THE DEVELOPMENT OF DNA VACCINES CONTAINING GENES EXPRESSING SPECIFIC ENTEROTOXIGENIC *ESCHERCHIA COLI* ANTIGENS AND CYTOKINES FOR THE ENHANCEMENT OF ANTIBODY PRODUCTION

BY

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A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department of Animal Science
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ABSTRACT

Immunization using DNA vaccines, a promising new technology, has facilitated a powerful new approach to vaccinology as it offers a unique means of stimulating and enhancing immune responses. The objectives of this study were to develop DNA vaccines containing genes capable of expressing enterotoxigenic *Escherichia coli* (ETEC) virulence factors, antigens and specific cytokines, as genetic adjuvants, for the enhancement of antibody production in mice and chickens.

DNA vaccines were synthesized by insertion of the ETEC K88 *fae*G or K99 *fan*C genes into an expression plasmid. These vaccines, when injected into mice and chickens, induced an immune response. Also the injection of both the DNA vaccine and the corresponding protein injection greatly enhanced antibody titers in both mice and chickens compared to that obtained with DNA or protein alone.

A plasmid vector encoding both the K88 *fae*G antigen gene and a cytokine gene (Interleukin-4, IL-4) co-expressed both proteins and stimulated a high level of anti-K88 fimbriae antibody response, while the plasmid co-expressing both the K88 fimbriae and IL-2 suppressed anti-K88 fimbriae antibody response. Interestingly, the interleukins

caused a shift in the type of immunoglobulin produced with IL-4 producing a greater concentration of the IgG1 isotype and IL-2 producing a greater concentration of the IgG2a isotype.

A new plasmid that encodes both the K88 fimbrial gene (*fae*G) and chicken Interleukin-6 (IL-6), as a genetic adjuvant, increased the production of specific antibodies against the K88 fimbriae antigen in chickens compared to that obtained with the vaccine encoding only the *fae*G. In addition, the vaccine containing both genes expressed both the K88 fimbriae and chIL-6 proteins yielding a positive immunochemical band at 32 kDa with the anti-K88 fimbriae antibody and positive bands at approximately 36 and 32 kDa with the anti-chIL-6 antibody.

These data demonstrate that a DNA vaccine alone can stimulate an antibody response to ETEC antigen and that this response can be enhanced by co-injection of an antigen or by immunization with a DNA vaccine containing the gene of interest and a genetic adjuvant such as the IL-4 gene in mice and IL-6 in chickens. DNA vaccines offer many advantages relative to protein immunization as they are also safer, less evasive, provide long lasting immunity and are less expensive.

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FOREWORD

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LIST OF ABBREVIATIONS

APCs = antigen-presenting cells

cAMP = cyclic adenosine 5'-monophosphate

cDNA = cyclic deoxyribonucleic acid

Cyclic GMP = cyclic guanosine monphosphate

ELISA = enzyme-linked immunosorbent assay

ETEC = enterotoxigenic *Escherichia coli*

FCA/FIA = Freund's complete/incomplete adjuvant

GM-CSF = granulocyte-macrophage colony-stimulating factor

HCMV = human cytomegalovirus

HIV = human immunodeficiency virus

i.d. = intradermally

i.m. = intramuscularly

Ig = immunoglobulin

IL = interleukin

LT = heat-labile

MHC = major histocompatibility complex

NK cells = natural killer cells

O.D. = optical density

PBS = phosphate-buffered saline

PCR = polymerase chain reaction

SDS-PAGE = sodium dodecyl sulfate-polyacrylaminde gel electrophresis

ST = heat-stable

Th-cells = helper T-cells

GENERAL INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) strains are able to cause infections of animals and humans. This pathogen has recently been identified by the World Health Organization (WHO) as one of the targeted pathogens for the control by vaccination (Sansonetti, 1998). In has been shown that ETEC-mediated diarrheal diseases in addition to affecting humans also affect agricultural animals, especially cattle and pigs. Because calves and piglets are particularly susceptible, the agricultural industry suffers a sizeable loss of livestock each year from outbreaks of these diseases. In one of the surveys of pre-weaning disease in pigs, diarrhea had the highest morbidity and represented 11% of the pre-weaning mortality with ETEC being the primary and sole infectious cause (Alexander, 1994). A second disease, post-weaning diarrhea, is a major cause of economic losses to swine industry from both mortality and reduced growth rates and is the most common cause of post-weaning mortality in many farms, killing 1.5 to 2% if pigs weaned (Hampson, 1994).

The strains of ETEC that are associated with intestinal colonization are those that express the K88, K99, 987P, F41 and F18 fimbrial adhesions and enterotoxins (Sta, Stb,

LT and VTe), (Ojeniyi *et al.*, 1994). These adhesions are located in the rod-like pili (fimbriae) that extend from the *E.coli* and are bound to specific receptors on the intestinal wall. Among the different ETEC, those expressing the K88 fimbrial antigen are the most prevalent form of *E.coli* infection, being found world—wide wherever pigs are raised in high numbers (Rapacz and Hasler-Rapacz, 1986). There are significant concerns that diarrhea in neonatal and post-weaning pigs will become more serious in the future, given the trend towards large herds, early weaning, increased incidence of antibiotics resistance in microorganisms and pressure by regulatory agencies to ban or reduce the use of antibiotics in feeds. Thus, there is a need to develop alternate strategies to control this organism in human and veterinary medicine, particularly in swine industry.

One of the alternative strategies for the controlling the pathogens is vaccination. Traditional vaccines fall into two broad categories: attenuated, non-pathogenic live infectious material; and killed, inactivated, or subunit preparations. Although most of vaccines produce a diverse and persistent immune response, they present safety concerns or may mutate to become infectious.

Thus to control diarrheal diseases in animals and humans, vaccination against specific strains of ETEC using vaccines based on the traditional vaccines could be an

alternative strategy. Use of vaccines based on immunogenic material of *E.coli*, selected from pili, pili proteins and *E.coli* antigens for controlling diarrheal disease in livestock has been reported by several researchers (Marquardt *et al.*, 1999; Isaacson, 1994). There are several reported on the preparation of genetic vaccines based on colonization factor antigens (CFAs) of ETEC (Alves *et al.*, 1998b; Alves *et al.*, 1999a; Alves *et al.*, 1999b). Use of a genetic vaccine containing specific genes coding for antigens of pathogenic *E.coli* and/or other pathogenic microorganisms provides an efficient, cost-effective and safe alternative for producing antibodies which can be used in controlling diarrheal and other infectious diseases. This technology allows specific genes to be expressed in non-infecting vectors such as plasmids (Chattergoon *et al.*, 1997). The sequence of a plasmid insert can be manipulated to present all or part of the genome of an organism of interest, thereby tailoring the immune response to the pathogen. Also, genes that lead to undesired immunogenic inhibition or autoimmunity can be altered or deleted. Furthermore, genes that encode important immunologic epitopes can be included whereas those that confer pathogenicity or virulence can be excluded. This flexibility can be exploited to include sequences of multiple epitopes within a protein or across divergent sequences from different strains.

It is known that the immune response to genetic vaccine can also be manipulated by altering the conditions under which the vaccine is administered (Lewis and Babiuk, 1999; Weiner and Kennedy, 1999). These conditions include the method of delivery (through gene gun, microneedles, liposomes, microspheres), route of administration (intradermal, intramuscular, intranasal and oral), presence of co-administered cytokines, chemokines or costimulatory molecules, and the administration of an endotoxin-free vaccine.

However, it is becoming increasingly clear that the quality of the immune response is as important as its quantity in determining the outcome of infectious disease. In particular, the balance between Th1 and Th2 responses and between CTL and antibody isotypes, can be critical. For this reason, the use of cytokines to enhance immune responses to vaccines is an area of growing interest. The use of cytokines as adjuvants has focused on their presentation at a high local concentration in depot form with the antigen to steer the immune response selectively toward the desired outcome (Raz *et al.*, 1993; Chow *et al.*, 1998; Wortham *et al.*, 1998). Novel synergies between cytokines have been uncovered. A single dose depot administration of cytokines with the antigen also has the advantages of a lower risk of systemic side effects compared to that obtained by the

systemic administration of cytokines, which has been fraught with difficulties.

One highly attractive and effective alternative approach for the control of pathogens, including *E.coli*, is the use of therapeutic antibodies. These antibodies can be produced in any animal and can be administered orally to another animal to control a specific disease. The advantage of using these antibodies is that they provide a safe, highly effective, simple and sustainable means of controlling pathogens. Such a treatment will not result in the development of resistant strains of pathogens, would spare the use of antibiotics and could be relatively inexpensive. Antibodies can be obtained from several sources including the colostrums of lactating animal, blood of animals, transgenic plants and animals, recombinant microorganisms and chicken eggs. Specifically, chickens produce eggs non-invasively and due to the phylogenic distance, the adjuvant does not cause severe responses as it does in mammals. It has also been shown that the production and maintenance of high levels of specific antibodies over a long period of time are possible in laying hens. Also, it is now possible to obtain antigen specific antibodies from egg-yolk of hyper-immunized hens (Marquardt *et al.*, 1999; Yokoyama *et al.*, 1992).

The objectives of this study, as discussed subsequently, were to demonstrate that DNA plasmids can be constructed that are able to elicit an immune response against

specific virulence factor from *E.coli* in mice, as a model, and in the yolk of hyperimmunized hens and that this response can be enhanced by co-expression of genetic adjuvants, such as cytokines, and more specifically certain interleukins.

LITERATURE REVIEW

1. Enterotoxigenic *Escherichia coli* (ETEC)

Enterotoxigenic *Escherichia coli* (*E.coli*) (ETEC) are an important cause of diarrhea in piglets (Shipley *et al.*, 1981). The diarrhea occurs just after birth, in 1 to 7-day-old piglets, as neonatal diarrhea. Post-weaning diarrhea occurs just after weaning in 22 to 49-day-old piglets. Such strains of ETEC can produce the heat-labile enterotoxin (LT), any of the heat-stable enterotoxins (STa, STb), or any combination of the three, and most of the recognized virulence factors of enterotoxigenic *E.coli* including the fimbrial adhesions (pili) K88(F4), K99(F5), 987P(F6) and F41 which facilitates the adhesion (colonization) of the organism to the small intestine (Orskow and Orskow, 1966).

1.1 Fimbrial adhesions of *E.coli* K88 (F4) and K99 (F5) strains

Most pathogenic *E.coli* strains produce fimbrial adhesions. The K88 and K99 fimbrial adhesions which are associated with diarrhea in piglets are plasmid encoded and are rod-like structures. Three antigenic variants of K88 fimbrial adhesion (K88ab, K88ac, an2d K88ad) have been identified (Guinee and Jansen, 1979; Orskow *et al.*, 1964). Each variant shares a common antigen (a) and expresses one of three variant-specific antigens

(b, c, or d respectively) (Hu *et al.*, 1993; Klemm, 1985). K88 fimbriae are filamentous structures that enable ETEC to bind to a receptor in the mucus layer of the intestine. Their binding capacity can be determined by hemagglutination, a simple assay in which *E.coli* strains and erythrocytes of different species are mixed on slides or in microtiter plates (Jones and Rutter, 1974; Hacker *et al.*, 1985).

The major fimbrial component of K88 fimbriae was first described by Mooi and de Graaf (1979). Analysis of purified K88 on sodium dodecyl sulfate-polyacrylaminde gels (SDS-PAGE) showed a single protein band with an apparent molecular weight of 23 to 26 kDa depending on the K88 variant isolated (Mooi and de Graaf, 1979). As the K88 fimbria has a length of 0.1 – 1 μm and a diameter of 2.1 nm, it is clear that one K88 fimbria consists of hundreds of these identical protein subunits (Klemm, 1985) which build up the body of the fimbria. Analysis of the K88 gene cluster revealed that the major fimbrial protein was encoded by the *fae*G gene, and consequently was designated FaeG with an exact calculated molecular weight of 27,540 Da (Mooi *et al.*, 1984; Turnes *et al.*, 1999).

The K99 fimbriae are also a pilus (filamentous) adhesion that mediates the attachment of ETEC K99 strains to the small intestines of neonatal calves, lambs, and

piglets (Jones and Isaacson, 1983). Fimbriae extracts obtained from bovine and porcine ETEC strains K99 have been analyzed by SDS-PAGE, immunoblotting, and haemagglutinating activity (De Graaf *et al.*, 1984). The major protein, a 17 kDa band, was identified as the fimbrial subunit for K99 fimbriae (De Graaf *et al.*, 1984; Vazquez *et al.*, 1996). The gene, *fan*C, is the major subunit polypeptide of K99 (F5) fimbriae of ETEC strains (Roosendaal *et al.*, 1987; Lee and Isaacson, 1995; Ogunniyi *et al.*, 2002)

1.2 Enterotoxins

Enterotoxins produced by ETEC include the LT (heat-labile) toxin and/or the ST (heat-stable) toxin, the genes for which may occur on the same or separate plasmids. The LT enterotoxin is very similar to cholera toxin in both structure and mode of action. It is an 86 kDa protein composed of an enzymatically active (A) subunit surrounded by 5 identical binding (B) subunits. It binds to the same identical ganglioside receptors that are recognized by the cholera toxin, and its enzymatic activity is identical to that of the cholera toxin (Spangler, 1992).

The ST enterotoxin is actually a family of toxins which are peptides having molecular weights of about 2,000 daltons. Their small size may explain why they are not inactivated by heat. ST causes an increase in cyclic GMP in host cell cytoplasm leading

to the same effects as an increase in cAMP. STa is known to act by binding to a guanylate cyclase that is located on the apical membranes of host cells, thereby activating the enzyme. This leads to secretion of fluid and electrolytes resulting in diarrhea (Alexa *et al.*, 1997).

2. The antibiotics

2.1 Antibiotics use in animals

The term "antibiotics" is usually used to describe a medicine which destroys or inhibits the growth of bacteria. The continued responsible use of antibiotics has given man and animals freedom from many diseases and has greatly facilitated the safe and efficient production of livestock.

In animal medicine, antibiotics are used in three main ways: to treat an individual or an outbreak of disease; to prevent outbreaks occurring; and to enable farm animals to derive optimum benefit from their food. In the United States, livestock producers routinely use 20,000 tons of antibiotics each year, also 40 to 80 percent of the antibiotics applied on the farm are considered to be unnecessary (Hays *et al.*, 1981). Canadian livestock producers also utilize large quantities of antibiotics. Most of the antibiotics used

in the feed industry are used as growth promoters (Brufau, 2000).

2.2 A serious problem: Use of antibiotics as feed additives

Bacteria may become resistant to a given antibiotic by either mutation or by acquiring a gene from other bacteria that are already resistant. Mutations under normal conditions do not become predominate unless they impart a competitive advantage to the host. Under the selective pressure of antibiotics killing non-resistant bacteria, mutation is more common. Eventually mutant bacteria emerge that are resistant and hence flourish and reproduce, creating a new resistant strain. Bacteria may also become resistant through gene transfer by routes known as 'conjugation', 'transformation' or 'transduction' where resistant bacteria transfer the genetic material necessary to become resistant to another type of bacteria (Davies, 1996).

Resistant bacteria that infect animals may directly transfer the resistant factor to human pathogens in various ways: direct contact of humans with animals, eating meat contaminated with resistant bacteria, eating eggs or milk that have not been properly pasteurized or cooked, and eating contaminated fruits or vegetables. This transfer of antibiotics resistance from an animal pathogen to a human pathogen has been well demonstrated and poses serious threat to the treatment of human diseases. An even more

serious problem in bacterial resistance is the development of multiple antibiotic resistances. Bacteria that become resistant to an antibiotic also have the ability to become resistant to many other antibiotics similar in structure to or in the same family as this antibiotic, or even in structurally unrelated antibiotics (Davies, 1994). The rising spread and emergence of resistant bacteria is progressively rendering the present antibiotics less and less useful. There are many reports where certain human pathogens can not be treated with any antibiotic as they have developed multiple antibiotics resistance (Chang and Roth, 2001; Ferber, 2000). Also, the rate of production of new antibiotics is slower than the rate of emerging antibiotic resistance.

2.3 Banning of the use of antibiotics for livestock production

As indicated above the widespread and indiscriminate use of antibiotics, especially as a growth promoter, has greatly increased the rate of which both animal and human pathogens are becoming resistant to most of the antibiotics used to treat human diseases. As a result certain enlightened countries such as those in the European Union have banned the use of antibiotics such as tylosin, vancomycin, virginiamysin, zinc carboadoz as feed additives (Brufau, 2000). The United States Senate is also introducing legislation to ban the use of antibiotics as feed additives (Schuff, 2003). Canada and,

hopefully, other countries such as China which uses massive amounts of antibiotics will also ban their use as feed additives. Such a ban would assist in increasing the efficacy of antibiotics as a human medicine but could result in serious production problems in the livestock industry. Therefore, other non-antibiotics treatments in the livestock industry must be found that will ensure a continued and inexpensive supply of high quality animal and poultry products that are safe to use. One of the best alternatives to antibiotics for the control of intestinal pathogens, which are the major cause of reduced productivity in the livestock industry, is the use of specific antibodies from the egg of hens hyperimmunized with specific virulence antigens. These antibodies when fed to young animals such as weaned and preweand pigs have been shown to be highly effective in the passive control of several intestinal diseases (Yokoyama *et al.*, 1992; Marquardt *et al.*, 1999), and as growth promoters (Owusu-Asiedu *et al.*, 2002, 2003).

3. The egg yolk antibodies

3.1 Producing pigs without antibiotics

Several alternative strategies can be used to replace the use of antibiotics as growth promoters for pigs and for the control of disease. An important overall factor is to

maintain clean, sanitary facilities and particularly to avoid transferring of animals from one producer to another. This latter precaution is often not possible. Several different dietary strategies can be utilized to reduce the pathogenic load including the addition to the diet of pigs of organic and inorganic acids, oligosaccharides, and probiotics. Various combinations of these treatments have been shown to produce limited growth promotion and protection against pathogens. Also they have not been shown to be highly effective at controlling intestinal pathogens such as enteropathogenic *E.coli* or *Salmonella*.

One highly attractive and effective alternative approach for the control pathogens that infect the intestinal tract is to use therapeutic antibodies for passive immunity. These antibodies can be produced in any animal and can be administered orally to another animal to control a specific disease. The advantage of using antibodies is that they will provide a long term and sustainable means of controlling pathogens. Such treatment is highly effective, would not result in the development of resistant strains of microorganisms, would spare the use of antibodies and could be relatively inexpensive to use. Antibodies can be obtained from several sources including the colostrums of cow's milk, blood of animals, transgenic microorganisms and finally the yolk of the laying hen. Antibodies from the colostrums of the lactating animal are only produced over a short

period of time. Antibodies from spray-dried plasma proteins are probably highly effective but currently no information has been published on the ability of this product to counteract different intestinal pathogens. Current technology for monoclonal antibody production is prohibitively expensive. They, nevertheless, have been shown to be highly effective at controlling certain diseases. The ultimate goal is to provide a libray of antibodies with nearly endless specificity and without dependence on animals or their cells for their synthesis. These latter procedures, which will involve the production of antibodies in microorganism or plant, are only being initiated and currently cannot be sue for large scale antibody production. Among all of the different possibilities, the use of chicken egg-yolk antibodies to control intestinal pathogens appears to be one of the best alternatives.

3.2 Immunoglobulin Y

Chickens protect their offspring by transferring maternal antibodies from serum to egg yolk. The properties of the 7S Ig species from avains are slightly different from those of mammalian IgG, and are called IgY (Leslie and Clem, 1969). The protein has a molecular weight of 180,000, which consists of two subunits: a heavy chain of 67,000 – 70,000 and a light chain of 22,000 – 30,000 (Leslie and Clem, 1969; Song *et al.*, 1985).

IgY is relatively heat stable without loss in antibody activity and stable at pH levels, above 4 (Shimizu *et al.*, 1988).

3.3 The advantages of egg yolk antibody

Egg yolk as an alternative source of polyclonal antibodies have several advantages over other source of antibodies such as spray dried-porcine plasma (SDPP), serum antibodies from rabbits and colostrums from immunized dams.

The quantitative advantage of raising IgY has been presented by Gottstein and Hemmeler (1985). They showed that the amount of purified IgY produced in one month from a hen is 18 times higher than that of IgG production in a rabbit. Also the egg yolk antibodies do not activate mammalian complement factors (Larsson *et al.*, 1992), which can be of a great advantage in case of assay development for components in mammalian serum samples and it does not bind to protein A (Kronvall *et al.*, 1974) or protein G (Åkerström *et al.*, 1985). Another advantage is that IgY antibodies are selectively passed to the egg yolk in large amounts and, therefore, no IgM and IgA impurities can be found in IgY preparations (Schade *et al.*, 1997). Moreover, due to the phylogenetic distance between birds and mammals (Jensenius *et al.*, 1981), chickens produce more specific antibodies against mammalian antigens than do mammals. Therefore, IgY is superior to

serum antibody for certain applications as it yields a much higher level of specific antibodies (Orlans, 1967; Ross *et al.*, 1974), it is relatively easy to purify (Akita and Nakai, 1992) and its production costs are low (Polson and Von Wechmar, 1980).

3.4 Use of egg yolk antibodies to control intestinal diseases

The application of egg yolk antibody for controlling diseases has been proven in previous studies (Yokoyama *et al.*, 1992; Marquardt *et al.*, 1999; Shin *et al.*, 2002; Owusu-Asiedu *et al.*, 2002). These authors have shown that neonatal and early-weaned piglets that received egg yolk antibodies were protected against ETEC infection. In the case of *Helicobacter pylori* infection, *H. pylori*-associated gastritis could be successfully treated by orally administered IgY-Hp antibodies (Shin *et al.*, 2002).

4. DNA vaccines

A new stage in vaccine development is nearing clinical application with several clinical trials in humans having been successful. This new vaccine is a DNA vaccine that may open the way to preventing diseases for both animals and humans including the possibility of controlling infection with the human immunodeficiency virus (HIV). This promising new technology has facilitated a powerful new approach to vaccinology as it offers a unique means of stimulating and enhancing immune responses.

Observations in the early 1990s concluded that plasmid DNA could exert a direct effect on animal cells (Tang *et al.*, 1992). This sparked exploration of the use of DNA plasmids, which are small circular rings of bacterial DNA, to induce immune responses in animals and humans. The DNA, which encodes antigenic proteins, is inserted into the cell by direct injection. In this way, the gene sequence of a desired antigen is delivered the host in a non-replicating plasmid vector. Immunization with a DNA vector has produced a strong and broad-based immune response. DNA vaccines represent a unique means of stimulating both cell-mediated and humoral immunities, which are resistance to disease organisms (Lewis and Babiuk, 1999).

4.1 Expression plasmid

DNA vaccines consist of the foreign gene of interest cloned into a bacterial plasmid. The plasmid DNA is engineered for optimal expression in eukaroytic cells. Requisites include (a) an origin of replication allowing for growth in bacteria (it provides large copy numbers in bacteria with high yields on purification); (b) a bacterial antibiotic resistance gene (this allows for plasmid selection during bacterial culture; the ampicillin resistance, the most common resistance gene used for studies in mice, is precluded for use in humans, and kanamycin is often used); (c) a strong promoter for optimal

expression in mammalian cells (for this, virally derived promoters such as from cytomegalovirus (CMV) or simian virus 40 provide the greatest gene expression); and (d) stabilization of mRNA transcripts, achieved by incorporation of polyadenylation sequences such as bovine growth hormone (BGH) or simian virus 40.

4.2 Advantages of DNA vaccine

DNA vaccines have a number of advantages over more classical vaccines (Table 1). DNA vaccines mimic live attenuated vaccines and live recombinant proteins by producing the immunizing material in the host. However, DNA vaccines are unlike live viral vaccines in that they do not cause infections (Lai and Bennett. 1998; Weiner and Kennedy, 1999). DNA vaccines are subunit vaccines in that they express only one, or a subset of proteins, from a pathogen. Another advantage of DNA vaccines is that they can result in long-term protection. The multiple inoculations of inactivated vaccines require frequent immunizations to rise and maintain immunity (Galambos and Sewell, 1995). Moreover, DNA vaccines can induce both cell-mediated and humoral immune responses, and can evoke the desired immune response to the targeting antigen by the simultaneous use of a genetic adjuvant (cytokine, CpG dinucleotide and chemokines), and the selection

Table 1. Comparison of various vaccines (Weiner and Kennedy, 1999).

Vaccine approach	Helper T-cell				Strengths	Weakness
	Th1	Th2	CTL	Ab		
Live attenuated	?	?	+	+	Cellular immunity Humoral immunity	Safety
Whole inactivated		+	–	+	Easy to prepare Low cost	Limited immunization
Virus like particles		+	–/+	+		Limited immunization Difficult to preparation
Protein subunit/peptides		+	–/+	+	Humoral immunity	Poor cell immunity Difficult to preparation High cost
Plasmid DNA	+	+	+	+	Cellular immunity Humoral immunity Easy to preparation Low cost	Limited experience

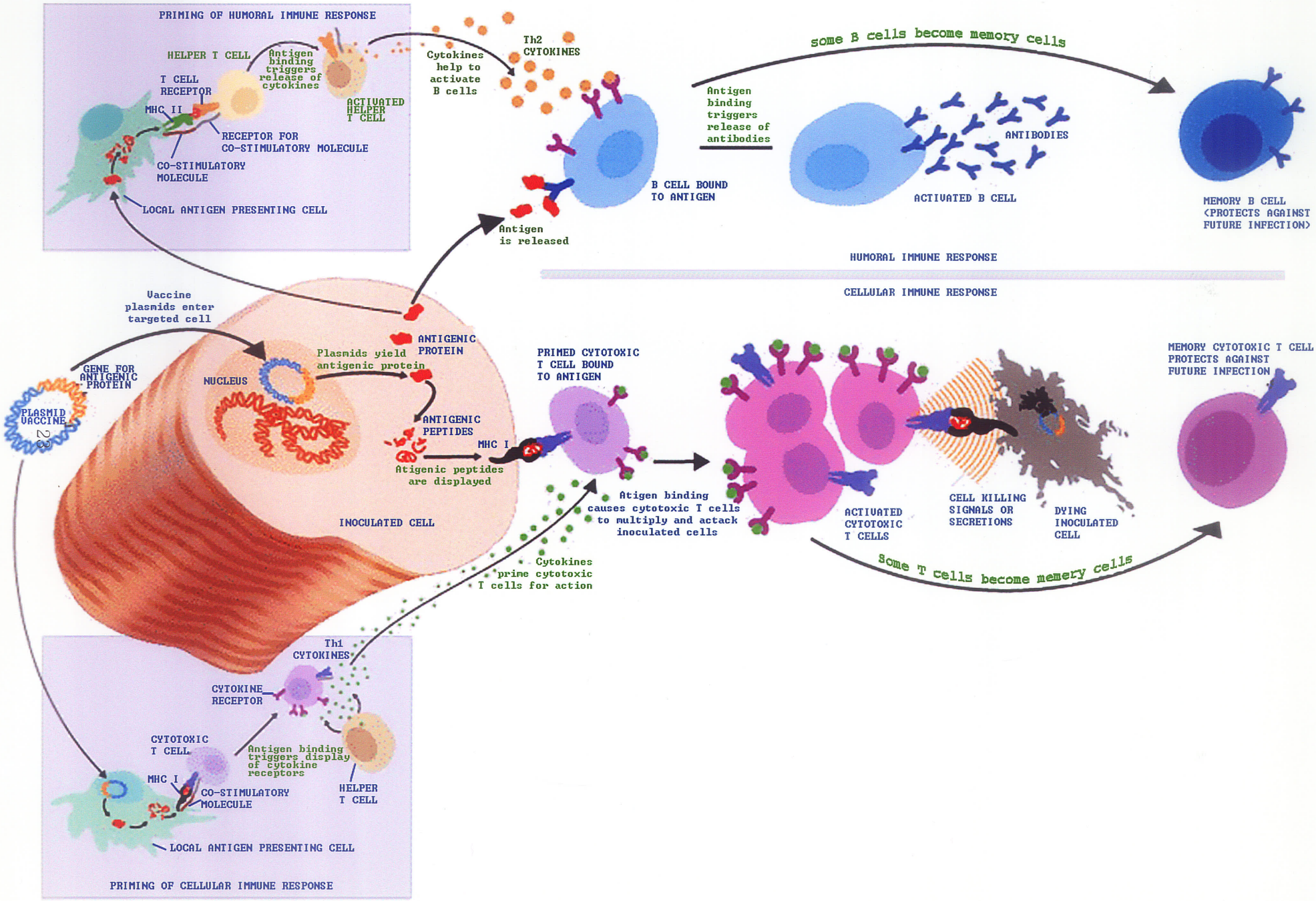
of vectors and delivery methods (Weiner and Kennedy, 1999; Gurunathan *et al.*, 2000). Furthermore, DNA vaccines may be applied to the immunization of infants, if maternal antibodies do not interfere (Lai and Bennett. 1998). DNA vaccines are also relatively inexpensive to produce, are heat-stable and can be handled and stored under conditions that would destroy recombinant proteins. In addition, multiple vectors encoding several antigens can be delivered in a single administration and DNA vaccines can induce major histocompatibility complex (MHC) class I-restricted CD8+ T-cell responses and MHC II-restricted CD4+ Th2-cell responses (Chattergoon *et al.*, 1997).

4.3 Mechanism of antigen presentation

DNA vaccines elicit both cellular and humoral immune responses by activating two different functions of the immune system (Fig. 1) (Weiner and Kennedy, 1999). DNA plasmids when injected into muscle begin to yield copies of antigenic proteins or to release antigenic proteins after uptake by antigen-presenting cells (APCs).

There are two ways to send information from antigenic proteins in humoral response. In the first, antigenic proteins that are released from vaccinated DNA plasmids directly bind to the B-cells stimulating them to secrete antibody molecules after an analysis of the antigenic protein's characteristics or to stimulate other offspring of the B-

Fig 1. Action of DNA vaccine. Illustrating the two different immune responses against a DNA vaccine; the humoral immune response and cell-mediated immune response (Weiner and Kennedy, 1999).


PRIMING OF HUMORAL IMMUNE RESPONSE
HELPER T CELL
Antigen binding triggers release of cytokines
T CELL RECEPTOR
ACTIVATED HELPER T CELL
MHC II
RECEPTOR FOR CO-STIMULATORY MOLECULE
CO-STIMULATORY MOLECULE
LOCAL ANTIGEN PRESENTING CELL
Cytokines help to activate B cells
Th2 CYTOKINES
Antigen is released
B CELL BOUND TO ANTIGEN
Antigen binding triggers release of antibodies
ANTIBODIES
ACTIVATED B CELL
some B cells become memory cells
MEMORY B CELL (PROTECTS AGAINST FUTURE INFECTION)
HUMORAL IMMUNE RESPONSE
CELLULAR IMMUNE RESPONSE
Vaccine plasmids enter targeted cell
GENE FOR ANTIGENIC PROTEIN
PLASMID VACCINE
NUCLEUS
Plasmids yield antigenic protein
ANTIGENIC PROTEIN
ANTIGENIC PEPTIDES
MHC I
Atigenic peptides are displayed
INOCULATED CELL
PRIMED CYTOTOXIC T CELL BOUND TO ANTIGEN
Atigen binding causes cytotoxic T cells to multiply and actack inoculated cells
ACTIVATED CYTOTOXIC T CELLS
CELL KILLING SIGNALS OR SECRETIONS
DYING INOCULATED CELL
MEMORY CYTOTOXIC T CELL PROTECTS AGAINST FUTURE INFECTION
Some T cells become memery cells
Cytokines prime cytotoxic T cells for action
Th1 CYTOKINES
CYTOKINE RECEPTOR
CYTOTOXIC T CELL
Antigen binding triggers display of cytokine receptors
MHC I
CO-STIMULATORY MOLECULE
HELPER T CELL
LOCAL ANTIGEN PRESENTING CELL
PRIMING OF CELLULAR IMMUNE RESPONSE

cells to become memory cells that will quell the pathogen if it circulates outside cells or is involved in future infections. A secondary method of sending information is the use of APCs to ingest antigen molecules, partially digest them and display the resulting peptides on MHC class II molecules. The T helper cells, when they receive the information from the peptides, will trigger the release of cytokines from activated Th2-cells. These cytokines from the Th2-cells help to activate the B-cells.

In the cellular immune response, digested antigenic peptides are displayed on MHC I molecules for recognization of the information presented by the antigenic peptides. The antigenic peptides that are bound to the cytotoxic T-cells stimulate the multiplication of the cytotoxic T-cells, the killing of infected cells, and send signals toward other T-cells. Some activated T-cells will also become memory cells, ready to kill cells infected by the pathogen in the future. The MHC I molecules are also involved in the cellular immune response when DNA plasmids are directly taken up by local APCs. The encoded antigens, after ingestion of the plasmids by professional APCs, are synthesized and the antigen fragments are presented on MHC class I molecules. The cytotoxic T cells must recognize these signals and also be stimualted by Th1 type cytokines from Th1-cells.

5. Cytokines

Cytokines are peptides or glycopeptides, secreted by host cells, which influence the behavior of other host cells or of the cells that produced them. Typically, cytokines are produced and secreted in low amounts, and act mostly at short range. Most cytokines can be produced by various cell types, although a particular cell type may represent the major source of a given cytokine. Monocytes/macrophages, B cells and T lymphocytes, particularly T helper cells, are the major cytokine-producing cells.

Some of the cytokines are called interleukins to indicate that they mediate communications among leukocytes. However, many of the interleukins (IL-1, IL-12, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, and GM-CSF) also affect other cell types. They sometimes have very different functions but mostly work together and support each other.

Molecular biology has provided a range of pure recombinant cytokines, which have been tested as modulators of specific immune responses. The functions of interleukins have been extensively studied in mammals (Table 2), and some of these have been used in combination with DNA vaccines as adjuvants (Chow *et al.*, 1998; Wortham *et al.*, 1998; Scheerlinck *et al.*, 2001).

Table 2. Source and function of various cytokines.

Cytokines	Source	Functions
IL-1	macrophages, epithelial cells	T cell and macrophage activation, B-cell growth
IL-12	macrophages, B-cells	activates NK and T-cells, induces Th1-like CD4 T-cells
IL-2	activated T-cells	stimulates differentiation and proliferation of T-cells promotes proliferation and Ig secretion by B-cells
IL-4	T and B-cells, macrophages	Induces T and B-cells proliferation, induces Ig class switching in B-cells to IgE and IgG1, upregulates MHC II on B-cells and macrophages
IL-6	T-cells, monocytes, macrophages	Induces differentiation and proliferation of T-cells and B-cells
IL-10	T-cells, macrophages	Suppress macrophages, Th1 lymphocyte function
GM-CSF	T-cells, B-cells	Promotes the proliferation, maturation

5.1 Cytokines as a genetic adjuvant

The potential advantages gained by using novel vaccine adjuvant are numerous. These include enhancement of the immunogenicity of weaker immunogens, such as highly purified or recombinant antigens; reduction of the amount of antigen or the frequency of booster immunizations needed to provide adequate protection; and improvement of efficacy of vaccines used in newborns, the aged, and in immuno-compromised individuals.

Cytokines as an adjuvant also can promote T or B-cell proliferation and cell-mediated immunity. They can stimulate not only MHC class I restricted CD8 cytotoxic T lymphocyte (CTL) responses but humoral immune responses by MHC class II when used with vaccine antigens that cannot achieve this alone or when formulated with alum.

5.2 Cytokines in clinical trials in mammalians

IL-2 is synthesized and secreted primarily by activated T-lymphocytes, particularly CD4+ Th-cells. It acts as helper (growth) factors in B, T-cells and NK cells, and plays a critical role in the immune system. In addition, IL-2 enhances cytotoxicity by NK cells, giving rise to lymphokine-activated killer cells that may be cytotoxic to some cancer cells, and stimulates antibody synthesis by B-cells.

IL-2 is widely used as an immuno-adjuvant to elicit a strong cellular immune response. Co-injection of IL-2 has been shown to enhance humoral and cellular immune responses to an exogenous protein including a significant enhancement of the activity of Th 1-cells, an increased production of immunoglobulin (Ig) G2a antibody, as well as a marked inhibition of Th 2-cells and decreased production of IgG1 antibody (Chow *et al.*, 1998). A high immunoglobulin G1 (IgG1) antibody response was stimulated by the injection of a vector that contained both the IL-2 gene and a foreign gene (Wortham *et al.*, 1998). They found that the adjuvant effect of cytokine-expressing vectors was dependent upon co-injection with a plasmid encoding the foreign gene, as injection of the two plasmids separately had no effect on the magnitude of specific immune responses. This indicates that co-localization of cytokines and antigens at the site of the immune interaction was important for the observed cytokine adjuvant effect (Chow *et al.*, 1998)

IL-4 is an apleiotropic cytokine produced mostly by some Th-cells, but also by mast cells and basophils. Its effects on different cell types include the induction of B-cell proliferation, induction of Ig class switching in B-cells to IgE and IgG1 production, up-regulation of cell-surface molecules including MHC class II on B-cells and macrophages leading to enhanced antigen-presenting capacity by those cells, and inhibition of

macrophage-mediated antibody-dependent cellular cytotoxicity. IL-4 plays a critical role in the priming of Th 2-cells; as a result, IL-4 is important for the production of specific antibodies from B-cells.

Muscle injection with a cDNA encoding IL-4 selectively increased IgG1 levels but did not alter the cellular immune response (Raz *et al.*, 1993). Moreover, co-injection of the IL-4 gene significantly enhanced the development of specific Th 2-cells and increased production of IgG1 antibodies, whereas Th 1 differentiation and IgG2a production were suppressed (Chow *et al.*, 1998).

IL-6 is a critical factor in end-stage differentiation of B-cells into IgA-secreting plasma cells. It is vital for maintenance of mucosal IgA responses. IL-6 also stimulates early proliferation of mucosal T-cells or augments B-cell growth and/or immunoglobulin production. Co-administration of DNA encoding IL-6 enhanced the protective immune responses in mice, and induced specific serum IgG and IgA production (Larsen *et al.*, 1998). A significantly (10 to 50 fold) higher specific antibody titer was obtained in mice when one plasmid carrying both the IL-6 gene and a foreign gene was injected compared to that in mice injected with a plasmid expressing only foreign protein (Steidler *et al.*, 1998). These cytokines are also involved in the regulation of isotype switching by B-cells

(Kaiser, 1996).

Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) has a variety of effects on immune responses. It induces MHC class II antigen expression on the surface of macrophages, and enhances dendtritic cell (APCs) maturation and migration. It results in a localized inflammation at the injection site. Animal and human studies suggest that administration of GM-CSF can increase antibody titers to foreign antigens (Weiss *et al.*, 1998; Wortham *et al.*, 1998). Co-injection of a new plasmid PyCSF1012 with a plasmid encoding murine GM-CSF increased protection against malaria (increased to 58%) as it increased antibodies to PyCSP of the IgG1, IgG2a, and IgG2b isotypes, but not IgG3 or IgM (Weiss *et al.*, 1998). The GM-CSF fusion, which co-expressed the foreign antigen and the cytokine, stimulated predominantly IgG1 antibody production while the level of IgG2a was extremely low (Wortham *et al.*, 1998). These results suggest that the GM-CSF fusion stimulated a predominant Th 2-dominated response.

5.3 Chicken cytokines

Many of the chicken cytokine studies are based on studies in the mammalian system as comparatively little is currently known about avian cytokines. Based on their functional activities, several avian cytokines with activities homologous to those of

mammalian cytokines have been described (Kaiser, 1996; Schneider *et al.*, 2001). Although some of these have been partially purified, very few have been characterized at the molecular level (IL-2, IL-6, IL-8, and IL-10) (Kaiser and Mariani, 1999; Schneider *et al.*, 2001; Kaiser *et al.*, 1999; Sunyer *et al.*, 1996).

The main problems in cloning the gene encoding for avian cytokines include: (1) recombinant mammalian cytokines have little or no effect in avian bioassays because there is only few of recombinant cytokine which was cloned; (2) there is little or no cross-reactivity of antibodies to mammalian cytokines in the avian system; and (3) there is a paucity of specific avian bioassays for cytokine activity. However, even though cytokines from avian (chickens) and mammals have a low degree of identity between genes, cytokines show surprisingly little species specificity.

The application of delivery methods for the effective induction of humoral immunity in chickens will be facilitated by combining current knowledge on the mechanism for the function of cytokines with techniques for the use of recombinant chicken cytokines and naked DNA constructs. Such an approach will enhance specific egg yolk antibody production, thereby providing specific protection against diseases.

HYPOTHESES

The following hypotheses are made;

1. A DNA vaccine (plasmid DNA) encoding either K88 *fae*G or K99 *fan*C fimbrial genes from enterotoxigenic *E.coli* will elicit a humoral immune response in chickens in a manner similar to that previously shown for mice.

2. The simultaneous injection of two different types of vaccines (a DNA and a protein), are capable of eliciting the same anti-fimbrial antibody response, will synergistically enhance the level of antibody produced by either when injected alone.

3. A DNA vaccine encoding both the fimbrial gene (*fae*G) and a cytokine gene (IL-2 or IL-4) in a plasmid will express both the K88 fimbriae and the cytokine (IL-2 or IL-4) in COS-M6 cells.

4. The same DNA vaccines as outlined in three will stimulate the production of specific anti-fimbrial antibodies when injected into mice. The antibody titer will be greater and longer lasting than that achieved with the DNA vaccine encoding only the fimbrial gene.

5. Immunoglobulin isotypes and levels of cytokines in the serum of injected mice will change according the type of vaccine and the kind of adjuvants used.

6. A DNA vaccine encoding the K88 *fae*G and a newly sequenced chicken cytokine (chIL-6) gene will stimulate the secretion of both the K88 fimbrial protein and IL-6 protein in COS-M6 cells and the production of an enhanced level of anti-K88 antibodies in the yolk of eggs of immunized hens compared to that achieved by a DNA vaccine containing only the K88 *fae*G gene.

OBJECTIVES

The overall objectives of this thesis will be to test the hypotheses as outlined above. The detailed objectives are as follows;

(1) to demonstrate that DNA vaccines can be used to produce high amounts of specific antibodies in the yolk of eggs over a relatively long period of time using two routes of injection, intramuscular (i.m.) or intradermal (i.d.).

(2) to determine if a combined protein and DNA vaccination would further enhance the production of a high and sustained amount of antibody in the yolk of the egg.

(3) to demonstrate that a plasmid containing either the K88 *fae*G gene and the IL-2 gene or the K88 *fae*G gene and the IL-4 gene will expresses both K88 fimbriae and cytokine in COS cells while plasmids containing either the K88 *fae*G gene, the IL-2 gene or the IL-4 gene also will only express each protein separately.

(4) to test the possibility that a genetic adjuvant can be used to enhance the production of specific antibodies against ETEC (enterotoxigenic *Escherichia coli*) K88 fimbriae in mice after co-injection with the plasmid cDNA(s). This study is to compare the effects of injecting plasmids capable of expressing either IL-2 or IL-4, and K88 fimbriae protein on

the induction of humoral immune response in mice as measured by the level of IgG1 and IgG2a anti-K88 antibody titer.

(5) to prepare genetic constructs in *E.coli* that have the ability to expresses both the K88 fimbriae and IL-6 proteins in COS cells.

(7) to compare the effects of two different DNA vaccines (a DNA vaccine and a DNA vaccine coupled to a genetic adjuvant) on the ability of the chicken to produce anti-K88 antibodies in the yolk of the egg. The ability of these vaccines to stimulate antibody production is also to be compared with that obtained by the use of a purified fimbrial antigen (K88 fimbriae) that is to be isolated from a wild strain of *E.coli* K88.

(8) to characterize the expressed proteins from the constructed plasmid using an ELISA test and an immunoblotting assay.

MANUSCRIPT I

Production of an enhanced level of chicken egg-yolk antibodies against enterotoxigenic *Escherichia coli* by the combined use of a protein and a recombinant DNA vaccine

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ABSTRACT

Induction of humoral immune responses by administration of a vaccine consisting of naked plasmid DNA has been an important strategy for vaccine development against several pathogens. The objectives of this study were to demonstrate that DNA vaccines that have been encoded with K88 *fae*G or K99 *fan*C genes from enterotoxigenic *Escherichia coli* can produce high humoral antibody responses in the egg yolk of laying hens and in the serum of mice. Mice and laying hens were injected in three different ways, DNA alone, protein alone, and DNA plus protein, and the antibody titers in serum and egg yolk were compared. The DNA plus the protein injection greatly enhanced antibody titer in both mice and chickens compared to that obtained with DNA or protein alone. Also the antibody titer of DNA plus a protein injection after 24 wk was nearly equal to the maximum values obtained after 8 wk with either alone. The data demonstrate that DNA vaccine can elicit a strong and long-lasting immune response in both chickens and mice; furthermore, the DNA plasmid combined with a protein injection has an ability to enhance the production of antigen-specific antibody.

INTRODUCTION

The fimbrial antigens of enterotoxigenic *Escherichia coli* (ETEC) that are associated with intestinal colonization have been extensively investigated with respect to their genetic, chemical and immunological properties. Traditional protein vaccines have been widely used with promising results in the control of colibacillosis in animals (Gyles and Mass, 1987; Husband and Serman, 1979; Isaacson, 1994). In passive immunization experiments, chicken egg yolk antibodies have been shown to be highly effective at protecting neonatal animals against fatal enteric colibacillosis (Marquardt, 1999; Yokoyama *et al.*, 1992). These antibodies have proven to be as effective as other treatments such as antibiotics or spray dried animal plasma (Marquardt *et al.*, 1999). Currently, egg yolk antibodies have been only produced using a protein antigen (mostly fimbrial antigen) with the adjuvant invariably being Freund's complete or incomplete adjuvant (FCA or FIA). Some of the disadvantages of using fimbrial protein vaccinations are that the antigen is expensive to isolate from the wild type organism, several booster shots are required in order to maintain a high antibody titer in the egg, and Freund's adjuvants must be used if high titers are to be obtained. However, it is undesirable to use

Freund's adjuvant as it not only produces undesirable side effects in the animal but it is also relative expensive to use.

Recently, administration of DNA plasmids has proven to be an effective means of antigen expression in vivo for the generation of both long-lasting humoral and cellular immune responses (Casey *et al.*, 1998; Gurunathan *et al.*, 2000). Several researchers (Alves *et al.*, 1998a; Alves *et al.*, 1998b; Alves *et al.*, 1999a; Alves *et al.*, 1999b; Lasaro *et al.*, 1999; Turnes *et al.*, 1999) have demonstrated that a DNA vaccine is able to elicit an antibody response over a period of up to one year. DNA vaccines, therefore, have the potential of providing an attractive alternative to protein vaccines for antibody production especially in the egg of the laying hens. It is conceivable that they could also be used in conjugation with protein vaccines to stimulate a high and sustained level of antibody production resulting in a reduced need for several booster injections with the protein antigen.

An objective of this study was to demonstrate if DNA vaccines can be used to produce high amounts of specific antibodies in the yolk of eggs over a relatively long period of time using two routes of injection, intramuscular (i.m.) or intradermal (i.d.). A second objective was to determine if a combined protein and DNA vaccination would

further enhance the production of a high and sustained amount of antibody in the yolk of the egg. This is important as the curative property of the antibody is directly related to total amount of antibody present. It is hypothesized that the initially high concentration of antigen in the protein vaccine would stimulate a large initial immune response while the continuous release of the antigen from the DNA would stimulate the immune system to continuously maintain a high content of the specific antibody. It is intended that in future studies these antibodies will be fed to young pigs to control colibacillosis in a manner similar to that obtained with antibodies produced from protein vaccines alone (Marquardt, 1999; Yokoyama *et al.*, 1992). Initial studies were carried out with a mouse model so as to ensure that the DNA vaccines would elicit antibody responses similar to those obtained by other researchers (Alves *et al.*, 1998a).

MATERIALS AND METHODS

Bacterial strains and growth conditions. Plasmid *p*I294, having the K88 fimbrial gene (*fae*G) (Mooi *et al.*, 1984; Turnes *et al.*, 1999) at the *Hind* III site of *p*BR322, and plasmid *p*I297, having the K99 fimbrial gene (*fan*C) (Lee and Isaacson 1995; Ogunniyi *et al.*, 2002; Roosendaal *et al.*, 1987) at the *Bam* HI site of *p*BR322

vector (Isaacson, 1985), were provided by Dr. R.E. Isaacson (University of Illinois, Urbana, IL, USA). *E.coli* NM522 *sup*E *thi* Δ (*lac-proAB*) *hsd-5*[F' *proAB lacI* ^{q} *lacZ* Δ *15*] was used for the transformation (Mead *et al.*, 1985). Transformed bacteria were grown at 37°C overnight in Luria broth or Luria agar (Difco, USA) with ampicillin (100 μg/ml) for the transformation experiments and for isolation of plasmid DNA.

Plasmid construction. The plasmid *p*SLIATPs, carrying the human cytomegalovirus (HCMV) immediate-early promoter with an intron (intron *a*) was provided by Dr. LA Babiuk (University of Saskatchewan, Saskatoon, Canada). The vector included a multiple cloning site for insertion of coding sequences, as described below, downstream of the human cytomegalovirus intermediate early promoter (HCMV) (Braun *et al.*, 1997).

The fimbrial gene, encoding the fimbriae protein, was amplified by the polymerase chain reaction (PCR) using as template the DNA of the ETEC plasmids *p*I294 and *p*I297 and two pairs of synthetic oligonucleotide primers containing restriction sites for *Nhe* I and *Bam* HI. The K88 sense primer is 5'AGGGGTTTATGCTAGCAAA AAGACT 3', and anti-sense of K88 primer is 5' GGATCCGGATCCTTAGTAATAAGT 3'. The K99 sense primer is 5' CTACTAGCT AGCATCTTAGGT 3', and anti-sense of

K99 primer is 5' CGCAATGTAGGATCCTTTAAATA 3'). DNA sequences of the fimbrial genes K88 and K99 from *E.coli* were obtained from the GenBank (www.ncbi.nlm.nih.gov/entrez) for primer design. PCR was performed following standard procedures (Sambrook *et al.*, 1989) in a thermocycler with the following program; 30 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min, and an extension step at 72°C for 5 min at the end of the cycle. The product of the PCR reaction was isolated on a 0.8% agarose gel (Sambrook *et al.*, 1989) and recovered with glass beads (Bio 101. Inc., Carlsbad, CA). Following digestion with *Nhe* I and *Bam*H I nucleases, the fragments were inserted with *p*SLIATPs also cut with *Nhe* I and *Bam*H I. These plasmids, named *p*UM8 and *p*UM9, containing the genes that express the K88 and K99 fimbrial antigens, respectively, were used to transform competent *E.coli* NM522 (Mead *et al.*, 1985). The transformed cells were cultured on Luria agar-plates containing ampicillin, and selected colonies of *E.coli* containing the *p*UM8 and *p*UM9 were cultured for 18 h at 37 °C in 5 mL of Luria broth with ampicillin. The plasmid DNA was purified (Sambrook *et al.*, 1989), and the concentration of plasmid DNA was determined at 260 nm. Before injection, the plasmid was precipitated in ethanol, washed with 70% (vol/vol) ethanol, and dissolved in sterilized saline.

Purification of fimbriae. *E.coli* K88ac and K99 were cultured in 1L of Luria broth as described previously (Erickson *et al.*, 1992; Fang *et al.*, 2000). Cultures were centrifuged at 4°C and 3000 *g* for 15 min, and the pellets were resuspended in 5 mL of PBS (phosphate buffered saline, pH 7.2). The precipitates were heated to 60°C in a water bath for 30 min to release the fimbriae from the bacteria and, while still hot, were blended for 10 min at high speed using an IKA-ULTRA-TURRAX T25 homogenizer (Jacke & Kunkel GmbH & Co IKA Labortechnik, Staufen, Germany). The insoluble bacterial residue was removed by centrifugation (14,000 x *g*, 4°C, 15 min), followed by filtration through a 0.45 μm membrane filter (Millipore Corp, Milford, MA 01757, USA). Citric acid (2.5%) was added to the supernatant to a pH of 4.0 and the fimbriae were allowed to precipitate at 4°C for 2 h, after which the precipitated fimbriae were collected by centrifugation (14,000 x *g*, 4°C, 15 min). The precipitated fimbriae were resuspended and dissolved in 5 ml of PBS followed by precipitation with 2.5% citric acid to pH 4.0. The pH 4.0 precipitation and pH 7.2 solublization steps were repeated three times. The final pellet was dissolved in 0.1 *M* PBS (pH 7.2) and store at –20°C at a concentration of about 1.0 mg/mL fimbriae protein.

Immunization of mice. The dosages selected for mice when injected with fimbrial protein and the corresponding plasmid that expresses its gene were based on the results of previous researchers (Alves *et al.*, 1998a; Alves *et al.*, 1999a; Lewis and Babiuk, 1999). Doses in the range of 100 μg DNA/mouse have been routinely used. A total of 50 CD-1 female mice (4 to 6 wk old) were purchased from the University of Manitoba. Forty of the mice were randomly assigned to eight groups, each containing five mice. The treatments were: i.m. and i.d. injections of DNA alone (four groups; two for K88 and two for K99), i.m. injections of protein alone (two groups; one for K88 and one for K99), and i.m. injections of DNA plus protein (two groups; one for K88 and one for K99). The mice in each DNA group were injected i.m. or i.d. at one site at 2 wk intervals with 100 μg of plasmid DNA dissolved in 100 μL of PBS (pH 7.2) using a 27 gauge needle. This procedure was similar to that used by Lasaro *et al.* (1999). For protein immunization, each mouse was injected i.m. twice at 2 wk intervals at two sites. Each mouse at each time period was injected with 100 μg of purified fimbrial protein in 50 μL of PBS emulsified in an equal volume of Freund's complete adjuvant (first injection, Sigma). The second boost of the fimbrial protein (100 μg) was mixed with Freund's incomplete adjuvant (second injection, Sigma). For mixed immunization, the mice were

injected with both plasmid DNA in PBS and fimbrial protein emulsified in Freund's complete adjuvant or incomplete adjuvant each at the same time. The dosages were the same as for DNA or protein when administered alone. In addition, there were two control groups of two mice each to serve as negative controls. One group was injected with 100 μg of *p*SLIATPs (vector plasmid) and one with only 100 μL of 0.85% saline per mouse. Blood (300 μL) was collected from the tail at 2 wk intervals after immunization and immediately incubated at 4°C for 2 h and centrifuged to obtain serum. Serum was stored at -20°C until assayed for antibody titer.

Immunization of laying hens. A total of four experiments were conducted using Single Comb White Leghorn laying hens. Exp. 1 to 3 involved the injection of different amounts of several fimbrial proteins while Exp. 4 involved the administration of different combinations of two DNA vaccines and their corresponding fimbrial proteins.

In Exp. 1 to 3, a total of 40, 60 and 160 hens were randomly assigned to two, three and eight treatment groups, respectively, with each treatment group consisting of two replicates of 10 hens. In Exp. 4, a total of 80 hens were assigned to 10 treatment groups with eight replicates (eight hens per group). Four of the treatment groups received the K88 vaccine which was administered i.m. or i.d. as a DNA vaccine, i.m. as fimbrial

protein alone, or i.m. as DNA plus protein (i.m.) in a manner similar to that outlined for the mouse experiment. The same four treatments were replicated with a second fimbrial type, K88 (DNA, i.m.; DNA. i.d.; fimbrial protein alone, i.m., and DNA plus fimbrial protein, i.m.). Controls included two groups of eight chickens each. They were injected i.d. with the basic expression vector alone (eight chickens) and 0.85% saline alone (eight chickens). A detailed description for each experiment including of the number of replicates, hens per replicate, time and amount of antigen injected and time of collection of eggs is outlined in Table 3. Chickens in Exp. 1 to 4 that were immunized with the protein vaccine were injected at four sites with the fimbrial protein in 0.5 mL PBS (pH 7.2) emulsified in an equal volume of Freund's complete adjuvant (0 d). They were then injected one or two times at 2 wk intervals after the initial immunization with one-half the amount of fimbrial protein used in the first injection. The proteins for the second and/or third injections were dissolved in 0.5 mL PBS and emulsified in the equal volume of Freund's incomplete adjuvant. DNA (100 μg/chicken/time period) was administered at two sites in 1ml of saline using a 27 gauge needle. The injection protocol for the DNA plus protein immunization was a combination of the two individual protocols.

Table 3. Injection schedule of Single Comb White Leghorn laying hens.

Exp.	Trt No.	No of replicate (hens /replicate)	Type of antigen[a]	Specific fimbrial type	Injection wk (age of laying hens)[b]			Antigen injected (ug/ml)[c]			Wk after injection (hen age)[b]
					1st	2nd	3rd	1st	2nd	3rd	
1	1.1	2(10)	protein	K88	0(20)	2(22)	4(24)	500	250	250	5(25)[d]
	1.2	2(10)	protein	K88	0(20)	2(22)	4(24)	1000	500	500	5(25)[d]
2	2.1	2(10)	protein	K88	0(20)	2(22)	4(24)	500	250	250	5(25)[d]
		2(10)	protein	K99	0(20)	2(22)	4(24)	500	250	250	5(25)[d]
		2(10)	protein	987P	0(20)	2(22)	4(24)	500	250	250	5(25)[d]
	2.2	2(10)	protein	K88	0(20)	2(22)	4(24)	250	125	125	5(25)[d]
		2(10)	protein	K99	0(20)	2(22)	4(24)	250	125	125	5(25)[d]
		2(10)	protein	987P	0(20)	2(22)	4(24)	250	125	125	5(25)[d]
	2.3	2(10)	protein	K88	0(20)	2(22)	4(24)	1000	500	500	5(25)[d]
		2(10)	protein	K99	0(20)	2(22)	4(24)	1000	500	500	5(25)[d]
		2(10)	protein	987P	0(20)	2(22)	4(24)	1000	500	500	5(25)[d]
3	3.1	2(10)	protein	K88	0(30)	2(32)	4(34)	500	250	-	5(25)[d]
	3.2	2(10)	protein	K88	0(30)	2(32)	4(34)	300	150	-	5(25)[d]
	3.3	2(10)	protein	K88	0(30)	2(32)	4(34)	200	100	-	5(25)[d]
	3.4	2(10)	protein	K88	0(30)	2(32)	4(34)	100	50	-	5(25)[d]
4	4.1	8(1)	protein	K88	0(20)	2(22)	-	500	250	-	0(20) - 40(60)
	4.2	8(1)	DNA(i.m.)	K88	0(20)	2(22)	-	100	100	-	0(20) - 40(60)
	4.3	8(1)	DNA(i.d.)	K88	0(20)	2(22)	-	100	100	-	0(20) - 40(60)
	4.4	8(1)	Protein+ DNA(i.m.)	K88	0(20)	2(22)	-	500 100	250 100	- -	0(20) - 40(60)
	4.5	8(1)	protein	K99	0(20)	2(22)	-	500	250	-	0(20) - 40(60)
	4.6	8(1)	DNA(i.m.)	K99	0(20)	2(22)	-	100	100	-	0(20) - 40(60)
	4.7	8(1)	DNA(i.d.)	K99	0(20)	2(22)	-	100	100	-	0(20) - 40(60)
	4.8	8(1)	Protein+ DNA(i.m.)	K99	0(20)	2(22)	-	500 100	250 100	- -	0(20) - 40(60)
	4.9[e]	8(1)	Vector	–	0(20)	2(22)	-	100	100	-	0(20) - 40(60)
	4.10[e]	8(1)	Saline	–	0(20)	2(22)	-		–		0(20) - 40(60)

a. Two types of vaccines were used, one being a fimbrial protein antigen [K88(F4), K99(F5) and/or 987P(F6) of ETEC] and the other a DNA vaccine capable of expressing either the K88 or K99 fimbrial gene. All antigens were injected i.m. except for two of the DNA treatment groups (Treatments 4.3 and 4.7) which were injected i.d.

b. Values indicate the time (wk) of first, second or third injections. Values in brackets represent the age of laying hen in wk at the time of each injection.

c. Each protein antigen was emulsified in an equal volume (0.5 mL) of Freunds complete (first injection) or incomplete (second and third injections) adjuvant prior to injections. DNA vaccines were dissolved in saline (1 mL) prior to injection.

d. Eggs were collected over a 2 d period on d 6 and 7 of the 5th wk and one egg from each of 10 hens was pooled to form a replicate. There were two replicates/ treatment.

e. Treatments 4.9 and 4.10 served as controls (expression vector alone or 0.85% saline alone).

One egg per laying hen was obtained from each hen in Exp. 1 to 3 over a 2 d period at the times indicated in Table 3. Each treatment consisted of two replicates (each replicate consist of a cage of 10 hens). Over a 2 d period, 10 eggs were collected from each hen in each replicate. In these experiments, the eggs were cracked, the yolks were separated from the white, eggs from the same replicate were pooled and mixed and the mixture was freeze-dried and used for the ELISA. In Exp. 4, eggs yolks were collected from each hen and the wet yolk from each egg was used for ELISA. The freeze-dried powder from Exp. 1 to 3 was diluted 20-fold in PBS (pH7.2) while the yolks from Exp. 4 were diluted 10-fold in PBS (pH 7.2) to measure antibody titer using the ELISA. The initial concentration of yolk dry matter from all experiment, after dilution, would have been approximately the same since yolk contains approximately 50 % dry matter. Blood was collected from the wing vein (4 mL), incubated at 37°C for 1 h and centrifuged to obtain serum. Serum was stored at -20°C until analyzed. The mice and chickens were cared for in accordance with the guidelines established by the Canadian Council on Animal Care (CCAC, 1993).

Detection of antibodies by ELISA. Enzyme-linked immunosorbent assay (ELISA) with purified fimbrial antigen was used to analyse the serum of immunized mice

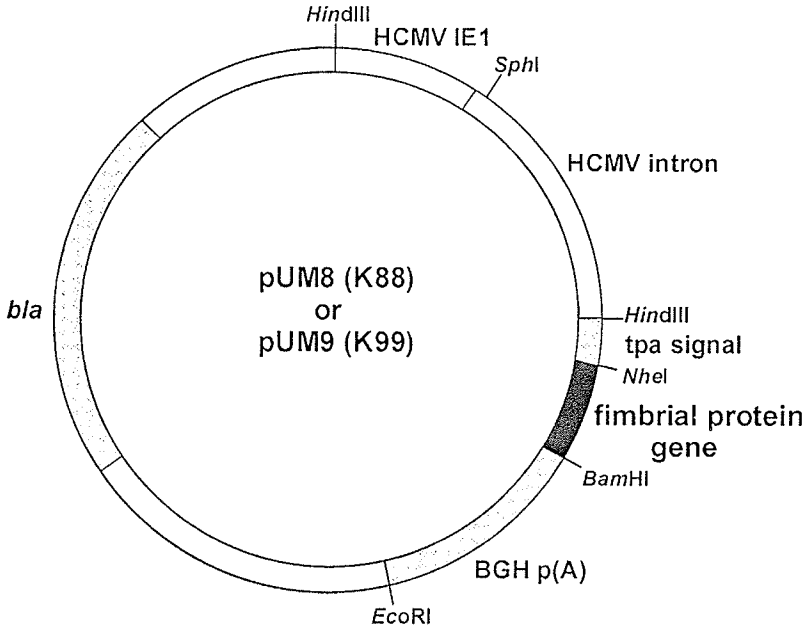
or egg yolk of immunized chicken. The procedure was a modification of that reported by Kim *et al* (1999). Wells of Microtest III flexible assay plates (Falcon 3911) were coated overnight at 4°C with 0.1μg of the fimbrial antigen suspended in 100 µL of PBS. The plates were washed with pH 7.2 PBS- 0.05% Tween 20 and then blocked overnight at room temperature with 5% (wt/vol) skim milk in PBS followed by washings with PBS-T. The washed plates were inoculated with dilutions of mouse serum or egg yolk (100 µL) and kept for 2 h at 37°C. After washing the plates were incubated with 100 µL of alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Inc., diluted 1 in 5,000) or alkaline phosphatase-conjugated rabbit anti-chicken IgY (Jackson ImmunoResearch Inc., diluted 1 in 5,000), the second indicator antibodies, for 2 h at 37°C. The reaction was visualized after 40 min at 37°C using the substrate tablets (p-Nitrophenyl Phospate, Sigma N9389). The absorbances of the reactions were determined at 405 nm in a microplate reader (Bio-Rad, Model 3550, Richmond, CA, USA, 94804). The titer was the dilution of antibody required to give one-half of the maximum absorbency reading (Harlow and Land, 1998; Kim *et al.*, 1999; Marquardt *et al.*, 1999). Assays at different times were corrected using standard samples containing known K88 or K99 antibody titers.

Statistical analysis. The design in all experiments was a Completely Randomized Design (CRD) with Exp. 1 to 3 having 10 hens per replicate and 2 replicates per treatment (10 eggs from the 10 hens were pooled for analysis). In Exp.4, the replicates consist of individual hens housed separately and eggs from these hens were analyzed separately. Data from each collection period were analyzed separately using the GLM procedure (SAS/STAT, 1989), with the significance of the differences ($P<0.05$) between the means of antibody titer being determined using the Student *t*-test at different time intervals (Fig. 3 and 4).

RESULTS

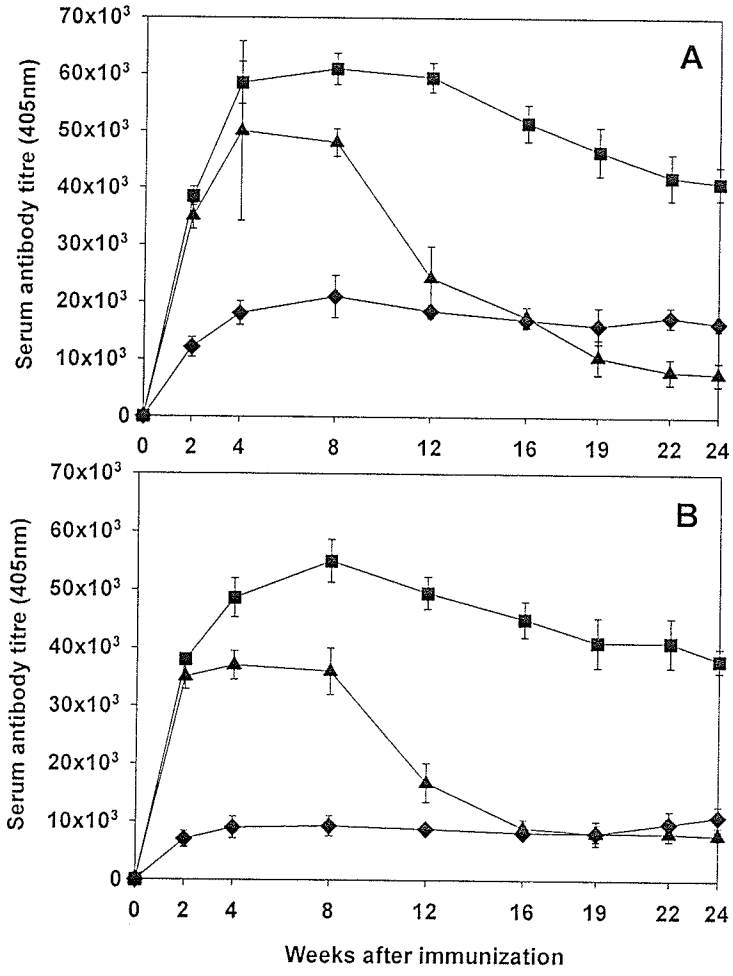
Construction of *p*UM8 and *p*UM9. The DNA vaccines were constructed by creating plasmids that would express K88 or K99 fimbriae proteins in transfected eucaryotic cells. The *p*UM8 plasmid expressing the K88ac colonization factor antigen contains a fragment of approximately 0.85kb between *Nhe* I and *Bam*H I sites. The plasmid *p*UM9 contains a fragment of about 0.55kb from *E.coli* strain K99 at the same site as *p*UM8 (Fig. 2).

FIG. 2. Schematic representation of *p*UM8 and *p*UM9 plasmid. The K88 or K99 fimbrial genes were obtained by cleavage of the PCR-generated fragment with *Nhe* I and *Bam* HI restriction endonuclease, and were then cloned with the remaining BGH p(A) encoding gene.


HindIII
HCMV IE1
SphI
HCMV intron
pUM8 (K88)
or
pUM9 (K99)
bla
HindIII
tpa signal
NheI
fimbrial protein
gene
BamHI
BGH p(A)
EcoRI

Immune responses in mice. The time course changes in anti-K88 and K99 fimbrial protein antibody titers in mice that were administered DNA plasmid alone, fimbrial protein alone or both DNA and fimbrial protein are outlined in Fig. 3. The results show that when either fimbrial protein was injected i.m. alone, there was a dramatic increase in the titer of both K88 and K99 antibodies within 4 wk of the first immunization followed by a gradual decrease in titer over the following 24 wk. This pattern of response is typical of that for mice injected with protein antigens (Harlow and Lane, 1998). Mice injected i.m. with only DNA reached a peak titer within 6 to 8 wk (increases from 0 to 20,000; $P<0.05$) and only slightly decreased ($P>0.05$) over the next 16 to 18 wk. This pattern is also typical of mice injected with different DNA vaccines (Alves *et al.*, 1998a; Braun *et al.*, 1997). The maximum titer obtained with DNA alone was about three - (K88) or four - (K99) fold less than that obtained with fimbrial protein alone. However, after 24 wk, the titers obtained with both types of vaccines were similar. Intradermal injection of the DNA vaccine yielded a 2.5-fold lower titer ($10,068 \pm 781$, $P<0.05$) than that obtained with the i.m. injection of DNA ($25,583 \pm 1,650$). Therefore, intradermal injection is not recommended. The combined injection of both DNA and protein yielded the highest titers. These values were reached within 8 wk

FIG. 3. Serum antibody responses of immunized mice as determined by ELISA. Antibodies against K88 (A) and K99 (B) fimbrial antigen were measured by the ELISA using serum of mice injected i.m. twice each at 0 and 2 wk with 100 μg aliquots of DNA (◆, DNA alone), i.m. twice each with 100 μg aliquots of emulsified fimbrial protein (▲, protein alone) or with both injections (2 × 100 μg of DNA and 2 × 100 μg of protein, ■, combination). Blood samples were collected at the indicated periods. The data for each time represent the mean of values (± SE) for each of five blood samples.


A
B
Serum antibody titre (405nm)
70x10³
60x10³
50x10³
40x10³
30x10³
20x10³
10x10³
0
0 2 4 8 12 16 19 22 24
Weeks after immunization

of injection and then decreased by less than 35 % over the following 16 wk. The titers obtained after 24 wk with the combined injections were approximately 3 to 4-fold higher ($P<0.05$) than those obtained when either K88 or K99 DNA or fimbrial protein were injected alone. Other researchers have demonstrated that the immune responses generated by DNA vaccination may be enhanced by protein injection (Gurunathan *et al.*, 2000). In one of these studies it was shown that antibody production could be substantially increased in monkeys vaccinated with DNA encoding an HIV-1 envelope protein followed by a protein boost (Letvin *et al.*, 1997). The time course change, however, was not established. In the present study, no antibodies were detected against K88 and K99 in mice prior to injection and in mice administered the plasmid without the fimbrial genes. Also there was no cross-reactivity between the K88 or K99 antigens and the corresponding K99 or K88 antibodies.

The influence of the dose of DNA on the immune response was not investigated in the current study. However, in another study (Cho *et al.*, 2003) we observed that two different dosages (40 and 100 μg) of either the protein antigen (K88 fimbriae) or the DNA vaccine (*p*UM8), administered each at two time periods, had no effect on the anti-K88 antibody titer in the serum of immunized mice over the entire 16 wk time course of

the study. Alves *et al.* (1999b) also followed the antibody response in mice immunized with different doses of a DNA vaccine encoding for *E.coli* K88 fimbriae. They reported that the long lasting IgG response (52 wk after immunization) tended to reach similar values regardless of whether the dose was 50 or 200 μg DNA or whether one or two injections were administered. The second dose of DNA at 2 wk after the initial injection resulted in a transient booster effect on the immunoglobulin levels. In the current study, a dose of 100 μg DNA was injected at zero time and after 2 wk. This should have ensured a maximal long term response.

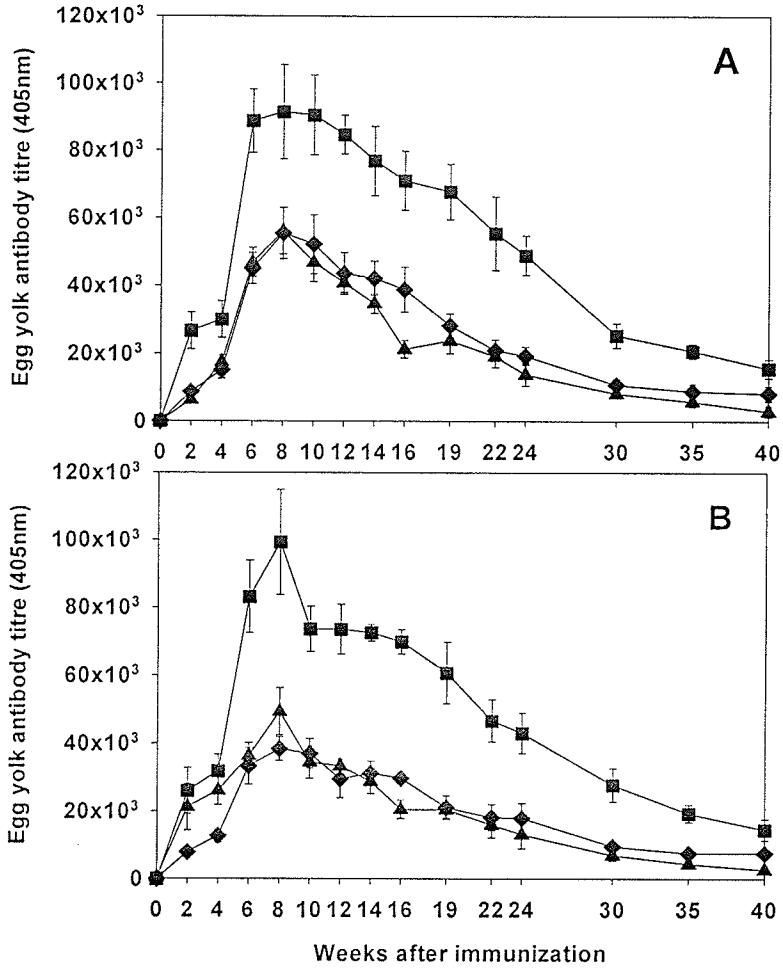
These data demonstrate that DNA immunization can induce an antibody response on mice, that antibody titer is sustained for at least 24 wk after the initial immunization and that antigen alone gives a much higher initial response than does DNA alone, which declines to lower levels 16 to 24 wk after injection. The highest and longest sustained titers were obtained when both antigen and DNA were injected. The results also suggest that there was an additive response initially up to eight wks but that the response after 12 to 24 wk was synergistic as both injections yielded considerably higher titers than either one alone. This pattern of response, as indicated above, was also similar to that obtained by other researchers with other antigens. The reason for this effect was not established

but it may be attributed to the duration and amount of antigen presented to the antigen presenting cells (APCs) with the two types of vaccines (Cohen *et al.*, 1998; Gurunathan *et al.*, 2000; Harlow and Lane, 1998). The amount of antigen delivered to the APCs initially was probably much greater when protein was injected compared to DNA. This could account for the difference in the initial antibody titers. In contrast, there would have been very little residual antigen available to the APCs cells after several wks when protein alone was injected, whereas a small amount of the fimbrial protein would have been continuously expressed by the injected DNA. The large initial injection of protein and the continued small production of antigen by the DNA vaccine would have provided conditions favorable for the continued high production of antibodies, in contrast, to that achieved by protein injection alone.

Immune responses of chicken. Three preliminary experiments were carried out to establish the optimal dose of fimbriae required to obtain maximal antibody titer. In Exp. 1, a dose of 500 and 1000 μg of K88 fimbrial antigen followed by two other injections of the antigens yielded antibody titers in the dried yolk of 33,500 ± 3182 (SE) and 30,000 ± 2828 (P=0.052). In Exp. 2, the three different antigens were injected simultaneously each at three different dose levels (250, 500 and 1500 μg). The antibody

titers ± SE in the dried yolk after the hens were initially injected with 250, 500 and 1500 μg of the antigen followed by two other injections were 18,000 ± 961, 18,000 ±1516, and 18,000 ± 1140 (P>0.05) for the K88 fimbrial protein, respectively. The average treatment values for K99 were 9,000 ± 1414 (P>0.05) and 6,000 ± 1732 (P>0.05) for the 987P fimbrial antigen. In the third study, hens were immunized with 100, 200, 300 or 500 μg of K88 fimbrial antigen in the first injection and one-half the amount in the second injection. The corresponding antibody titers ± SE were 66,000 ± 7056, 54,000 ± 3962, 53,000 ± 1378 and 59,000 ± 1483 (P>0.05), respectively. Collectively the results from the four experiments demonstrate that the anti-K88 antibody titer was not greatly influenced by the amount of antigen injected. Initial injection of between 100 and 1500 μg K88 fimbrial proteins followed by one or two subsequent booster injections at one-half the concentration essentially yielded the some antibody titers within each given experiments. Result from Exp. 1, however, suggests that at very high doses of antigen (1,500 μg/ first injection) compared to 500 μg resulted in a slight decrease in antibody titer (10%). An initial dose of 500 μg K88 or K99 fimbrial antigens was selected for Exp. 4 as this dosage, on the basis of the above results, should have been sufficient to elicit a maximal response.

FIG. 4. Egg yolk antibody response of immunized laying hens as determined by ELISA. Chicken egg yolk antibodies against K88 (A) and K99 (B) fimbrial antigen were measured by the ELISA using 10 times diluted egg yolk from chicken injected i.m. with two × 100 μg of DNA (◆, DNA alone), or injected i.m. with 2 × 500 μg and 1 × 250 μg of emulsified fimbrial proteins (▲, protein alone) or injected with 2 × 100 μg of DNA and 2 × 500 μg and 1 × 250 μg of proteins (■, combination). Injections were on day 0 and 14. Egg yolk samples were collected at the indicated periods and the egg yolks were isolated. The data for each time point represent the mean (± SE) for each of eight egg yolks.


A
B
Egg yolk antibody titre (405nm)
120x10³
100x10³
80x10³
60x10³
40x10³
20x10³
0
0 2 4 6 8 10 12 14 16 19 22 24 30 35 40
Weeks after immunization

The pattern of response of antibody production in the yolk of eggs from laying hens immunized with K88 or K99 fimbrial antigens, a DNA plasmid that can express these antigens or both the fimbrial antigens and the corresponding DNA vaccine are shown in Fig. 4 (Exp. 4). The pattern of response was similar for each antigen (K88 and K99) and to that obtained with the mouse model. These results demonstrate that the injection of the DNA vaccine in combination with the fimbrial protein enhanced specific antibody production 2 to 2.5-fold compared to that obtained when either DNA or protein was injected alone. Also the combined injection yielded a titer after 24 wk that was nearly equal to the maximum values obtained after 8 wk with either alone. The antibody titer after 40 wk was almost 10-fold higher for the combined injection compared to that obtained with the protein injection alone. At 40 wk, DNA alone also yielded antibody titers that were at least four fold greater than those obtained with protein alone. This apparent synergistic effect cannot be attributed to an effect of an initially higher concentration of the protein antigen provided by both the protein and the DNA because the preliminary studies demonstrated that an injection of three times more antigen (1,500 compared to 500 μg/ hen, Exp. 1) not only did not increase the antibody titer but actually caused it to decrease slightly. In contract and as demonstrated for mice, a combined

injection of both a DNA plus a protein vaccine were able to enhance antibody titer, particularly over a longer period of time compared to that obtained by protein alone. This may be attributed to the fact that a protein injection alone cannot sustain a high titer alone, irrespective of the amount of antigen initially injected, since its half-life in the hen is limited (Harlow and Lane, 1998). As a result, the immune system is not continuously stimulated and therefore antibody titer for the specific antigen decreases over time. However, in the presence of a DNA vaccine, a sufficient amount of the antigen is produced over a long period of time to maintain antibody titer at a level higher than that achieved by the protein or DNA vaccines alone. It is not known if there would have been a further enhancement of the synergistic effect if a higher dose of DNA had been used. No K88 or K99 antibodies were detected in pre-immune serum, or in the eggs of chicken injected with only saline or the expression vector alone.

The concentration of anti-K88 immunoglobulin in the serum of chickens 8 wk after injection with antigen only were two fold greater than that present in the yolk (263026 ± 15365 vs 91,428±14051). These data suggest that high sustained antibody titers can be produced in both the blood of laying hens and the yolk of their eggs following only two injections of both DNA and the corresponding protein. These

antibodies should therefore be highly effective at controlling ETEC pathogeneses in weaned pigs in a manner similar to that demonstrated by Marquardt *et al.* (1999) using antibodies from hen immunized only with the fimbrial antigen.

DISCUSSION

The current studies demonstrate that a DNA vaccine, when injected into a laying hen, can be used to elicit an antibody response in the yolk of an egg against two ETEC fimbrial genes that expresses the K88 and K99 fimbrial proteins and that this response can be enhanced when the protein and DNA antigens are simultaneously injected. In this study two doses of the DNA or protein were used for the production of antibodies in both mice and laying hens. Additional research must, therefore, be carried out to optimize the immune response of laying hens when immunized with a DNA vaccine. Included would be further time dose response studies on the effects of both DNA, protein and a combination of injections on antibody titers; studies on an alternate system of delivery of antigen and the modulation of the response to DNA injections using genetic adjuvants (Cohen *et al.*, 1998; Gurunathan *et al.*, 2000). A particularly promising field of study may be through the co-administrations of biological adjuvant such as cytokines. It has been

shown that DNA vaccines when used in conjunction with an appropriate genetic adjuvant can increase the production of immunoglobulin by a factor of more than sevenfold (Raz *et al.*, 1993). It is, therefore, conceivable that a DNA fimbrial vaccine for *E.coli* when combined with a genetic adjuvant such as the interleukin gene would yield sustained and much higher antibody titers than that obtained by use of a protein antigen.

The ability to readily produce large quantities of a DNA vaccine in the laying hen that is inexpensive, safe, environmentally friendly, sustainable, innocuous to the host animal and highly effective (Cohen *et al.*, 1998; Gurunathan *et al.*, 2000; Marquardt, 1999) should provide economic and social benefits to the animal feed industry. Especially important is the production of antibodies in the egg of the chicken for the therapeutic and prophylactic treatment of intestinal diseases in domestic livestock. This new technology should provide further stimulus to an infant industry which is currently involved in the use of egg yolk antibodies to not only control intestinal disease but also food borne pathogens. This is particularly important since the most widely used alternative, the use of antibiotics, may not be available in the future because of antibiotic resistance or the banning of their use in the feed industry by government agencies.

The data demonstrate that DNA vaccines against K88 and K99 fimbrial proteins can be used to elicit antibody responses in both mammalian (mice) and avian (chicken) species. They also demonstrate that DNA vaccines plus its encoded antigen, when injected into mice or chickens, have the ability to elicit the production of a much higher titer of the antibodies in the blood of mouse and blood or the egg-yolk of laying hens than that obtained by injection of either the DNA or protein alone. Chicken egg-yolk antibodies that are produced by a combined injection of both DNA and the antigen protein could provide a good, noninvasive source of antibodies for passive immunization of pigs or other animals against ETEC and other intestinal infections. They offer an attractive, environmentally friendly alternative to the use of antibiotics.

MANUSCRIPT II

Enhanced antibody responses in mice injected with a DNA vaccine that co-expresses both cytokine and *Escherichia coli* K88 fimbrial antigen

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The University of Manitoba.

ABSTRACT

Several vaccines were investigated experimentally in an enterotoxigenic *Escherichia coli* K88 model system. In this study, we show that the efficacy of a DNA vaccine can be improved by simultaneous expression of a cytokine, interleukin-4 (IL-4). Plasmid vectors encoding the K88 *fae*G antigen gene alone or fused to genes expressing IL-2 or IL-4 were constructed and compared for their potential to induce the K88 fimbriae antigen-specific immune response in mice injected intramuscularly. The K88 fimbriae protein and each of the cytokines were secreted following transfection of cells in culture with these plasmids. The plasmid expressing both K88 fimbriae and IL-4 stimulated the highest anti-K88 fimbriae antibody response, while the plasmid expressing both K88 fimbriae and IL-2 suppressed anti-K88 fimbriae antibody response. The simultaneous injection of plasmids expressing K88 fimbriae and IL-4 separately or of the single plasmid expressing only K88 fimbriae produced an intermediate response. Interestingly, the interleukins caused a shift in the type of immunoglobulin produced with IL-4 producing a greater concentration of the IgG1 isotype and IL-2 producing a greater concentration of the IgG2a isotype. The data demonstrate that DNA vaccines that can expresses both a foreign protein and a cytokine or a foreign protein alone can provide an

alternative approach to the use of conventional protein vaccines for the production of a desirable specific antibody and that they can maintain a high titer antibody response.

INTRODUCTION

Cytokines are peptides or glycopeptides, secreted by host cells, which influence the behavior of other host cells or of the cells that produced them, and cytokines act mostly at short range. Typically, cytokines are produced and secreted in low amounts. Some of the cytokines are called interleukins to indicate that they mediate communications among leukocytes. Recent studies have shown that cytokines are effective when used with either peptide vaccination or DNA immunization (Kim *et al.*, 2001; Kim *et al.*, 2000; Scheerlinck *et al.*, 2001). The use of cytokines to enhance immune responses to vaccines is an area of growing interest. Two cytokines; Interleukins 2 (IL-2) and 4 (IL-4) are able to induce T-helper (Th) 1 and Th 2 type immune responses. In particular, IL-4 effects the induction of B-cell proliferation, a switch in immunoglobulin (Ig) class in B-cells to IgE and IgG1 production, the up-regulation of cell-surface molecules including MHC (Major Histocompatibility Complex) class II on B-cells and macrophages, leading to enhanced antigen-presenting capacity by those cells, the down-regulation of the expression of some T-cell and macrophage-produced cytokines, and inhibition of macrophage-mediated antibody-dependent cellular cytotoxicity. Collectively, IL-4 plays a critical role in the priming of Th 2-cell, and is

important for the production of specific antibody from B-cells. The results demonstrate that muscle injection with a cDNA encoding IL-4 selectively increased IgG1 levels but did not alter the cellular immune response (Raz *et al.*, 1993). In addition, other results indicate that co-injection of the IL-4 gene significantly enhanced the development of specific Th 2-cells and increased production of IgG1 antibody, whereas Th1 differentiation and IgG2a production were suppressed (Chow *et al.*, 1998; Kim *et al.*, 2001; Scheerlinck *et al.*, 2000; Sin *et al.*, 1999). On the other hand, the IL-2 is widely used as a genetic adjuvant for the enhancement of cellular immune response. Interestingly, only co-delivery of IL-2, not GM-CSF (granulocyte-macrophage colony-stimulating factor) or IL-4, stimulated IgG2a antibody responses, suggesting that IL-2 evokes the cellular immune response through the Th1-dependent pathway (Chow *et al.*, 1998; Chow *et al.*, 1997; Harvill *et al.*, 1996; Steidler *et al.*, 1998). Chow et al. (1998) found that the adjuvant effect of the cytokine-expressing vectors was dependent upon co-injection with the plasmid encoding foreign gene as inoculation of the two plasmids separately had no effect on the magnitude of specific immune responses. This indicates that co-localization of cytokines and antigens at the site of immune interaction are important for the observed cytokine adjuvant effect.

This study was designed to test the possibility that a genetic adjuvant can be used to enhance the production of specific antibodies against ETEC (enterotoxigenic *Escherichia coli*) K88 fimbriae in mice after co-injection with the plasmid cDNA(s). The immunigenicity of the DNA vaccines is compared to that obtained when a protein vaccine (K88 fimbriae) is injected in the presence of a strong but undesirable adjuvant (Freund's adjuvant). Also the study compares the effects of IL-2 and IL-4 for the induction of a humoral immune response. The results demonstrate that injections of genes encoding IL-2 and IL-4 induce systemic immunological effects that are specific functions of the respective cytokine proteins.

MATERIALS AND METHODS

Mice. Six-wk-old CD-1 and BALB/c mouse, were housed in temperature-controlled, light-cycled facilities at the University of Manitoba. Their care was under the guidelines of Canadian Council on Animal Care (CCAC, 1993).

Bacteria. *Escherichia coli* NM522 *sup*E *thi* Δ (*lac-proAB*) *hsd-5*[F' *proAB lacI*^{q} *lacZ* Δ *15*] was used for plasmid preparation (Mead *et al.*, 1985). Transformed bacteria were grown at 37°C overnight in Luria broth or Luria agar (Difco, USA) with ampicillin (100

μg/mL) for the transformation experiments and for isolation of plasmid DNA.

Plasmid construction. The *p*SLIAgDs expression vector, carrying the human cytomegalovirus (HCMV) immediate-early promoter with an intron (intron *a*) was provided by Dr. LA Babiuk (University of Saskatchewan, Saskatoon, Canada). This expression vector, which was used for cloning and expression of the proteins, contains the origin of replication from *E. coli*, a gene for ampicillin resistance, and a regulated promoter (HCMV) with an intron (intron *a*) (Braun *et al.*, 1997) that appears to function in efficient transport of the mRNA out of the nucleus. Also, it has the signal sequence from gD protein of bovine herpesvirus, and there is a 3' region from bovine growth hormone present following the insertion site which will take care of the polyadenyaltion..

The *fae*G gene (887 bp), encoding the K88 fimbriae protein, was amplified by the polymerase chain reaction (PCR) using as the template the DNA of the ETEC plasmids *p*I294 (Isaacson, 1985) and two pairs of synthetic K88 oligonucleotide primers containing restriction sites for *Nhe* I and *Bam* HI. The K88 sense primer was 5' AGGGGTTTATGCTAGCAAAAAGACT 3', while the anti-sense primer was 5' GGATCCGGATCCTTAGTAATAAGT 3'. DNA sequences for primer design of the K88 *fae*G gene from *E. coli* was obtained from the GenBank (www.ncbi.nlm.nih.gov/entrez).

PCR was performed following standard procedures (Sambrook *et al.*, 1989) in a thermocycler with the following program; 30 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min, and an extension step at 72°C for 5 min at the end of the cycle. The cDNA of *fae*G was inserted into the *Nhe* I and *Bam* HI sites of *p*SLIAgDs to generate plasmid *p*UM8 (Mead *et al.*, *et al.*, 1985). Mouse IL-2 (ATCC 37553) and IL-4 (ATCC 37561) cDNAs were inserted individually into *p*UM8 at the *Bam* HI site generating *p*UM82 containing the K88 and the IL-2 genes and *p*UM84 containing the K88 and the IL-4 genes. Also IL-4 cDNA alone was inserted into *p*SLIAgDs at the *Bam* HI site to produce *p*UM4.

Analysis of protein expression. Each of the plasmid constructs (1 μg) were transiently transfected in duplicate into 5 X 105 COS-M6 cells (Green monkey's kidney cell, GIBCO-BRL, Canada) using the Lipofectamine-Plus reagent according to the supplied protocol (GIBCO-BRL, Canada). Three days post-transfection, the supernatants were harvested and used to determine if plasmid constructs had expressed the proteins. Supernatants from COS-M6 cells transfected with plasmids encoding K88 or IL-4 were analyzed by ELISA using specific antibodies raised against these proteins.

Preparation of purified *E. coli* K88+ fimbriae (K88 fimbriae). A local strain of

hemolytic ETEC K88 bacteria was identified as being K88 (Kim *et al.*, 1999) and the K88+ fimbrial antigen (K88 fimbriae) was purified using the method of Fang *et al.* (2000). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Laemmli, 1970) was used to check purity and molecular weight. ELISA confirmed its identity (Kim *et al.*, 1999).

Experimental design, immunization of mice. Two different animal experiments were carried out. The first experiment was to compare the enhancement of humoral immune response to the K88 fimbrial antigen in mice injected with different vaccines containing the K88 fimbrial gene plus different combinations of the IL-2 or IL-4 adjuvants. The second experiment was designed to examine the enhancement of humoral immune response using plasmids containing the IL-4 gene as a genetic adjuvant, and the responses were also to be compared to those obtained when the antigen was injected in the presence of Freund's adjuvants. Experiments 1 and 2 are very similar designs; only two differences are that the treatments of experiment 1 involved the injection of *p*UM82 plasmid instead of the injection of K88 fimbrial proteins in the treatments of experiment 2; also the experiment 2 has two different dosages (40 and 100μg of plasmids or proteins) injected.

In the first experiment, six-wk-old CD-1 mice were divided into five groups of six mice. At days 0 and 14, groups 2 to 4 were injected intramuscularly (i.m.) with a 27-gauge needle at two different sites in both thigh muscles with a total of 100μg of plasmid DNA (*p*UM8, *p*UM82 and *p*UM84; groups 2 to 4, respectively) dissolved in 100 µL of normal saline. Group 1 was injected with 100µL of normal saline. Group 5 was injected with a mixture (plural) of 100μg of *p*UM8 and 100μg of *p*UM4 dissolved in 100µL of normal saline. Serum was collected from the mice every two wks after the primary immunization.

In the second set of experiment, six-wk-old BALB/c mice were divided into five groups of 16 mice with one-half of each group receiving one dose (40μg) and the other half a higher dose (100μg) of the vaccine. Groups 2, 3, and 4 were injected intramuscularly with different plasmid DNA's (*p*UM8, *p*UM84 and a mixture of *p*UM8 plus *p*UM4, respectively) each at 0 (primary immunization) and 6 wks as outlined in Table 4. Group 5 was injected intramuscularly with a mixture of K88 fimbrial antigen and Freund's complete (FCA; 1st injection) or incomplete (FIA; 2nd injection) adjuvant as outlined in Table. 4. Group 1 was injected intramuscluarly with a plasmid DNA (*p*SLIAgDs) that did not express K88 fimbriae or cytokine at the same time periods as for

Table 4. Experimental design for mice trials.[1]

Group No.	Experiment 1		Experiment 2	
	Plasmid	Dose (μg)	Plasmid	Dose (μg)
1	Saline	100	Saline	40 or 100
2	*p*UM8	100	*p*UM8	40 or 100
3	*p*UM82[2]	100	*p*UM84[2]	40 or 100
4	*p*UM84[2]	100	*p*UM8+*p*UM4[3]	40/40 or 100/100
5	*p*UM8+*p*UM4[3]	100/100	K88 protein	40 or 100

[1] Plasmids were injected intramuscularly at 0 and 6 wks, each wk in a 100 µL of saline. K88 proteins in Experiment group 5 was diluted to 100 µL of PBS and mixed with 100 µL of FCA (wk 0) or FIA, as a booster injection at wk 6.

[2] Plasmid *p*UM8 with cytokine gene in the same vector.

[3] K88 *cfa* I and IL-4 genes were encoded in the separate vectors

the other groups. Serum was collected from the mice at 0, 2, 4, 6, 10, 12, 14, and 16 wks after the primary immunization.

Antibody assays and isotype analysis. The enzyme-linked immunosorbent assay (ELISA) with purified fimbrial antigen was used to analyse the sera of immunized mice for antibody titer. The procedure was a modification of that reported by Kim et al (1999). Wells of Microtest III flexible assay plates (Falcon 3911) were coated overnight at 4°C with 0.1 μg of the purified fimbrial antigen suspended in 100 μL of PBS. The plates were washed with pH 7.2 PBS- 0.05% Tween 20 and then blocked overnight at room temperature with 5% (wt/vol) skim milk in PBS followed by washings with PBS-T. The washed plates were incubated with dilutions of mouse serum (100 μL) and kept for 2 h at 37°C. After washing the plates were incubated with 100 μL of alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Inc., diluted 1 in 5,000), the second indicator antibodies, for 2 h at 37°C. The reaction was visualized after 40 min at 37°C using the substrate tablets (p-nitrophenyl phosphate, Sigma N9389). The absorbances of the reactions were determined at 405 nm in a microplate reader (Bio-Rad, Model 3550, Richmond, CA, USA, 94804). The titer was the dilution of antibody required to give one-half of the maximum absorbency reading (Harlow and Lane, 1998;

Kim *et al.*, 1999; Marquardt *et al.*, 1999). Assays at different times were corrected using a standard samples containing known K88 antibody titer.

The isotype ELISA was performed as described above. For determination of the relative levels of IgG1 and IgG2a, a goat anti-mouse IgG1-alkaline phosphatase conjugate and a goat anti-mouse IgG2a-alkaline phosphatase conjugate (Biocompare, Inc., CA, USA), respectively, were substituted for anti-mouse IgG-alkaline phosphatase in the standard ELISA assay..

Cytokine ELISA. Cytokines in culture supernatant and serum were determined by ELISA using purified anti-IL-2 and anti-IL-4 as capture antibodies and the corresponding biotinylated antibodies as reporter antibodies according to supplier recommendations (PharMingen, San Diego, CA). Primary capture antibodies and biotinylated reporter antibodies were used at a concentration of 2 and 1ug/mL, respectively. The enzyme substrate used was TMB microwell peroxidase (Kirkgaad & Perry Labs. Inc, Gaithersberg, MD). Purified recombinant IL-2 and IL- 4 (PharMingen) was used as a standard (Shehab *et al.*, 1996).

Statistical analysis. Statistical analysis was done by the paired Student *t* test and analysis of variance (ANOVA) (SAS/STAT, 1989). Values for *p*UM8 alone or K88

fimbriae protein immunization were compared with values for cytokine co-injection groups. $P<0.05$ was considered significant.

RESULTS

Expression in COS cells. The DNA vaccines were constructed by creating a plasmid that would express the K88 fimbrial antigen and mouse IL-2 or IL-4 in transfected eucaryotic cells. The *p*UM8 plasmid contained a DNA fragment capable of expressing the K88 fimbriae of approximately 0.85 kb (Fig. 5). The *p*UM82 plasmid contained the 0.85 kb K88 *fae*G gene and the 1.0 kb mouse IL-2 gene. The *p*UM84 plasmid also contained the 0.85 kb K88 *fae*G gene in combination with the 0.8 kb mouse IL-4 gene. The *p*UM4 plasmid contained the IL-4 gene alone.

COS-M6 cells were transiently transfected with each of the vectors to determine if the constructed plasmids could produce the K88 fimbriae and the cytokines. The concentrations of the K88 antigen expressed in COS-M6 cells three days after transfection ranged from 21 to 45 ng/mL (Table. 5). Transfection with *p*UM8 yielded the highest of expression (45 ng/mL). The concentrations of IL-2 and IL-4 were more than 20 fold lower than that of the K88 fimbriae and were expressed in all plasmids containing

FIG. 5. Restriction map of *p*UM82 or *p*UM84. The IL-2 or IL-4 DNA was inserted into the site of *BamH* I at pUM8.

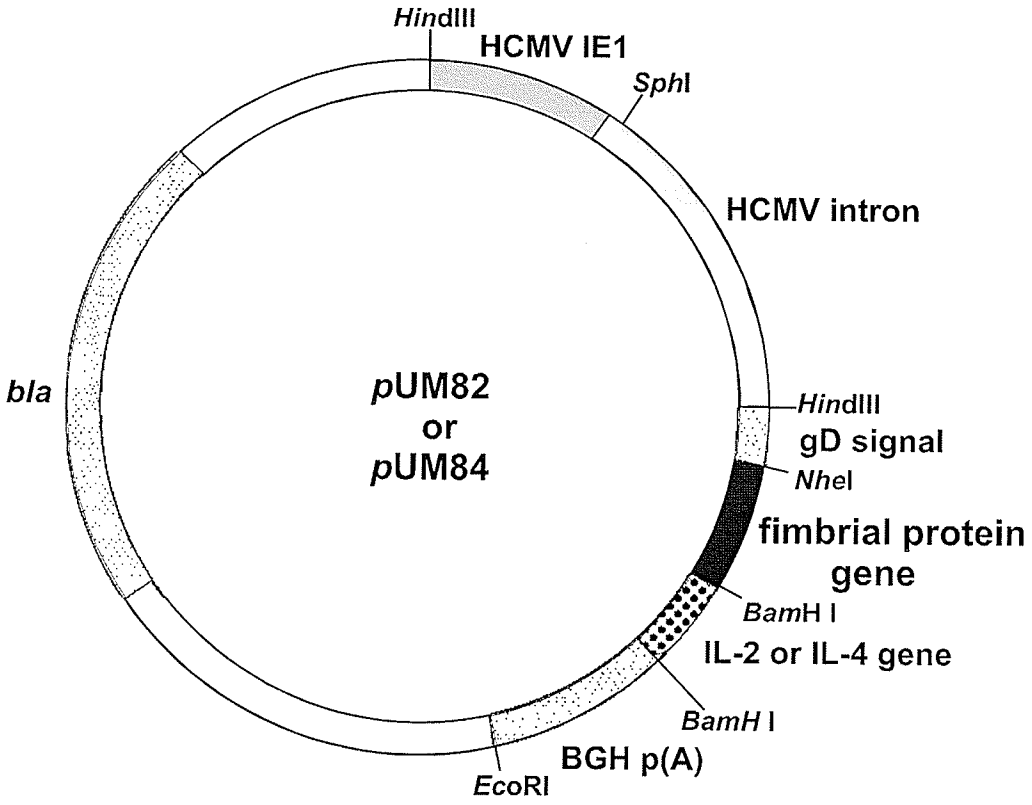

HindIII
HCMV IE1
SphI
HCMV intron
bla
pUM82
or
pUM84
HindIII
gD signal
NheI
fimbrial protein
gene
BamH I
IL-2 or IL-4 gene
BamH I
BGH p(A)
EcoRI

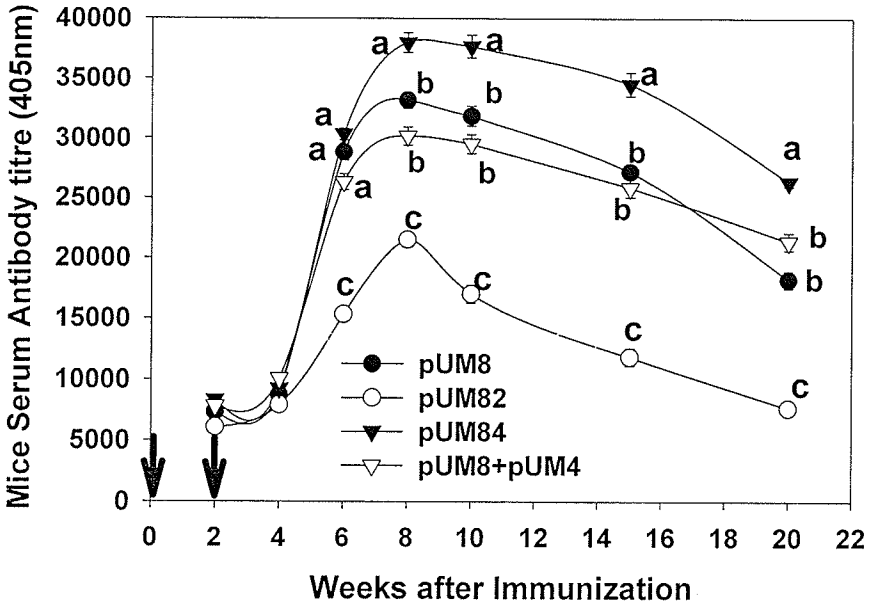
Table. 5. The expression of K88 and Cytokines in the tissue after transfection of COS-M6 cells.

Transfected plasmid DNA	K88 (ng/mL)	Cytokines (ng/mL)	
		IL-2	IL-4
*p*UM8	45	-	-
*p*UM84	31	-	1.2
*p*UM8 + *p*UM4	33	-	2.1
*p*UM82	21	1.4	-
*p*SLIAgDs (control)	-	-	-

the IL-2 and IL-4 genes. Plasmids that did not contain the fimbrial or cytokine genes did not express the corresponding proteins (data not shown).

Effects of genetic adjuvants on anti-K88 antibody responses. In the first experiment, we determined the effects of i.m. cytokine and fimbrial antigen gene injections on the anti- K88 antibody response. The pattern of antibody response over a 20 wk period is illustrated in Fig. 6. The highest anti-K88 antibody titer following the injection of the different plasmids was obtained with *p*UM84, expressing both the K88 fimbriae and IL-4. The antibody titers between wks 8 and 20 were approximately 17% higher ($P<0.05$) at wk eight and 43% higher ($P<0.05$) at wk 20 compared to those obtained with the plasmid containing only the K88 gene (*p*UM8). The simultaneous injection of a mixture of two plasmids (*p*UM8 + *p*UM4) each capable of separately expressing the K88 antigens and IL-4 yielded similar antibody titers over the entire study to that obtained by injection of the plasmid capable of expressing only the K88 antigen (*p*UM8), $P>0.05$. Injection of the plasmid containing genes capable of expressing both the K88 and IL-2 (*p*UM82) depressed antibody titer by 34% at wk 8 ($P<0.05$) to 60% at wk 20 compared to values obtained by injection of the plasmid capable of expressing only the K88 antigen (*p*UM8). The anti-K88 antibody titer for the control group receiving

FIG. 6. Serum anti-K88 antibody response in mice injected at wks 0 and 2 of experiment 1 with the *p*UM8 plasmid capable of expressing the K88 antigen, the *p*UM82 plasmid capable of expressing the K88 antigen and IL-2, the *p*UM84 plasmid capable of expressing the K88 antigen and IL-4, and a mixture of the *p*UM8 + *p*UM4 plasmids each capable of separately expressing the K88 antigen and IL-4. Values are a mean ± SE of 6 mice. Error bars smaller than symbols are not shown. Values for wks 2 to 4 were not different ($p>0.05$). Values for each treatment group for each wk from 6 to 20 having different letters (a, b, and c) were significantly different ($P<0.05$). The two arrows represents the times of the first and second injections.

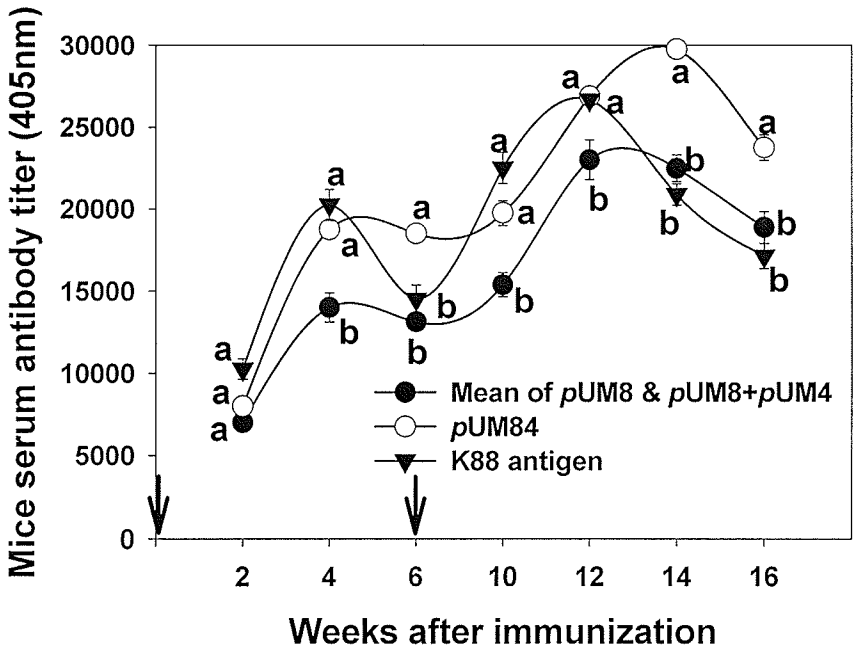

Mice Serum Antibody titre (405nm)
40000
35000
30000
25000
20000
15000
10000
5000
0
0
2
4
6
8
10
12
14
16
18
20
22
Weeks after Immunization
pUM8
pUM82
pUM84
pUM8+pUM4

only saline was essentially zero over the entire experiment. (absorbency value of less than 0.01 at 405 nm, data not shown).

The objectives of experiment 2 were to confirm results obtained in experiment one, to examine the time course change in antibody titer when two different concentration of the vaccines were injected and when injection periods for the vaccines were separated by 6 rather than 2 wks as in experiment one, and to compare titers obtained with the DNA vaccines with those obtained using the antigen (K88 fimbriae) in the presence of a strong adjuvant (FCA and FIA).

The antibody titer for all time points and all vaccines were not affected ($P>0.05$) by amounts (40 or 100μg) of vaccine injected (data not shown). The values for the two dosages were therefore pooled. The pattern of change when *p*UM8 (K88 gene) and when *p*UM8 + *p*UM4 (IL-4 gene) were mixed and injected together were the same ($P>0.05$) over the entire time course of the study. As a result, the data were pooled and means were compared with those obtained for the other two treatments (Fig. 7). The trends for the treatments (*p*UM8 and *p*UM8 + *p*UM4) were the same as for experiment one. The pattern of antibody titer change for mice injected with *p*UM84 (K88 fimbriae and IL-4 genes) was also the same as for either *p*UM8 or *p*UM8 + *p*UM4, although the antibody titer for

FIG. 7. Serum anti-K88 antibody response in mice in the experiment 2 injected at wks 0 and 6 with *p*UM84 (plasmid expressing both the K88 antigen and IL-4), the K88 fimbrial antigen, and the mean of *p*UM8 (plasmid expressing the K88 antigen) and *p*UM8 + *p*UM4 (plasmids separately expressing the K88 antigen and IL-4, respectively). Results from injecting *p*UM8 (K88 gene) and simultaneously injecting the *p*UM8 + *p*UM4 were pooled as there were no differences ($P > 0.05$) between these values for any of the time periods. Values are a mean ± SE of 16 mice for *p*UM84 and the K88 antigen and 32 for the other comparison (mean of *p*UM8 and *p*UM8 + *p*UM4). Values for each treatment group for each wk having different letters (a, b) were different ($P<0.05$). Error bars smaller than symbols are not shown. The two arrows represents the times of the first and second injections.

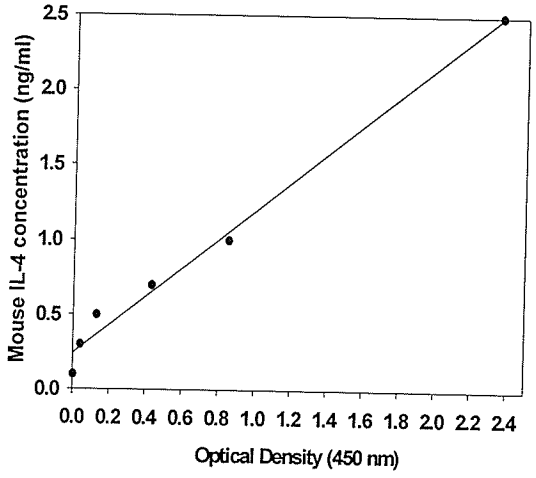

Mice serum antibody titer (405nm)
30000
25000
20000
15000
10000
5000
0
2
4
6
10
12
14
16
Weeks after immunization
Mean of pUM8 & pUM8+pUM4
pUM84
K88 antigen

mice injected with *p*UM84 was 64 % higher between wks 6 and 16 than the titer in mice injected with *p*UM8 and *p*UM8 + *p*UM4 ($P<0.05$). The pattern of antibody titer change for mice injected with *p*UM84 fluctuated less than the pattern obtained when the K88 fimbriae antigen was directly injected. Also, the *p*UM84 yielded antibody titers that were lower at wk 4 ($P<0.05$), the same at wks 2, 10 and 12 ($P>0.05$) and 30 % higher at wks 6, 14 and 16 ($P<0.05$) than those obtained with the K88 antigen. The serum of mice injected with saline or the *p*SLIAgDs vector did not contain anti-K88 fimbriae antibodies (data not shown).

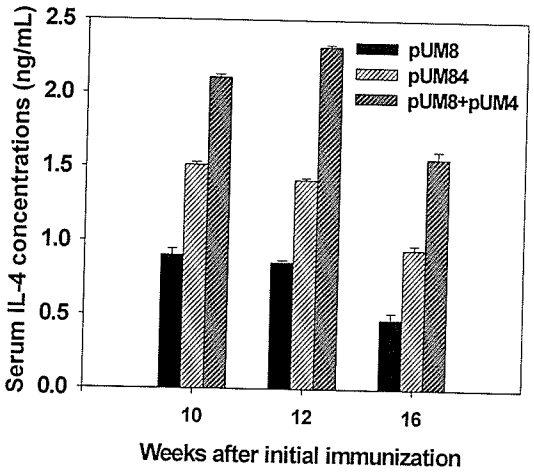
Cytokine mouse IL-4 production. The concentrations of IL-4 in the serum of mice injected with the higher dose of the plasmids were determined in experiment 2 at 10, 14, and 16 wks after the first immunization using a modified indirect ELISA method. These serum samples were assayed immediately because mouse IL-4 has a short half-life. A standard curve was established using the commercial mIL-4 at concentrations of 0.1, 0.3, 0.5, 0.7, 1.0, and 2.5 ng/mL. The regression equation was $f(y) = 0.242 \text{ X } f(x) + 0.954$, $R^2 = 0.99$, $P<0.05$ (Fig 8A).

The concentration of IL-4 in the serum ranged from 0.5 – 2.3 ng/mL for all treatments between 10 and 16 wks after the first injection with the trends over time being

FIG. 8. A; A standard curve of recombinant IL-4 for the measurement of serum IL-4 concentration. Values are a mean of duplicate analysis. B; The effect of co-delivering cytokine genes with a DNA vaccine in the level of serum IL-4, *p*UM4 expressed IL-4, *p*UM8 expressed the K88 antigen, and *p*UM84 expressed both IL-4 and the K88 antigen. Values are a mean ± SE of serum from 8 mice. Values for each treatment group for each wk having different letters (a, b, and c) were different ($P<0.05$). Analyses of variance of values within each time period indicated that all values were significantly different ($P<0.05$).


Mouse IL-4 concentration (ng/ml)
2.5
2.0
1.5
1.0
0.5
0.0
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0 2.2 2.4
Optical Density (450 nm)

A


Serum IL-4 concentrations (ng/mL)
2.5
2.0
1.5
1.0
0.5
0.0
pUM8
pUM84
pUM8+pUM4
10
12
16
Weeks after initial immunization

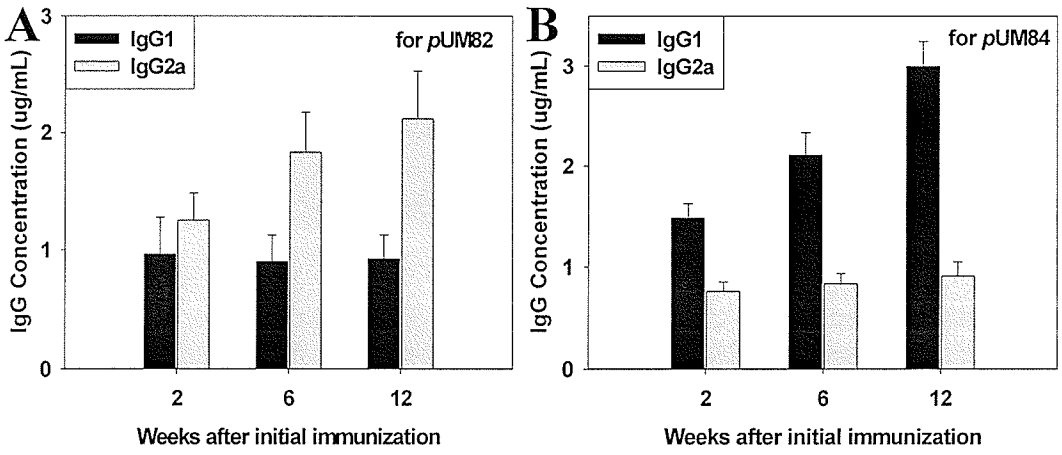
B

the same for all comparisons (Fig 8B). Injection of mice with *p*UM84 resulted a 34% higher value than that obtained with *p*UM8 (expressed only the K88 antigen), whereas co-injections of *p*UM8 plus *p*UM4 resulted in a 36% higher ($p<0.05$) average IL-4 concentration than with *p*UM84.

Serum concentrations of IgG1 and IgG2a. In experiment one, mice were vaccinated with *p*UM82 or *p*UM84 and blood was collected and analyzed for antibodies of immunoglobulin isotypes in wks 2, 6 and 12 after the first immunization (Fig. 9 A and B). The serum concentration of antibody IgG2a for all three time periods was greater ($P<0.05$) than that of IgG1 in mice vaccinated with *p*UM82. On wk 12, the concentration of antibody IgG2a was more than twice the concentration of IgG1. In contrast, the concentration of the antibody IgG1 subclass was higher ($P<0.05$) than that of IgG2a in mice vaccinated with *p*UM84. The relative concentration of antibody IgG1 tended to increase continuously over the time course of the study with its value being more than 3 fold higher ($P<0.05$) than that of IgG2a at 12 wks after the first immunization while the concentration of antibody IgG2a remained essentially constant over time.

In the second experiment, antibodies of IgG1 and IgG2a subclasses were detected in sera of all mice vaccinated with *p*UM84, K88 antigen protein plus a strong

FIG. 9. Total serum IgG1 and IgG2a isotypes concentration in Experiment 1 at 2, 6, and 12 wks after mice were initially injected with *p*UM82 (expressed both IL-2 and the K88 antigen, Fig 9A), and *p*UM84 (expressed both IL-4 and the K88 antigen, Fig 9B). Values are mean ± SE of serum antibody titer for 6 mice. Statistical analysis by the Student's *t*-test demonstrated the concentrations of two isotypes of immunoglobulin for each time period for both Fig. 9A and 9B were different at $P<0.05$ except at 2 wk in Fig 5A.

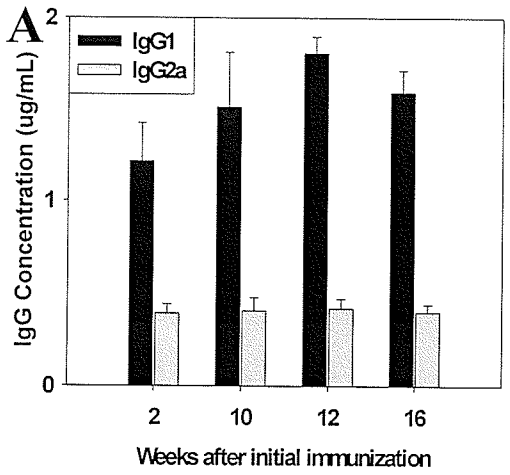

A
IgG1
IgG2a
for pUM82
IgG Concentration (ug/mL)
0
1
2
3
2
6
12
Weeks after initial immunization
B
IgG1
IgG2a
for pUM84
IgG Concentration (ug/mL)
0
1
2
3
2
6
12
Weeks after initial immunization

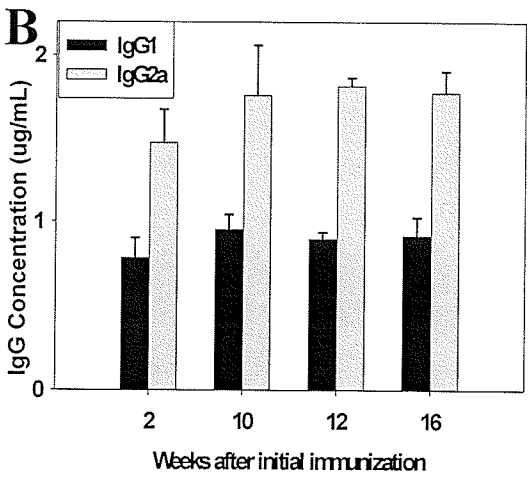
bacterial adjuvant (Freund's), and *p*UM8 at 2, 10, 12, 16 wks after the first immunization (Fig. 10 A, B, and C). IgG1 was the major antibody of IgG subclass elicited by immunization with purified protein (K88 fimbriae) in the presence of Freund's adjuvants (Fig 10A) or with *p*UM84 (Fig. 10C). In contrast, IgG2a was the major antibody of IgG subclass in mice vaccinated with *p*UM8 (Fig 10B). The content of IgG2a antibodies in all of these comparison were more than two fold higher than that of IgG1 ($P<0.05$) with the concentration of IgG1 remaining essentially constant over the time course of the study.

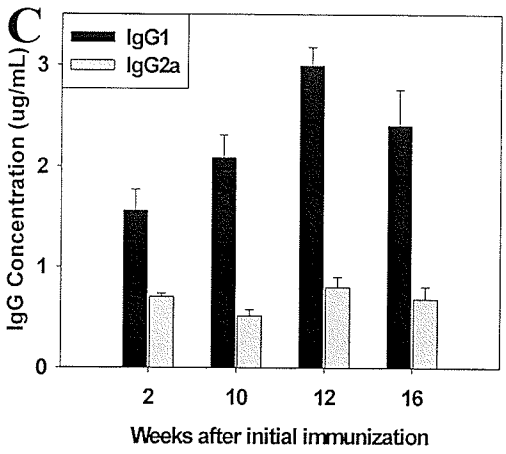
DISCUSSION

Vaccination of mice with a DNA vaccine encoding K88 fimbriae produces an antibody response against the expressed antigen and provides passive immune responses against ETEC K88 (Alves *et al.*, 1999; Alves *et al.*, 1998; Alves *et al.*, 1998; Alves *et al.*, 1999; Lasaro *et al.*, 1999). The type of specific antibody that is produced appears to be strongly correlated with the nature of the targeting antigen and other co-factors, such as the adjuvant. This is consistent with the recent finding that DNA encoding a foreign antigen and cytokine genes such as IL-4, IL-5, GM-CSF effectively enhanced the

FIG. 10. Total serum IgG1 and IgG2a isotypes concentrations in Experiment 2 at 2, 6, 12, and 16 wks after immunization, mice were initially injected with the K88 antigen containing Freunds complete adjuvant (A), *p*UM8 (expressed the K88 antigen, B), and *p*UM84 (expressed both IL-4 and the K88 protein, C). Values are mean ± SE of serum antibody titer for 8 mice injected with the high dose of vaccine. Statistical analysis by the Student's *t*-test demonstrated that the concentration of the two isotypes of immunoglobulin for each time comparison as shown in Fig. 10 A, B, and C were different at $P<0.05$.


A
IgG1
IgG2a
IgG Concentration (ug/mL)
0
1
2
2
10
12
16
Weeks after initial immunization


B
IgG1
IgG2a
IgG Concentration (ug/mL)
0
1
2
2
10
12
16
Weeks after initial immunization


C
IgG1
IgG2a
IgG Concentration (ug/mL)
0
1
2
3
2
10
12
16
Weeks after initial immunization

humoral response and confirms that the use of cytokines is preferable, suitable and safer (Scheerlinck *et al.*, 2001) than the use of other co-factors such as Freund's adjuvant.

The stimulation of antibody response in mice immunized with *p*UM84 compared to those immunized with *p*UM8 may be attributed, as suggested by other studies using a different vaccine, to an extension of the half-life of IL-4 and the co-expression of the antigen and the cytokine (Chen *et al.*, 1994; Harvill *et al.*, 1996). Also the enhanced antibody response after immunization with *p*UM84 may reflect the ability of IL-4 to directly stimulate growth of B-cell and the specific development of Th 2-cells (Maggi *et al.*, 1992).

Other researchers using a separate injection system, in contrast to the results obtained in this study and those reported elsewhere, reported that the adjuvant effect of the cytokine-expressing vectors was dependent upon co-injection of the cytokine plasmid with the plasmid encoding a foreign gene; inoculation of the two plasmids separately had no effect on the magnitude of specific immune responses, indicating that co-localization of cytokines and antigens at the site of immune interaction was important for the observed cytokine adjuvant effect (Chow *et al.*, 1998).

An interesting observation from the results reported here was that the different

profiles of IgG isotypes can be induced by different cytokines when linked to the same antigen suggesting that the cytokines are exerting subtle influences on the responding T-cell and B-cell population. It is known that production of the IgG1 isotype is induced by Th 2 type cytokines whereas the IgG2a isotype production is induced by Th 1 type cytokines (Coffman *et al.*, 1988). Likewise, injection of a vaccine capable of co-expressing both foreign protein and IL-4 stimulated a greater production of IgG1 than that of IgG2a, whereas injection of a similar vaccine containing the IL-2 gene or a plasmid without the cytokine gene induced a greater production of IgG2a than IgG1, not only in this study but in other studies with other antigens (Chow *et al.*, 1997; Raz *et al.*, 1993; Sin *et al.*, 1999). These results indicate that IL-4 has the ability to induce the switching of Ig subclasses to IgG1 and to stimulate B cells to produce more of the IgG1 subclass (Raz *et al.*, 1993).

In conclusion, the data presented here demonstrates that the co-expression of a Th 2 type cytokine and an antigen by a DNA vaccine can shift the immune responses toward a Th 2 profile, and increase the immunogenicity of targeting antigen. Moreover, these results strongly imply that the enhancement in humoral immune responses to B-cells could be achieved by using the IL-4 cytokine gene as an adjuvant in DNA

vaccination. Also, this study demonstrates that DNA vaccine co-expressed with the cytokine gene can replace the ordinary protein vaccine and, therefore, this strategy may be beneficial in the treatment of diseases caused by bacteria and certain virus infections.

MANUSCRIPT III

A plasmid DNA encoding chicken Interleukin-6 and *Escherichia coli* K88 *fae*G stimulates the production of K88 fimbriae antibodies in chickens

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The University of Manitoba.

ABSTRACT

Using enterotoxigenic *Escherichia coli* K88 as a model for K88 fimbriae plasmid development, we have previously shown that a DNA plasmid that encodes the K88 fimbriae protein can stimulate the production of anti-K88 fimbriae antibodies when injected into mice. We now report that a new plasmid that encodes the K88 fimbrial gene (*fae*G) stimulates the production of specific antibodies against the K88 fimbriae antigen in chickens. In addition, hens immunized with the plasmid expressing both the fimbriae and a cytokine chicken Interleukin-6 (chIL-6), as a genetic adjuvant, stimulated a slightly higher antibody titer compared with that obtained by the injection a plasmid encoding only the *fae*G gene especially 10 (19%, $P<0.05$) and 12 (27%, $P<0.05$) wks, respectively, after the secondary immunization. Also, co-expression of the K88 *fae*G and chIL-6 gene from the same plasmid maintained a high level of antibody titer for a longer period compared to that obtained with a plasmid expressing only the fimbrial protein and that obtained using K88 fimbriae directly as a protein antigen. Expression from both genes on the plasmid was confirmed in transfected COS-M6 cells, where proteins reacting with both the anti-K88 fimbriae antibody and the anti-chIL-6 antibody were observed. Therefore, DNA plasmids containing genetic adjuvants may offer many advantages

compared to those obtained by the use of protein immunization or a DNA plasmid without the genetic adjuvant. This study is the first example of using an avian cytokine (IL-6) for the enhancement of the immune response of the chicken.

INTRODUCTION

Diarrhea caused by enterotoxigenic (ETEC) *Escherichia coli* bacteria is a major cause of sickness and death of young animals. ETEC also cause significant economic losses in animal husbandry particularly among young animals directly after birth (neonatal diarrhea) and directly after weaning (post-weaning diarrhea) (Kim *et al.*, 1999; Marquardt *et al.*, 1999). However, ETEC infection of young animals can be prevented effectively by the oral administration of ETEC-specific antibodies. There are several different types of ETEC-specific antibodies, such as those present in spray-dried pig plasma (SDPP), purified antibodies from rabbit serum, antibodies from the colostrum of immunized dams and antibodies from the egg yolk of immunized hens. There are many advantages of using egg-yolk antibodies (IgY) for the therapeutic treatment of intestinal diseases such as those caused by ETEC including an ability to safely and economically produce an abundant supply of specific and highly effective antibodies (Marquardt *et al.*, 1999).

A novel immunization strategy, DNA or genetic vaccination, against ETEC strains has been reported to elicit both humoral and cellular immune responses in vivo in mice (Alves *et al.*, 1998; Alves *et al.*, 1999a; Alves *et al.*, 1999b; Lasaro *et al.*, 1999).

The encoded gene in the DNA plasmid drives the synthesis of specific foreign proteins within the vaccinated host to induce the production of specific antibodies without the associated risk of viral or bacteriological pathogenesis and an ability to produce a safe and sustained production of antibodies at a relatively low cost. They also are much less invasive than antigen plasmids particularly if Freund's adjuvants are used. A possible disadvantage of DNA plasmids is that they often do not stimulate the same level of antibody production as achieved with conventional antigen immunization and one of the limitations of DNA plasmids has been the lack of a strong humoral immune response. For this reason, the co-administration of cytokines has been explored as a genetic adjuvant to enhance an immune response (Chen *et al.*, 1994; Chow *et al.*, 1997; Chow *et al.*, 1998; Raz *et al.*, 1993; Scheerlinck *et al.*, 2001; Steidler *et al.*, 1996). Co-injection of cytokines such as Interleukin-2 (IL-2), IL-4, and GM-CSF with foreign proteins have been shown to successfully stimulate both cellular and humoral immune response in mammals, including humans. Unfortunately, DNA plasmids have not been widely used to elicit antibody production in hens in spite of many advantages of using these types of plasmids and using egg-yolk antibodies as therapeutic agents. Previous data from our laboratory, however, have demonstrated that mammalian interleukins, especially IL-2 and IL-4, are

ineffective in avians, probably because they are genetically distinct from the avian interleukins. IL-6, however, is a multifunctional cytokine in the avian immune system (Lynagh *et al.*, 2000) which possibly has functional properties similar to those of mammalian cytokines (IL-2, IL-4, and GM-CSF) and, therefore, may serve as an inducer of a high level of specific antibody production. Recently, Schneider *et al.* (2001) established the structure of chicken IL-6 (chIL-6) cDNA and elucidated some of its biological properties. The availability in our laboratory of plasmids that can express the K88 fimbriae in chickens and a plasmid encoding IL-6 cDNA from chickens has facilitated this study as it was possible to combine a DNA plasmid for expression of K88 fimbriae (*p*UM8) with a genetic adjuvant (IL-6 cDNA) from chickens. Previous studies in our laboratory (Cho *et al.*, 2003) and by other researchers with mice have demonstrated that the DNA plasmid and the genetic adjuvant not only must be co-delivered to the animal but that their genes must be expressed from the same plasmid. Under such conditions, the interleukins were able to modify the level and type of antibody produced (Wortham *et al.*, 1998; Cho *et al.*, 2003). Therefore, in this study, the chIL-6 and the K88 *fae*G genes were combined on the same expression vector (*p*UM86) to study the effect of co-expression on anti-K88 antibody production and to compare the

efficacy of the genetic adjuvant.

MATERIALS AND METHODS

Chickens. Twenty-week-old Single Comb White Leghorn laying hens were housed in the temperature-controlled, light-cycled facilities at the University of Manitoba. Their care was under the guidelines of Canadian Council on Animal Care (CCAC, 1993).

Plasmid construction (*p*UM8 and *p*UM86). *Escherichia coli* NM522 *sup*E *thi* Δ (*lac-proAB*) *hsd-5*[F' *proAB lacI*^{q} *lacZ* Δ *15*] was used for the plasmid preparation (Mead *et al.*, 1985). Transformed bacteria were grown at 37°C overnight in Luria broth or Luria agar (Difco, USA) containing ampicillin (100μg/mL) for the transformation experiments and for isolation of plasmid DNA.

The *p*SLIAgDs expression vector, carrying the human cytomegalovirus (HCMV) immediate-early promoter with an intron (intron *a*) was generously provided by Dr. LA Babiuk. This plasmid was constructed by Babiuk's group using procedures outlined by Braun *et al.*(1997) and van Drunen Little-van den Hurk *et al.* (1998). This expression vector, which was used for cloning and expression of the proteins, contains the *E. coli* origin of replication, a gene for ampicillin resistance in *E. coli*, and a regulated promoter

(HCMV) with an intron (intron *a*) that appears to function in efficient transport of the mRNA out of the nucleus. Also, it has the signal sequence from gD protein of bovine herpesvirus, and there is a 3' region from bovine growth hormone present following the insertion site which will take care of the polyadenyaltion.

The fimbrial gene from *E. coli* strain K88 (*p*I294, Isaacson, 1985), encoding the fimbriae protein, was amplified by the polymerase chain reaction (PCR) using as template the DNA of the ETEC plasmids *p*I294 and two pairs of synthetic primers containing restriction sites for *Nhe* I and *Bam* HI. The K88 sense primer was 5' AGGGGTTTATGCTAGCAAAAAGACT 3', while the anti-sense primer was 5' GGATCCGGATCCTTAGTAATAAGT 3'. The DNA sequence of the *E. coli* K88 *fae*G gene was obtained from GenBank (www.ncbi.nlm.nih.gov/entrez) for primer design. PCR was performed following standard procedures (Sambrook *et al.*, 1989) in a thermocycler with the following program; 30 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min, and an extension step at 72°C for 5 min at the end of the cycle. Taq polymerase was from Invitrogen Inc (Canada). The cDNA (887-bp) for the *fae*G gene was inserted into *Nhe* I and *Bam* HI sites of *p*SLIAgDs generating plasmid *p*UM8. Chicken IL-6 (chIL-6) cDNA (1142-kb) was excised from plasmid *p*cDNAI/chIL-6-5-1 (Schneider *et al.*, 2001)

using the restriction enzymes, *Bam*H I and *Sph* I and inserted into the *p*UM8 to generate plasmid *p*UM86. It has an internal ribosomal entry site at 23 -163 residues of chIL-6 cDNA. All plasmid DNA was purified using Qiagen kits following the manufacturer's recommendations (Qiagen Inc., Ontario, Canada).

Preparation of purified *E. coli* K88+ fimbriae. A local strain of hemolytic ETEC K88 bacteria was identified as being K88 (Kim *et al.*, 1999) and the K88+ fimbrial antigen (K88 fimbriae) was purified using the method of Fang *et al.* (2000). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Laemmli, 1970) was used to check purity and molecular weight. ELISA confirmed its identity (Kim *et al.*, 1999).

Immunization of chickens. Chickens were immunized intramuscularly as previous studies using the same pSLIAgD's expression vector demonstrated that this route yielded serum antibody titers similar to that obtained by use of the gene gun but somewhat lower than that achieved by intradermal administration (i.d.), (Braun *et al.*, 1997). I.d. injections were not used as preliminary studies in our laboratory demonstrated that i.d. injection of *p*UM8 did not yield higher titers compared to those obtained using i.m. injections of the same plasmid (data not shown).

A total of 64 hens were assigned to 8 treatment groups with eight replicates (eight hens per group). Each chicken was administered a DNA plasmid or protein at zero time (primary injection) and boosted with a secondary dose of the same materials 6 wks after the first immunization. Treatments one and two were i.m. (intramuscular) injections of either 150 or 300μg of *p*UM8, treatments 3 and 4 were i.m. injections of either 150 or 300μg of K88 fimbriae protein, and treatments 5 and 6 were i.m. injections of either 150 or 300μg of *p*UM86. The chickens in each DNA treatment group (*p*UM8 or *p*UM86) were injected i.m. into the pectoral muscle at two sites at each time interval with either 150 or 300μg of plasmid DNA dissolved in 300 µL of PBS (pH 7.2) using a 25 gauge needle. For protein immunization, each chicken was injected i.m. twice at two sites. Each chicken at each time period was injected with either 150 or 300 μg of purified fimbrial protein in 300 µL of PBS emulsified in an equal volume of Freund's complete adjuvant (first injection, Sigma). The second boost of the fimbrial protein (either 150 or 300μg) was mixed with Freund's incomplete adjuvant (Sigma). Controls included two groups of eight chickens each (treatments 7 and 8). They were injected i.m. with 300 μg of *p*SLIAgDs vector in 300 µL of phosphate buffered saline (PBS) or 0.85% saline alone (eight chickens for each group).

Blood was collected from the wing vein (4 mL), incubated at 37°C for 1 h and centrifuged at 1,500 g for 20 min to obtain serum. Serum was stored at -20°C until analyzed. Also, eggs were collected every 2 wks after the first immunization, and the yolk was separated from the white prior to analysis and diluted 10-fold in phosphate buffered saline (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.2) to measure antibody titer using the ELISA.

Purification of chIL-6. A fragment of chIL-6 cDNA was digested by restriction enzymes *Bam* HI and *Xho* I from *p*cDNAI/chIL-6-5-1 for protein expression. The chIL-6 DNA fragment was inserted between the *Bam* HI and *Xho* I restriction sites of the prokaryotic expression vector *p*Bluescript KS M13+ to generate *p*KS6. Expression of this construct in *E. coli* strain NM522 was induced by 0.1 mM IPTG (isopropyl-beta-D-thiogalactopyranside, Sigma, USA) and yielded over-expressed chIL-6 which was secreted internally into the bacteria. The overall procedure was similar to that reported by Ehn *et al.* (2001). The bacterial pellet from 60 mL of culture medium was collected by centrifugation at 14,000g for 20 min, resuspended in 20 mM Tris-HCl, pH 8.0, and then disrupted by sonication for 3 min in an ice/water bath to release the proteins. The supernatant containing the soluble IL-6 protein was filtered through a 0.45 μL filter

(Millipore Corp., Bedford, MA). Supernatant samples were analyzed by SDS-PAGE.

A total of 3.3 g of solid $(NH_4)_2SO_4$ was added slowly to the 30 mL protein solution under magnetic stirring at room temperature until a salt concentration of 19 % saturation was obtained. The suspension was centrifuged at 40,000 *g* for 20 min. The protein pellet was suspended in 30 mL of 20 mM Tris-HCl, pH 8.0, and the suspension was stirred at room temperature for approximately 30 min. Insoluble material was removed by a second step of centrifugation at 40,000 *g* for 20 min followed by dialysis against the 20 mM Tris-HCl buffer, pH 8.0. The ammonium sulfate precipitate contained chIL-6 of a relatively high purity. All fractions were analyzed by SDS-PAGE. The purified IL-6 (100 μg/mL) was emulsified with Freund's complete adjuvant (1st injection; 100 µL/mouse) and Freund's incomplete adjuvant (2nd injection; 100 µL /mouse) and injected i.m. into mice at four sites to produce the specific antibody. Serum was collected at 4 wks after the first immunization, and stored at -20 °C for development of the ELISA.

Analysis of protein expression in COS-M6 cells. Plasmid constructs (1 μg) were transfected in duplicate into 5 X 10^5 COS-M6 cell using the Lipofectamine-Plus reagent according to the supplied protocol (GIBCO-BRL, USA). The supernatants were harvested at three days post-transfection and they were analyze by ELISA using specific

antibodies raised against these proteins to check for expression.

Antibody assays in the egg yolk. Enzyme-linked immunosorbent assay (ELISA) with the purified fimbrial antigen was used to analyze the egg yolks from immunized hens for anti-K88 fimbrial antibody titer. The procedure was a modification of that reported by Kim *et al* (1999). Wells of Microtest III flexible assay plates (Falcon 3911) were coated overnight at 4°C with 0.1 μg of the fimbrial antigen suspended in 100 µL of PBS. The plates were washed with pH 7.2 PBS- 0.05 % Tween 20 (PBST) and then blocked overnight at room temperature with 5 % (wt/vol) skim milk in PBS followed by washings with PBS-T. The washed plates were inoculated with the dilutions of egg yolk (100 µL) and kept for 2 h at 37°C. After washing the plates were incubated with 100 µL of alkaline phosphatase-conjugated rabbit anti-chicken IgY (Jackson ImmunoResearch Inc., diluted 1 in 5,000), as the second indicator antibodies, for 2 h at 37°C. The reaction was visualized after 40 min at 37°C using the substrate tablets (p-nitrophenyl phospate, Sigma N9389). The absorbencies of the reactions were determined with an ELISA plate reader fitted with a 405 nm filter. The titer was expressed as the dilution of antibody required to give one-half of the maximum absorbency reading (Harlow and Lane, 1998; Kim *et al.*, 1999; Marquardt *et al.*, 1999). Assays at different times were corrected using

standard samples containing known K88 antibody titer.

Cytokine ELISA. Cytokines in culture supernatant and serum were determined by indirect ELISA using mouse anti-chIL-6 as capture antibody and the corresponding alkaline phosphatase conjugated mouse IgG antibodies as reporter antibodies (Jackson ImmunoResearch Inc.) using a modification method of that used in commercial ELISA kits (Endogen). The general procedure outlined by Harlow and Lane (1998) was followed. The wells of a 96-well microplate (Falcon 3911) were coated by adding 100 μL of anti-mouse IL-6 (4 μg/mL), diluted in 0.05M bicarbonate buffer (pH9.6) and incubated overnight at 4°C. This concentration of antibodies was found to be optimal with regards to assay sensitivity and time required to complete the color reaction. The chIL-6 standards (0.1, 0.5, 1, 2, 5, 7, and 10 ng/mL) and the test samples in 100 μL of PBS buffer, pH 7.2, were added to the coated plate. The samples were incubated at 37°C for 2h, and then washed 3 times with PBST (PBS containing 0.05% of Tween20). A 100 μL mixture of the secondary antibody in PBS, pH7.2 (1 μg/mL of alkaline phosphatase conjugated rabbit anti-mouse IgG, Sigma) was added to each well and incubated at 37°C for 1h after three rinses with PBST. The enzyme substrate used was substrate tablets (p-Nitrophenyl Phospate, Sigma N9389). The optical density of the wells was determined at 405nm using

a plate reader.

SDS-PAGE and immunoblot analyses. Different protein fractions were analyzed by SDS-PAGE following the procedure of Laemmli (1970). The gels (4 – 15% gradients) were run under reducing conditions and stained with Coomassie brilliant blue (Sigma, USA) according to the suppliers' recommendations. For Western blotting, proteins were transferred to nitrocellulose by using a semidry transfer apparatus (BIO-RAD). Membranes were probed with chicken anti-K88 fimbriae antibody, or mouse anti-IL-6 antibody followed by goat anti-chicken IgY alkaline phosphatase-labeled antibody (Jackson ImmunoResearch Inc.) or goat anti-mouse IgG alkaline phosphatase-labeled antibody (Jackson ImmunoResearch Inc.).

Statistical analysis. Statistical analysis was done by the paired Student *t* test and analysis of variance (ANOVA) (SAS/STAT, 1989). Values for *p*UM8 alone or K88 fimbriae protein immunization were compared with values for cytokine co-injection group. The optical density for the chIL-6 assay was regressed against the log concentration of the chIL-6 standards using the REG procedures of SAS (SAS/STAT, 1989). $P<0.05$ was considered significant.

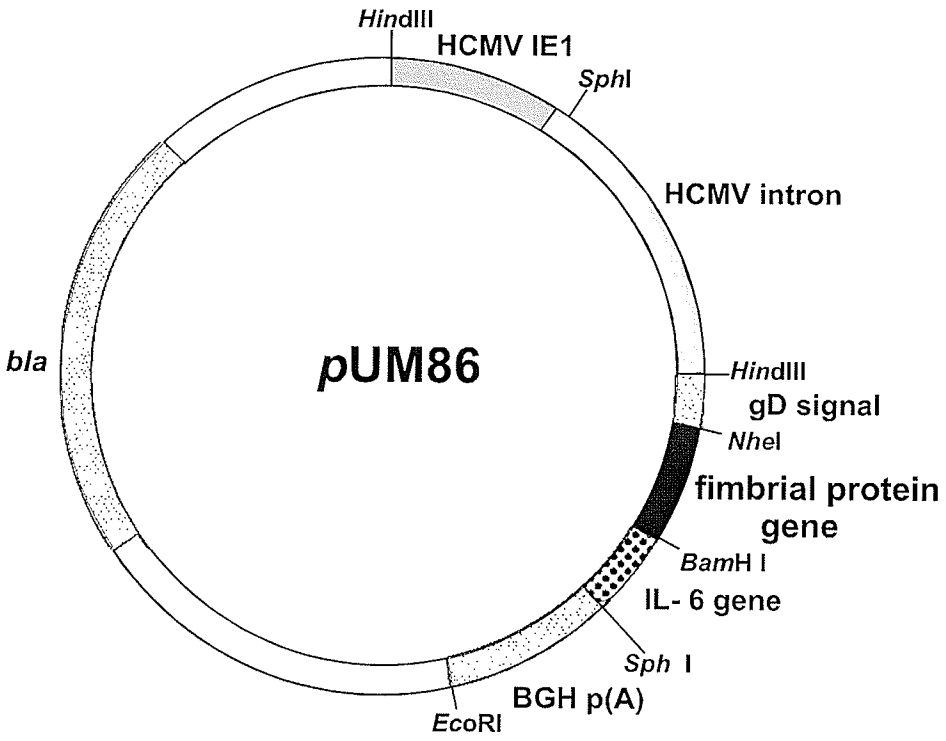
RESULTS

Expression of K88 fimbriae and cytokine in COS-M6 cells. DNA plasmids were constructed that were able to express the K88 fimbriae antigen and chicken IL-6 separately and together in transfected eukaryotic cells and in chickens. Plasmid *p*UM8 contains *fae*G, the gene for the K88 fimbriae antigen, and plasmid *p*UM86 contains the *E. coli* K88 *fae*G gene and the gene for chicken IL-6 (Fig. 11).

COS-M6 cells that were transiently transfected with the plasmids were analyzed by ELISA for the amounts of cytokine and K88 fimbriae present in the culture supernatant. The concentrations of K88 antigen ± SE expressed three days after transfection with *p*UM8 and *p*UM86 were 45.2 ± 6.0 and 33.1 ± 11.1 ng/mL, respectively. The concentration of IL-6 produced by transfection with *p*UM86 was 8.1 ± 1.1 ng/mL and was zero for *p*UM8.

K88 fimbriae and chIL-6 protein isolation. The K88 fimbriae were purified and shown to be of the correct molecular mass (29 kD) (Kim *et al.*, 1999). ChIL-6 was expressed in *E. coli* and purified. SDS-PAGE electrophoresis (Fig. 12A) showed that synthesis of chIL-6 was induced by IPTG (lane 1 versus lane 2), that centrifugation of the

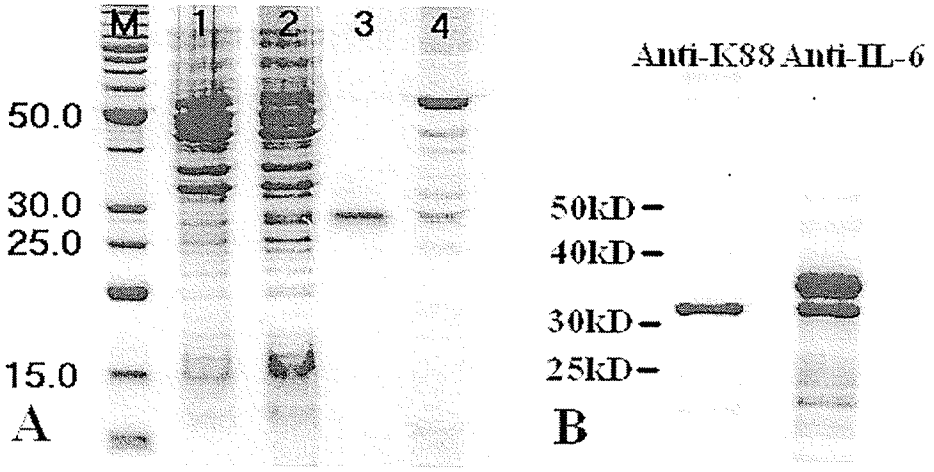
Fig. 11. Restriction map of *p*UM86; cDNA of chIL-6 was inserted between *Bam* HI and *Sph* I restriction sites of *p*UM8.


HindIII
HCMV IE1
SphI
HCMV intron
pUM86
bla
HindIII
gD signal
NheI
fimbrial protein gene
BamH I
IL- 6 gene
Sph I
BGH p(A)
EcoRI

crude extract removed a considerable amount of insoluble protein with most of the chIL-6 remaining in the supernatant (lane 2 versus lane 4), and that ammonium sulfate precipitation yielded a relatively pure preparation of chIL-6 (lane 3). The purity of chIL-6 was approximately 80%, and the molecular weight was approximately 27 kDa which was the same as reported by Schneider *et al.* 2001. The purified chIL-6 was injected into mice for antibody production.

The culture supernatant from COS-M6 cells, which were transfected with the *p*UM86 plasmid, was analyzed by Western blotting (immunoblotting) with anti-K88 fimbriae and anti-IL-6 antibodies (Fig. 12B). A band of protein with a molecular mass of approximately 32 kDa cross-reacted with anti K88 egg yolk antibody. This was somewhat larger than the expected 29 kDa for purified K88 fimbriae (data not shown) and may be the result of posttranslational modification. Similar modifications have been previously reported by other researchers (Wotham *et al.*, 1998). Probing with chIL-6 antibody revealed two bands at 36 and 32 kDa (Fig. 12B), also larger than the expected mass of 27 kDa of unmodified chIL-6 (Schneider *et al.* 2001). Immunoblot analysis of supernatant proteins from non-transfected COS-M6 cells did not yield positive bands for either chIL-6 or K88 fimbriae (data not shown).

Fig. 12. A: Commassie Blue stained SDS-PAGE gel showing a sample of purified chIL-6 from *E. coli* *p*KS6 strain; M: Protein Maker, 1: *E. coli* debris before induction, 2: *E. coli* debris after IPTG induction, 3: Purified chIL-6 after ammonium sulfate precipitation, 4: Crude chIL-6 after sonication and centrifugation. B: Western blotting of culture medium from COS-M6 cells transfected with *p*UM86 with anti-K88 and anti-chIL-6 antibodies.


M
1
2
3
4
50.0
30.0
25.0
15.0
A
Anti-K88 Anti-IL-6
50kD
40kD
30kD
25kD
B

Antibody responses in laying hens. The objectives of this study were to determine if a DNA plasmid that was capable of expressing the K88 fimbriae (*p*UM8) was able to stimulate antibody production in the yolk of eggs of vaccinated hens. The results were to be compared with those achieved by the direct vaccination of hens with the antigen (K88 fimbriae) emulsified in a strong adjuvant (Freund's). The latter protein immunization procedure has been a standard procedure, but has been strongly criticized by the Canadian Council of Animal Care since it produces undesirable effects in animals. A second objective was to determine if a genetic adjuvant (chIL-6 gene) when co-expressed with the K88 *fae*G gene would further enhance the response obtained with the fimbrial expression alone. A third objective was to determine if there was a dose response effect obtained by the administration of different amounts of the plasmids.

As indicated in Material and Methods, two doses of each plasmid were injected. The results demonstrated that there was no effect of plasmid dosage ($P>0.05$) on antibody titer with all values for each time period and for each plasmid being the same (data not shown). The dosage data were therefore pooled and presented as a single data set. The protein plasmid (K88 fimbriae) initially during wks 2, 4, and 6 of the experiment stimulated a much higher production of anti-K88 antibody in the egg yolk than that

produced by either of the DNA plasmids (*p*UM8 or *p*UM86) (Fig. 13). The approximate differences ($P<0.05$) in relative titers at 2, 4, and 6 wks were 50, 73, and 28%, respectively. However, by wk 8 there were no differences in antibody titers among all treatments ($P>0.05$) and no differences at 10 and 12 wks between hens immunized with either the antigen or *p*UM8. Hens immunized with the DNA plasmid containing the genetic adjuvant (*p*UM86) had 19 and 27% higher titers at 10 and 12 wks ($P<0.05$), respectively, compared to those injected with only the DNA plasmid (*p*UM8) and 35% higher ($P<0.05$) titer in wk 12 compared to those injected directly with the antigen. Chickens injected with either saline or the plasmid (*p*SLIAgDs) lacking the K88 *fae*G gene did not stimulate anti-K88 egg-yolk antibody production.

Cytokine chIL-6 production in the serum of laying hens. The objective of this study was to determine if the concentration of chIL-6 was affected by the type of plasmid administered. The concentration of chIL-6 was established using the formula; $f(y) = -0.125 \times f(x) + 1.23$, $R^2 = 0.93$ (Fig. 14A). The results demonstrate that, in general, control chickens and *p*UM8 injected chickens had similar IL-6 values and that the highest concentration of serum IL-6 was obtained in hens injected with *p*UM86 (Fig. 14B). Chickens injected with K88 protein and Freund's adjuvant also had higher IL-6

Fig. 13. Relative antibody concentration in the egg yolk of laying hens immunized on wks 0 and 6 with *p*UM8 (plasmid gene express K88 fimbriae), *p*UM86 (plasmid gene expresses both K88 fimbriae and IL-6), K88 fimbriae protein (protein antigen was injected in presence of Freund's complete and incomplete adjuvant) and control (average of saline and *p*SLIAgDs vectors). Values are a mean ± SE of number of analysis. Error bars smaller than symbols are not shown. Values within the same wk with different letters (a, b) are different ($P<0.05$).

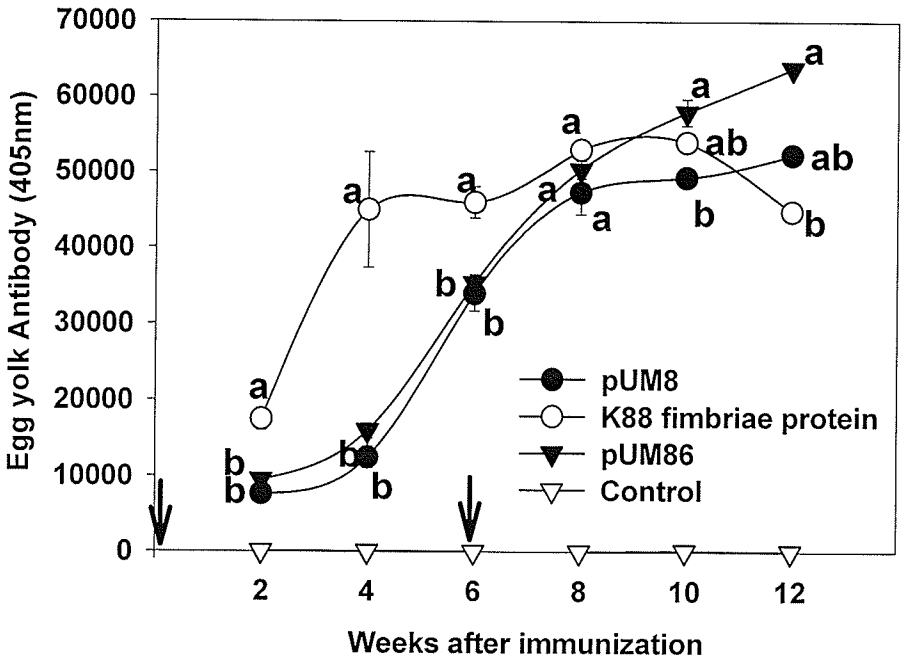
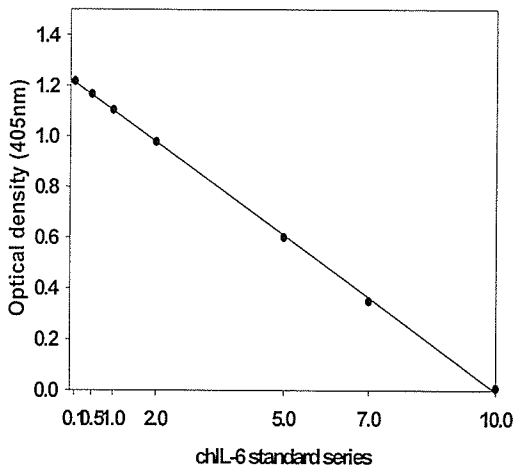
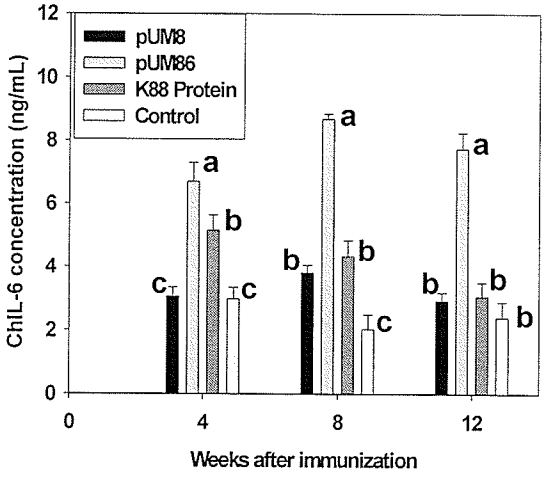

70000
60000
50000
40000
30000
20000
10000
0
Egg yolk Antibody (405nm)
2
4
6
8
10
12
Weeks after immunization
pUM8
K88 fimbriae protein
pUM86
Control
a
b
ab

Fig. 14. A; Standard curve for the determination of chIL-6 concentration in serum or the culture supernatant. The concentrations of chIL-6 were 0.1, 0.5, 1.0, 2.0, 5.0, 7.0, and 10 ng/mL. The number of replicates for each concentration of chIL-6 was 3. B; The level of IL-6 in the serum of laying hens immunized on wks 0 and 6 with *p*UM8, *p*UM86, K88 fimbriae protein, and control (*p*SLIAgDs). Values are a mean ± SE of number (6) of analysis. Values within the same wk with different letters (a, b and c) are different ($P<0.05$).


Optical density (405nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.1
0.5
1.0
2.0
5.0
7.0
10.0
chIL-6 standard series

A


ChIL-6 concentration (ng/mL)
12
10
8
6
4
2
0
pUM8
pUM86
K88 Protein
Control
a
b
c
a
b
c
a
b
b
0
4
8
12
Weeks after immunization

B

concentrations in wks 4 and 8 than those of the control but lower that those injected with pUM86 for all time periods.

DISCUSSION

The primary objectives of this study were to determine if DNA plasmids encoding K88 fimbriae and chIL-6 (*p*UM86) and a plasmid encoding just the K88 fimbriae (*p*UM8) could induce egg yolk antibody production and if chIL-6 as a genetic adjuvant could enhance the immune response in the chicken. The results indicate that each expressed protein from the two plasmids (*p*UM8 and *p*UM86) was immunogenic, and that immunization with the plasmid containing the artificial bicistronic operon enhanced the humoral immune responses to K88 fimbriae and increased the amount of IL-6 level in the serum. The pattern of antibody induction by the plasmid, lower than by protein antigen initially but higher later on, may have arisen because the initial amount of protein antigen available to stimulate immunity when the antigen was directly injected was probably higher than that provided by the DNA plasmid. However, the DNA plasmid was able to produce the K88 fimbriae for a longer period of time providing for longer term stimulation of antibody production.

The use of cytokines as agents to enhance both humoral and cell-mediated responses has been proven to be effective in many different experimental systems with mammals (Chen *et al.*, 1994; Chow *et al.*, 1997; Chow *et al.*, 1998; Raz *et al.*, 1993; Scheerlinck *et al.*, 2001; Steidler *et al.*, 1998; Harvill *et al.*, 1996). In one study, the injection of a plasmid that co-expressed both the antigen and IL-2 as fusion proteins enhanced the relative concentration of the anti-antigen antibody by a factor of 300% compared to that obtained by injection of the plasmid that only expressed the antigen (Harvill *et al.*, 1996). Cytokines, in addition to their role as enhancers of immune responses, can also influence the immunogenicity that is induced by direct linkage between the targeting protein and cytokine. This offers the advantage of specifically targeting the cytokine to the antigen-specific B-cell as well as prolonging the in vivo half-life of the cytokine (Chen *et al.*, 1994; Harvill *et al.*, 1996) Using this approach, we have shown that the antibody response to the bacterial K88 fimbrial protein when co-expressed separately with chIL-6 but not as a antigen-chIL-6 fusion protein resulted in a higher level of antibody production than that obtained with the K88 fimbriae protein without IL-6. The effect of the chIL-6 on the level of antibody production, however, was considerably less than that obtained with mammals using IL-2 or IL-4 as a genetic

adjuvants. The reason for the much less dramatic effect obtained with the IL-6 genetic adjuvant as used in the current study compared to that obtained in other studies with mammals was not established. This may be attributed to the inability of chIL-6 to stimulate antibody production in an manner similar to that obtained with IL-4 or it may be attribute to the fact that in the current study the plasmid expressed IL-6 and the K88 fimbrial protein separately, whereas, in the study by Harvill *et al.* (1996), they were coexpressed as a fusion proteins where they may have been more stimulatory. Additional research must be carried out to determine if one or both or neither of these factors are involved.

In addition, we show that the concentration of chIL-6 in the serum, which is enhanced when IL-6 is co-expressed with the antigen protein, in turn, may have influenced the entire immune system. Expressed IL-6 from plasmid DNA has been shown to stimulate the early proliferation of mucosal T-cells or augment B-cell growth and/or immunoglobulin production and induce specific serum IgG and IgA production (Larsen *et al.*, 1998). The enhanced production of IL-6 in chickens injected with the IL-6 producing plasmid may have further stimulated IgY production and therefore the production of high amounts of antibody in the egg yolk.

The results presented in this paper suggest that DNA plasmids have the potential to stimulate the production of a high level of specific antibodies in the egg yolk of immunized laying hens and when used in conjunction with a genetic adjuvant there appears to be a slight increase in antibody titer. Further research must be carried out to optimize dosages, injection times and relative concentration of foreign antigen to genetic adjuvant, to determine if other chicken cytokines are more effective, and to compare response obtained when the antigen and the cytokine are expressed separately or as a fusion protein. The technology offers many advantages compared to the use of protein immunization using antigen and a chemical adjuvant, such as a Freund's adjuvant. This study is the first example of using an avian cytokine for the enhancement of immune response in the chicken for production against avian diseases and for the production of the different types of egg yolk antibodies.

GENERAL DISCUSSIONS

DNA immunization is an important new vaccination strategy, that delivers DNA constructs encoding for a specific immunogen into the host (Pardoll and Beckerleg, 1995; Donnelly *et al.*, 1997). These expression cassettes transfect the host cells, and become the in vivo protein source for the production of antigen. This antigen then is the focus of the resulting immune response. This vaccination technique is being explored as an immunization strategy against a variety of infectious diseases including ETEC. Extensive experiments have shown that DNA vaccines are able to elicit humoral and cellular responses in vivo in a safe and well-tolerated manner in various model systems, including rodents and non-human primates.

Alves *et al.* (1998b) induced an immune response in mice by immunization with a gene capable of expressing the K88 antigen from *E.coli*. Turnes *et al.* (1999) induced the synthesis of protective antibodies in mice using a K88 *fae*G adhesin gene. These initial studies have demonstrated that DNA vaccines are able to elicit immune responses in a safe and well-tolerated manner. However, significant efforts have also focused on improving the immune potency of this technology. Thus, one strategy to enhance immune

responses for DNA-based vaccines is the use of molecular adjuvants (Kim *et al.*, 1998). Constructs of the molecular adjuvant can be co-administered along with immunogen to modulate the magnitude and direction (humoral or cellular) of the immune responses induced by the vaccine itself.

In order to focus the immune responses induced from DNA vaccines, others have investigated the co-delivery of cytokine gene adjuvants to modulate vaccine responses (Chen *et al.*, 1994; Chow *et al.*, 1998; Kim *et al.*, 1998; Kim *et al.*, 2000; Kim *et al.*, 2001; Larsen *et al.*, 1998; Weiss *et al.*, 1998; Wortham *et al.*, 1998). In this study, we also sought to determine whether the enhancement of immune responses observed in mice by the co-immunization of cytokine genes could also be achieved in chickens. DNA vaccines for K88 *fae*G alone were evaluate for their immunogenicity and compared to vaccines which also included IL-2 or IL-4 in mice and IL-6 cytokine cDNA constructs in poultry.

Interleukins from mammals and avian species are class specific, therefore, the mammalian ILs are ineffective in chickens (unpublished data). Recently, the cDNA sequence for chIL-6 was published (Schneider *et al.*, 2001) and a clone was made available to our laboratory. This IL-6 is known to stimulate humoral immune response

and therefore was used as a possible genetic adjuvant in this study. Other chIL may also have stimulating effect and these can be evaluated once their sequences have been reported.

The thesis involved three different manuscripts. The objectives of the first manuscript were to demonstrate if DNA vaccines can be used to produce high amounts of specific antibodies in the yolk of eggs over a relatively long period of time. A second objective was to determine if a combined protein and DNA vaccination would further enhance the production of a high and sustained amount of antibody in the yolk of the egg. The results demonstrated that DNA vaccine can elicit a strong and long-lasting immune response in both chickens and mice; furthermore, the DNA plasmid combined with a protein injection has an ability to enhance the production of antigen-specific antibody.

The objectives of the second manuscript were to test the possibility that a genetic adjuvant can be used to enhance the production of specific antibodies against ETEC (enterotoxigenic *Escherichia coli*) K88 fimbriae in mice after co-injection with the plasmid cDNA(s). Also the study was to compare the effects of IL-2 and IL-4 for the induction of a humoral immune response. The results demonstrated that the plasmid expressing both K88 fimbriae and IL-4 stimulated the highest anti-K88 fimbriae antibody

response, while the plasmid expressing both K88 fimbriae and IL-2 had considerably lower anti-K88 fimbriae antibody response. In addition, the interleukins caused a shift in the type of immunoglobulin produced with IL-4 producing a greater concentration of the IgG1 isotype and IL-2 producing a greater concentration of the IgG2a isotype.

The objectives of third manuscript were to evaluate the effect of chIL-6 as a genetic adjuvant for the enhancement of specific antibody production by the co-injection of both chIL-6 and the K88 *fae*G genes (*p*UM86). The results demonstrated that hens immunized with the plasmid expressing both the fimbriae and a cytokine chicken Interleukin-6 (chIL-6), as a genetic adjuvant stimulated a slightly higher antibody titer and maintained it for a longer period compared with that obtained by the injection of a plasmid encoding only the *fae*G gene. Co-expression of the K88 *fae*G and chIL-6 gene from the same plasmid compared to that obtained with a plasmid expressing only the fimbrial protein and that obtained using K88 fimbriae directly as a protein antigen.

Overall, we found many significant immunologic effects of co-delivering cytokine genetic adjuvants with DNA vaccines for K88 fimbriae. First, the use of synergistic combinations of cytokines was shown to greatly enhance our ability to selectively steer the actions of vaccines toward eliciting only the desired classes of

immune response. More importantly, we demonstrated the power of DNA delivery strategy in inducing significant systemic immune responses without apparent toxicity. We were in fact able to moderately suppress cellular response and dramatically increase the humoral response. Also, the application of DNA vaccines with genetic adjuvants is an alternative strategy for the production of antigen specific egg-yolk antibodies from hyper-immunized hens to the use of conventional procedures such as protein immunization with Freund's adjuvants. This latter procedure is considered to be unsuitable by the CCAC (1993). Moreover, we demonstrated that the co-delivery of the mouse cytokine (IL-4) and to a less or degree the chicken cytokine gene with DNA vaccine is important for the maintenance of high antibody production over relatively long periods of time. Additional studies are needed to further improve the potency and consistency of DNA vaccines, especially those that co-express a cytokine adjuvant.

The research has important implications for the development of the next generations of vaccines as they will provide a safe, effective and inexpensive means for controlling infectious diseases either actively or passively, especially for the production of therapeutic antibodies in the yolk of laying hens.

CONCLUSIONS

On the basis of the results obtained in this thesis it is concluded:

1. DNA vaccines encoding ETEC K88 *fae*G or K99 *fan*C genes evokes a humoral immune response in both mice and chickens and elicits a strong and long-lasting immune response in both chickens and mice
2. The combined injection of a DNA plasmid and the encoded protein further enhances the production of antigen-specific antibodies compared to that obtained by either alone and, therefore, provides a good strategy to maximize antibody production.
3. Co-expression of a foreign protein (antigen) and cytokine from the same plasmid enhances the production of antigen-specific antibody in both mice and chickens compared to that obtained with a plasmid expressing only the antigen.
4. The immunization of mice or chickens with a protein and Freund's adjuvant or with a DNA vaccine that co-expresses both the protein and IL-4 stimulates a high amounts of IgG1 antibody production while immunization with a DNA vaccine encoding only a foreign protein or a DNA plasmid encoding

both the foreign protein and IL-2 results in a shift of antibody isotype production from the IgG1 to the IgG2a subclass.

5. The DNA vaccines encoding the two genes (foreign protein and a cytokine) expressed the two proteins separately in COS-M6 cells after the transfection.
6. Injection of the DNA vaccine expressing chIL-6 enhanced the level of IL-6 in chicken serum
7. A DNA plasmid encoding both the foreign protein and chicken IL-6 enhanced the production of the specific antibody in the yolk of the egg compared to that obtained with the DNA vaccine encoding only the foreign protein.

Suggestions for future research are to:

1. further optimize the time of the second or third booster injections and the dosage required for maximizing the production of antibody;
2. further study the effect of combining the DNA and the protein vaccine on the magnitude of the immune response as measured by antibody titer (this approach could be of considerable benefit);
3. further examine the nature of expressed proteins from a DNA plasmid

encoding a foreign protein and a cytokine, (a comparison of the effects on antibody production following injection of hens with a plasmid that is able to either co-express two separate proteins (antigen and cytokine));

4. study the ability of a genetic adjuvant and a DNA vaccine to provide a greater antibody response in egg yolk than that achieved in this study using other cytokines;
5. determine if the same principles can be applied to other antigens for the control of other pathogens.

Overall, the results of this study suggest that DNA vaccines can be used to enhance specific antibody production, and that the co-expression of a cytokine with a foreign protein can further enhance the production of specific antibodies. Additional research will be required to identify unique cytokines to further improve the enhancement of antibody production in egg yolk.

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