of ricin B (RTB) was compared to cholera toxin for nasal delivery of antigen (T. Wilkins, Virginia Tech, personal communication). RTB was co-administered nasally to mice with ovalbumin as antigen. As shown in Figure 2.6, RTB had an adjuvant effect equal to complete cholera toxin in increasing the serum immune response to ovalbumin.

2.5.3. Production of ricin in tobacco

It has been shown that ricin protein can be produced in tobacco without killing the plant. Sehnke *et al.* (1994) introduced the cDNA encoding preproricin into tobacco under the control of the constitutive 35S promoter from cauliflower mosaic virus via *Agrobacterium tumefaciens*-mediated gene transfer and showed that the resulting plants were capable of processing the preproricin precursor into a fully active mature toxin. Western blot analysis of leaf extracts using anti-ricin A-chain (RTA) antibodies identified 34 and 32 kDa proteins that were electrophoretically indistinguishable from castor bean RTA. Analysis with anti-ricin B-chain (RTB) antibodies identified both a 34 kDa major band that comigrated with castor seed RTB, and a 30 kDa minor band. It was determined that the total ricin production was 0.25% of the soluble extractable protein and 0.0001% of the total plant wet mass.

Frigerio *et al.* (1998) reported the transient expression of a number of ricin A chain-encoding cDNA constructs in tobacco protoplasts. These workers presented evidence that the cellular fate of RTA varies depending on the form of toxin expressed within tobacco protoplasts and that the production of ricin as a precursor is essential for its routing to the vacuole and for protection of ricin-producing cells. It is only when RTA is synthesized as part of the preproricin molecule that it is delivered to its normal vacuolar destination.

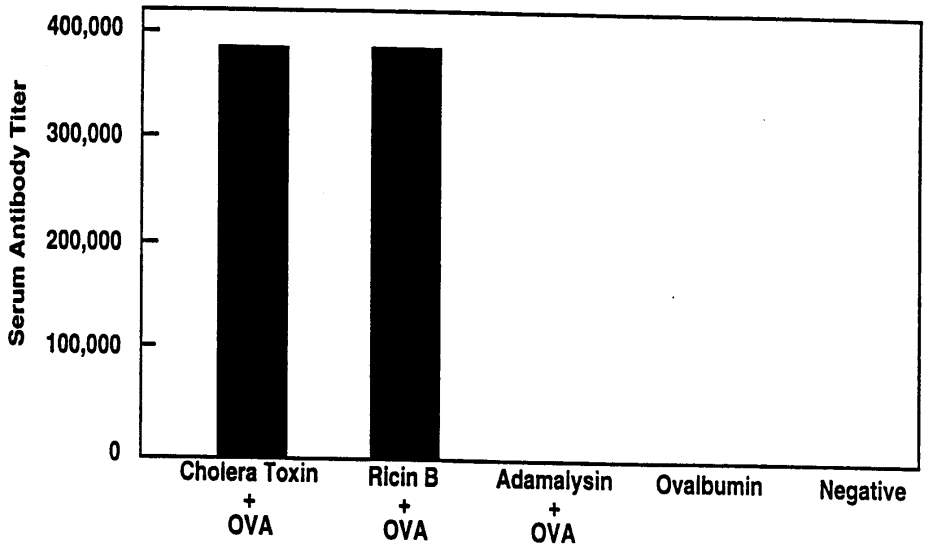

Fig. 2.6. Serum antibody response elicited using ricin B as an adjuvant. Mice were immunized with 1 μg of antigen (ovalbumin=OVA) and 1 μg of adjuvant. Animals were boosted on a 4-week schedule. Six days following the second boost serum antibody titers were determined. Titers shown are for the animal in each group exhibiting the highest titer. Negative, na ve animals; Ovalbumin, no adjuvant; Adamalysin, control toxin.

Free ricin A chain enters the lumen of the endoplasmic reticulum where it is efficiently glycosylated, but it is toxic to the cells and disappears with time in a brefeldin A-insensitive manner, suggesting reverse translocation to the cytosol and eventual degradation. Free ricin A and proricin are not secreted whereas free ricin B (expressed with an ER signal peptide) is found entirely in the extracellular medium. The coexpression of ricin A and B chain results in the formation of disulfide-linked, transport-competent heterodimers, which are secreted, with a concomitant reduction in the cytotoxicity. Table 2.5 summarizes these results.

2.6 Transgene expression strategies

2.6.1. Introduction

In any one cell at a particular time, only a subset of the total genetic information is expressed. Manifestation of a gene s function is a multistep process that starts at the chromosome of a cell in the form of transcription and ends at the production of a functional protein in the proper place and time. Thus, regulation can be applied at numerous points in this pathway. The early step of transcription initiation represents one such point of control. Typically, the regulatory elements that are responsible for directing specific transcription initiation reside within the 5 promoter region of the gene (Lam, 1994). Expression strategies include constitutive, inducible, and tissue specific (e.g. seeds, fruit, tubers) patterns for bioproduction of high valuable recombinant proteins (reviewed in Cramer *et al.*, 1999).

2.6.2. Cauliflower mosaic virus 35S promoter

Cauliflower mosaic virus (CaMV) has a double-stranded DNA genome within which two distinct promoters, producing 19S and 35S transcripts, have been identified (Franck *et al.*, 1980). Initial studies with transformed tobacco revealed that the 35S promoter appears to be active in most, if not all, of the tissues in the plant (Odell *et al.*, 1985). These observations suggest that the 35S promoter is a strong constitutive promoter and can be used as a general promoter element in plants.

Table 2.5. Cellular fate and effect of RTA, RTB and preproricin when expressed in tobacco protoplasts; SP=signal peptide (Frigerio *et al.*, 1998)

Gene	Product	Location	Effect
SP RTA	RTA	retrograde to cytosol	lethal
SP RTB	RTB	secreted	none
SP RTA SP RTB	RTA RTB	secreted	Low toxicity, probably some retrograde

Indeed, it has been one of the most commonly used promoters for expression of desired gene products in plant cells.

2.6.2.1. Functional analysis of the CaMV 35S promoter

A detailed deletion analysis of the 35S promoter has been carried out by Fang *et al.* (1989) with transgenic tobacco plants. In this study, only mature leaves of the transgenic plants were used for promoter activity quantitation. Results from a combination of 5 and 3 deletions suggested the following model of functional architecture for the 35S promoter. The region from —343 to —46 contains at least three domains of functional importance for leaf expression. The domain between —343 and —208is responsible for about 50% of the promoter activity. Deletion of the sequences between —208 and —90 further decreased the remaining activity about twofold. The third domain, from —90 to —46, is required for the synergistic activation observed with upstream sequences to drive high levels of expression. Promoters truncated at —90 or —105 have little activity above background. These results thus established that for maximal levels of expression in tobacco leaves, multiple sequence elements between —343 to —46 are required.

2.6.2.2. Tissue specificity of functional elements

The coding region of β-glucuronidase (GUS) was used as reporter gene in order to detect gene expression in tissue sections by histochemistry. Using this reporter gene, the —90 truncated 35S promoter was found to be active in roots of transgenic tobacco, while the upstream sequences (-343 to —90) directed preferential expression in leaves (Benfey *et al.*, 1989).

2.6.2.3. Promoter interaction with plant nuclear proteins

Since the activity of the 35S promoter does not depend on polypeptides encoded by the CaMV genome, host transcription factors must be responsible for its activity in plant cells. The first nuclear factor reported to bind to the 35S promoter was called Activating Sequence Factor 1 (ASF-1) and binds the sequence from —82 to —62. A second factor, ASF-2, binds to the —105 to —85 region of the 35S promoter (Benfey *et al.*, 1989).

2.6.3. Dual enhanced 35S promoter

A variant of the cauliflower mosaic virus 35S promoter with transcriptional activity approximately tenfold higher than that of the natural promoter was constructed by tandem duplication of 250 base pairs of upstream sequences. The duplicated region also acts as a strong enhancer of heterologous promoters, increasing the activity of an adjacent and divergently transcribed transferred DNA gene several hundredfold, and to a lesser extent, that of another transferred DNA gene from a remote downstream position (Kay *et al.*, 1987). This optimized enhancer element should be very useful for obtaining high levels of expression of foreign genes in transgenic plants. The double-enhanced 35S promoter was further modified by linking with the 5 -untranslated leader sequence from tobacco etch virus, which appears to function as a translational enhancer (Carrington and Freed, 1990).

2.6.4. MeGA™ promoter

Constitutive expression by the 35S promoter has significant limitations when commercial bioproduction in non-seed tissues is the goal. Proteins that accumulate to high levels may negatively impact yield or the overall health of the plant. High constitutive expression is also sometimes associated with co-suppression or gene silencing, resulting in little or no transgene product accumulation (Cramer *et al.*, 1999). The use of an inducible promoter appears to be an effective strategy for plant-based production of high-value proteins. The MeGA™ promoter is an inducible promoter that has been derived from the tomato *Hmg2* gene, which encodes one of the isoforms of 3-hydroxy-3-methylglutaryl CoA reductase (HMGR). In tomato (and tobacco), *Hmg2* encodes a defense-specific isoform of HMGR that is associated with the synthesis of sesquiterpenoid defense compounds (phytoalexin antibiotics). *Hmg2* shows very limited expression during normal growth and development but displays rapid, high-level local induction following wounding or treatment with various plant pathogens or chemical elicitors of the plant defense response (Weissenborn *et al.*, 1995). Thus the MeGA™ promoter is generally inactive during normal growth and development but shows rapid and strong gene activation in response to mechanical stress (wound induction or

Mechanical **G**ene **A**ctivation) or a variety of defense elicitors (Cramer and Weissenborn, 1997). This system has several advantages for high-level expression of therapeutic proteins. It separates biomass production from recombinant protein production minimizing the impact of 1) environmental factors on protein yield and quality and 2) possible deleterious effects of transgene expression or foreign gene accumulation on plant growth and development. In addition, the timing of protein extraction can be adjusted based on the stability of the particular gene product to optimize yield of fully active polypeptides (Cramer *et al.*, 1999).