A Plasmid DNA Encoding Chicken Interleukin-6 and *Escherichia coli* K88 Fimbrial Protein FaeG Stimulates the Production of Anti-K88 Fimbrial Antibodies in Chickens

P. Loewen,*[,1] S. H. Cho,* and R. R. Marquardt†

*Department of Microbiology; and †Department of Animal Science, University of Manitoba, 414 Buller Bldg., Winnipeg, Manitoba R3T 2N2, Canada

ABSTRACT Immunization using a plasmid to deliver an encoded protein for expression in situ as the antigen is a promising technology. A plasmid encoding the enterotoxigenic *Escherichia coli* K88 fimbrial protein FaeG when injected into chickens stimulates the production of antibodies against the fimbrial protein, similar to what has been observed in mice. The efficacy of a genetic adjuvant on fimbrial antibody production was tested by introducing the gene for chicken interleukin-6 in tandem with the *faeG* gene. Expression of both the fimbrial FaeG protein and chicken interleukin-6 protein was confirmed in COS-M6 cells. Slightly higher antiFaeG antibody titer in chickens was obtained compared with immunization with the plasmid encoding FaeG alone, especially at 10 (19%, $P < 0.05$) and 12 (27%, $P < 0.05$) wk, respectively, after the secondary immunization. Elevated antiFaeG antibody titer induced by chicken interleukin-6 and FaeG proteins expressed jointly persisted longer than when induced by FaeG protein alone. This is the first report of an avian cytokine enhancing an immune response, and confirms that coexpression of the antigen and adjuvant from a plasmid delivered by DNA immunization is an effective protocol.

(*Key words*: chicken interleukin-6, enterotoxigenic fimbrial protein FaeG, coexpression, antibody titer)

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INTRODUCTION

Diarrhea caused by enterotoxigenic *Escherichia coli* (ETEC) bacteria is a major cause of sickness and death in animals, causing significant economic losses in animal husbandry particularly among young animals directly after birth (neonatal diarrhea) and directly after weaning (postweaning diarrhea) (Kim et al., 1999; Marquardt et al., 1999). Enterotoxigenic *E. coli* 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, 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 to using egg yolk antibodies (IgY) for the therapeutic treatment of intestinal diseases such as those caused by ETEC, including the ease of safely and economically producing an abundant supply of specific, highly effective antibodies (Marquardt et al., 1999).

An immunization strategy involving DNA plasmid or genetic vaccination against ETEC strains has been reported to elicit humoral and cellular immune responses in mice (Alves et al., 1998, 1999a,b; Lasaro et al., 1999). A gene on the plasmid encoding the antigen is expressed, driving the synthesis of the antigen protein within the vaccinated host and this induces production of specific antibodies. The procedure avoids the risks of viral or bacteriological pathogenesis and allows for safe and sustained production of antibodies at relatively low cost. Antigens expressed from plasmid vaccines are much less invasive than protein-containing vaccines particularly when Freund's adjuvants are used. On the other hand, DNA plasmids often do not stimulate the same level of antibody production as conventional protein immunization and a strong humoral immune response is lacking. One solution that has been explored is the coadministration of a cytokine, either directly as the protein or as a genetic adjuvant, along with the antigen-encoding plasmid to enhance an immune response (Raz et al., 1993; Chen et al., 1994; Chow et al., 1997, 1998; Steidler et al., 1998; Scheerlinck et al., 2001). Coinjection of cytokines such as interleukin-2 (IL-2), IL-4, and granulocyte-macro-

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[1]To whom correspondence should be addressed: peter_loewen@umanitoba.ca.

Abbreviation Key: chIL-6 = chicken interleukin-6; ETEC = enterotoxigenic *Escherichia coli*; HRP = horseradish peroxidase; IgY = immunoglobulin Y; IL-2 or 4 = interleukin 2 or 4.

phage colony-stimulating factor with foreign proteins has successfully stimulated both cellular and humoral immune response in mammals, including humans.

Surprisingly, little effort has been made to apply genetic immunization techniques in chickens despite the obvious potential of using egg yolk antibodies as therapeutic agents. Mammalian interleukins, especially IL-2 and IL-4, are ineffective in avian species, whereas IL-6 is a multifunctional cytokine in the avian immune system (Lynagh et al., 2000), possibly with functions similar to those of mammalian cytokines, suggesting its use as an adjuvant for enhancing antibody production. Recently, Schneider et al. (2001) isolated and sequenced chicken IL-6 (chIL-6) cDNA, making possible the construction of an IL-6 genetic adjuvant. Coexpression of the antigen and genetic adjuvant from the same plasmid has been shown to be beneficial (Wortham et al., 1998). In this report, the efficacy of plasmids encoding K88 fimbrial protein FaeG, alone and in tandem with the gene for chicken IL-6, in producing antiFaeG antibody is described.

MATERIALS AND METHODS

Plasmid Construction

Escherichia coli NM522 *sup*E *thi* △ (*lac-proAB*) *hsd-5*[F′ *proAB lacI*^{q} *lacZ* △ 15] (Mead et al., 1985) was grown at 37°C in Luria-Bertoni medium[2] containing ampicillin (100 μg/mL) for plasmid selection. The *p*SLIAgDs expression vector was generously provided by L. A. Babiuk,[3] having been constructed using procedures outlined by Braun et al. (1997) and Van Drunen Little-van den Hurk et al. (1998).

The fimbrial gene *faeG* from *E. coli* strain K88 encoding the fimbrial protein was amplified from pI294 (Isaacson, 1985) using Taq polymerase,[4] the oligonucleotide primers AGGGGTTTATGCTAGCAAAAAGACT and GGATCCGGATCCTTAGTAATAAGT, containing restriction sites for *Nhe*I and *Bam*H I respectively, and 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. The 887-bp *faeG*-containing DNA was inserted into the *Nhe*I and *Bam*H I sites of pSLIAgDs to generate plasmid pUM8. A DNA fragment (1,142 bp) containing the gene for chicken IL-6 was transferred from plasmid pcDNAI/chIL-6-5-1 (Schneider et al., 2001) into pUM8 using *Bam*H I and *Sph*I to generate the plasmid pUM86. All plasmid DNA was purified using Qiagen kits.[5]

Preparation of Purified E. coli K88+ Fimbriae

A local strain of hemolytic ETEC K88 bacteria (Kim et al., 1999) was used as the source for K88+ fimbrial antigen (Fang et al., 2000). Sodium dodecyl sulfate-PAGE (Laemmli, 1970) was used to check purity and size, and identity was confirmed by ELISA (Kim et al., 1999).

Immunization of Chickens

Twenty-week-old Single Comb White Leghorn laying hens 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). Because initial studies (data not shown) determined that intramuscular immunization with pSLIAgD-derived plasmids produced antibody titers similar to intradermal or gene gun immunizations, all immunizations were carried out by intramuscular injection into the pectoral muscle at 2 sites at each time interval using a 25-gauge needle. One hundred fifty or 300 μg of plasmid was injected in a total volume of 300 μL of PBS, pH 7.2, and 150 or 300 μg of fimbrial protein was injected in 300 μL of PBS, pH 7.2, and emulsified with an equal volume of Freund's complete adjuvant[6] for the first injection or 300 μL of Freund's incomplete adjuvant[6] for the second immunization.

A total of 64 hens were assigned to 8 treatment groups with 8 replicates (8 hens per group). Each chicken was administered a primary intramuscular injection of DNA plasmid or protein at zero time and reimmunized with a second dose of the same material 6 wk later. Treatment groups 1 and 2 received 150 and 300 μg of pUM8, respectively; groups 3 and 4 received 150 and 300 μg of K88 fimbrial protein, respectively; and groups 5 and 6 received 150 and 300 μg of pUM86, respectively. Treatment group 7 received 300 μg of pSLIAgDs vector, and treatment group 8 received 300 μL of 0.85% saline alone.

Blood (4 mL) was collected from the wing vein, 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. Eggs were collected every 2 wk after the first immunization. Yolks were separated from the whites, and were diluted 10-fold in PBS for antibody titer determination using ELISA.

Purification of Chicken IL-6 and Antibody Preparation

A DNA fragment encoding chIL-6 was cloned from pcDNAI/chIL-6-5-1 into pBluescript KS M13+ using *Bam*H I and *Xho*I to generate pKS6. Protein expression in *E. coli* strain NM522 was induced by 0.1 m*M* isopropyl-beta-D-thiogalactopyranoside. The expressed chIL-6 that accumulated in the bacteria was purified using ammonium sulfate fractionation (Ehn et al., 2001). The purified chIL-6 (100 μg/mL) was emulsified with Freund's complete adjuvant for the first injection of 100 μL per mouse and rabbit and 2 wk later with Freund's incomplete adjuvant for the second injection of 100 μL per mouse and rabbit. The mice and rabbits were injected intramuscularly at 4 sites and serum was collected 4 wk after the

[2]GIBCO-BRL, Burlington, ON, Canada.
[3]University of Saskatchewan, SK, Canada.
[4]Invitrogen, Burlington, ON, Canada.
[5]Qiagen Inc., Mississauga, Ontario, Canada.
[6]Sigma Chemical Co., St. Louis, MO.

first immunization (2 wk after the second immunization), and stored at –20°C.

Analysis of Protein Expression in COS-M6 Cells

Plasmids were transfected in duplicate using 1 μg per 5 $\times 10^5$ COS-M6 cells using the Lipofectamine-Plus reagent. The supernatants were harvested at 3 d posttransfection and analyzed by ELISA for the presence of specific proteins.

Antibody Assays in the Egg Yolk

Egg yolks were analyzed for antiK88 fimbrial antibody titer using a modification of the method of Kim et al. (1999). Wells of Microtest III flexible assay plates[7] (Falcon 3911) were coated overnight at 4°C with 0.1 μg of the fimbrial antigen suspended in 100 μL of PBS. Incubation with 5% (wt/vol) skim milk in PBS was followed by washing with PBST **[AUTH QUERY: spell out PBST]**. The washed plates were inoculated with dilutions of egg yolk (100 μL) and kept for 2 h at 37°C. After washing, 100 μL of alkaline phosphatase-conjugated rabbit antichicken IgY,[8] diluted 1:5,000, was added as the indicator antibody. The plates were then incubated for 2 h at 37°C followed by addition of p-nitrophenyl phosphate and incubation for 40 min at 37°C. Quantification was performed at 405 nm, and titers were 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 titers of antiK88 fimbrial protein antibody.

Determination of Cytokine Levels

Cytokines in culture supernatant and serum were determined by modified direct ELISA using mouse antichIL-6 as capture antibody and horseradish peroxidase (HRP)[6] conjugated rabbit IL-6 antibodies, prepared in a 2-step glutaraldhyde reaction from purified serum (Harlow and Lane, 1998). The wells of a 96-well microplate[7] (Falcon 3911) were coated with 100 μL of mouse antichIL-6 (5 μg/mL), diluted in 0.05 *M* bicarbonate buffer (pH 9.6), and incubated overnight at 4°C. This concentration of antibodies was found to be optimal concerning 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, incubated at 37°C for 2 h and then washed 3 times with PBST. One hundred microliters of HRP-antichIL-6, diluted 1:20,000 to 1:40,000 in PBS, was added to each well and incubated at 37°C for 1 h before addition of 100 μL per well of indicator solution containing 400 μL of 0.6% 3,3′,5,5′-tetramethylbenzidine[6] in dimethylsulfoxide and 100 μL of 0.1% hydrogen peroxide in 25 mL of citrate-acetate buffer, pH 5.5. After incubation for 20 to 30 min at 37°C, the reaction was quenched by the addition of 50 μL of 2 *M* H_2SO_4 to each well and quantification was carried out at 450 nm. The standard curve allowed calculation of [chIL-6] in serum using the relationship $f(y) = 0.23 \times f(x) + 0.35$ ($R^2 = 0.98$), where f(y) is the titer response as A_{450}, and f(x) is the [chIL-6].

Western Immunoblot Analysis

Protein fractions were separated by SDS-PAGE (Laemmli, 1970) on 4–15% gradient gels and transferred to nitrocellulose using a semidry transfer apparatus. Membranes were probed with chicken antiK88 fimbrial antibody, or mouse antichIL-6 antibody followed by goat antichicken IgY alkaline phosphatase-labeled antibody[8] or goat antimouse IgG alkaline phosphatase-labeled antibody.[8]

Statistical Analysis

Statistical analysis was done by the paired Student *t*-test and ANOVA (SAS/STAT, 1989). 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 Fimbrial and Cytokine Proteins

DNA plasmids expressing the K88 fimbrial antigen alone (pUM8 containing *faeG*, encoding the K88 fimbrial antigen) and in combination with chicken IL-6 (pUM86 containing *faeG* and the gene for chicken IL-6) in transfected eukaryotic cells and in chickens were constructed (Figure 1). Expression of the fimbrial antigen and chIL-6 from the plasmids was confirmed in COS-M6 cells, where the concentration of K88 antigen 3 d after transfection with pUM8 and pUM86 was 45.2 ± 6.0 and 33.1 ± 11.1 ng/mL, respectively, and the concentration of chIL-6 was undetectable for pUM8 and 8.1 ± 1.1 ng/mL for pUM86.

Chicken IL-6 expressed in *E. coli* was purified through ammonium sulfate fractionation yielding a 27-kDa product (Figure 2, lanes a to d), the same as reported by Schneider et al. (2001). A Western immunoblot analysis of the culture supernatant from COS-M6 cells transfected with the pUM86 revealed a strong band of protein cross-reacting with antiK88 fimbrial antibody (Figure 2, lane e) and 2 bands cross-reacting with antichIL-6 antibody (Figure 2, lane f). The apparent sizes of both proteins expressed in COS cells were larger than when expressed in *E. coli*; FaeG increased to 32 from 29 kDa and chIL-6 increased to 32 and 36 kDa from 27 kDa, probably the result of posttranslational modification (Wortham et al.,

[7]Becton Dickinson Labware, Oxnard, CA.
[8]The Jackson Immunolaboratory, West Grove, PA.

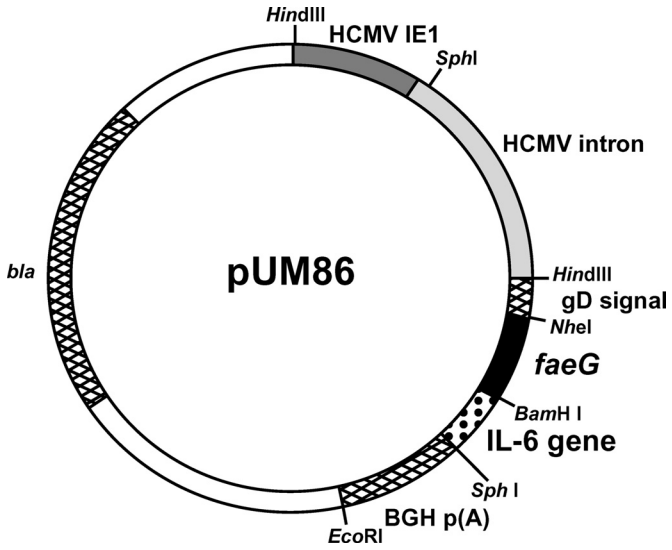

FIGURE 1. Restriction map of pUM86. A DNA fragment encoding chicken interleukin-6 (chIL-6) was inserted between the *Bam*H I and *Sph*I restriction sites of *p*UM8. The plasmid contains the *Escherichia coli* origin of replication and a gene for ampicillin resistance (*bla*) to allow preparation in *E. coli*, the regulated human cytomegalovirus immediate-early promoter (HCMV IE1) with an HCMV intron that enhances efficient transport of the mRNA out of the nucleus, the signal sequence from gD protein of bovine herpes virus, and a polyadenylation sequence 3′ to the gene insertion site (BGH p(A). The locations of the fimbrial protein gene (*fae*G) and chIL-6 genes are indicated.

1998). Immunoblot analysis of supernatant proteins from nontransfected COS-M6 cells did not reveal any cross-reacting bands for either chIL-6 or K88 fimbriae (data not shown).

The concentration of chIL-6 in the sera of laying chickens was increased at 8 wk following immunization with pUM86 (Figure 3) but not with pUM8. This level had dropped by 12 wk, but remained higher than that with pUM8. Fimbrial protein antigen in Freund's adjuvant caused an increase in chIL-6 equivalent to that of pUM86 at 12 wk.

Antibody Responses in Laying Hens

An initial review of the antiFaeG antibody titer in egg yolks revealed that there was no effect of plasmid or protein dosage ($P < 0.05$), allowing the data for 150 and 300 μg of plasmid or protein (treatment groups 1 and 2 receiving pUM8, groups 5 and 6 receiving pUM86, and groups 3 and 4 receiving K88 fimbrial protein) to be pooled into single data sets. The protein antigen produced a much faster response than either of the plasmid injections, which were very similar in the first 8 wk (Figure 4). At wk 2, 4, and 6, the protein antigen produced titers that were 2.0, 3.4, and 1.3 times higher ($P < 0.05$) than the plasmid titers, respectively. At 8 wk, all titers were about the same, and at 10 and 12 wk, no significant differences were obvious between the protein and pUM8 immunized groups. At wk 12, however, eggs from hens immunized with the pUM86 had titers 1.2 times higher ($P < 0.05$) than those from pUM8 immunization, and 1.4 times higher (P

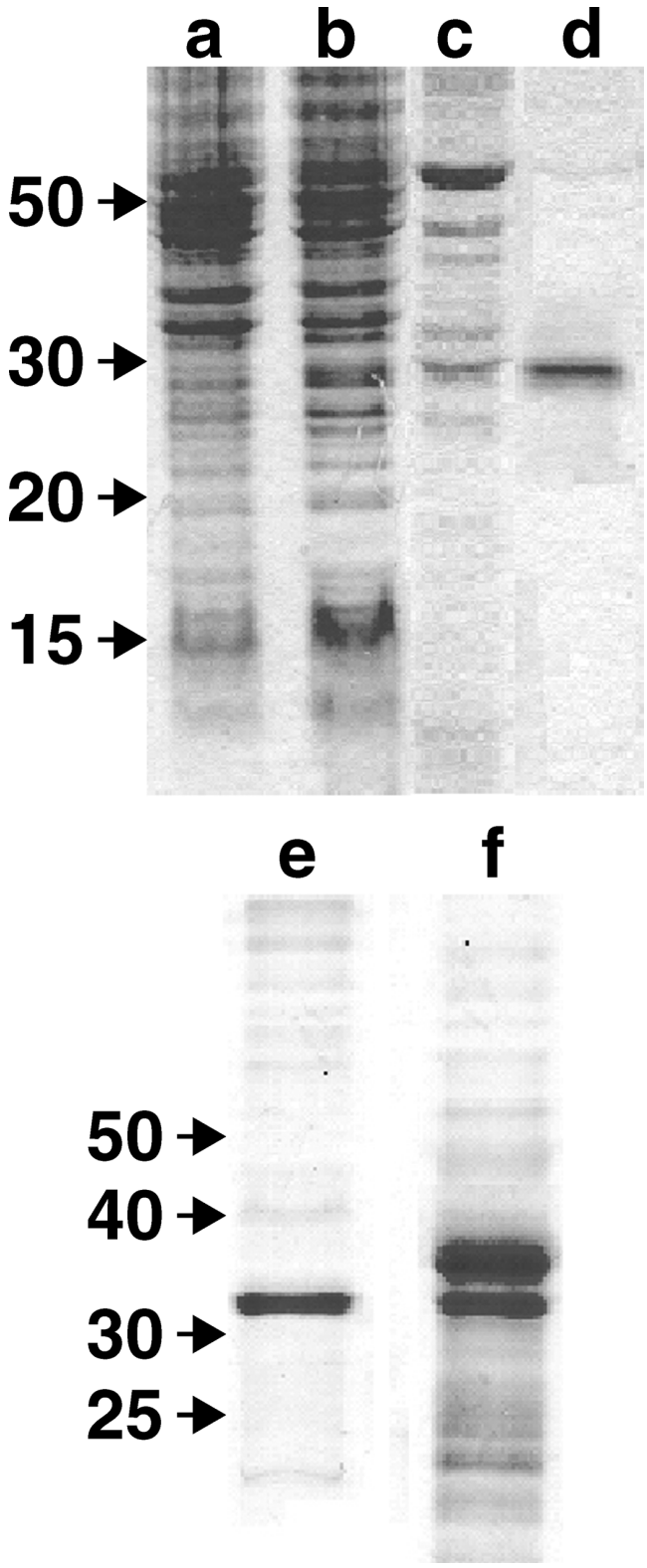

FIGURE 2. Sodium dodecyl sulfate-PAGE gel of various fractions from the purification of chicken interleukin-6 (chIL-6) expressed from pKS6. Molecular weight size markers (kDa) are indicated on the left. Lane a: *Escherichia coli* crude extract before induction; Lane b: *E. coli* crude extract after induction with isopropyl-beta-D-thiogalactopyranoside; Lane c: Crude chIL-6 after sonication and centrifugation; Lane d: Purified chIL-6 after ammonium sulfate precipitation; Lane e: Western blot of culture medium from COS-M6 cells transfected with pUM86 using antiK88 antibodies; Lane f: Western blot of culture medium from COS-M6 cells transfected with pUM86 using antichIL-6 antibodies.

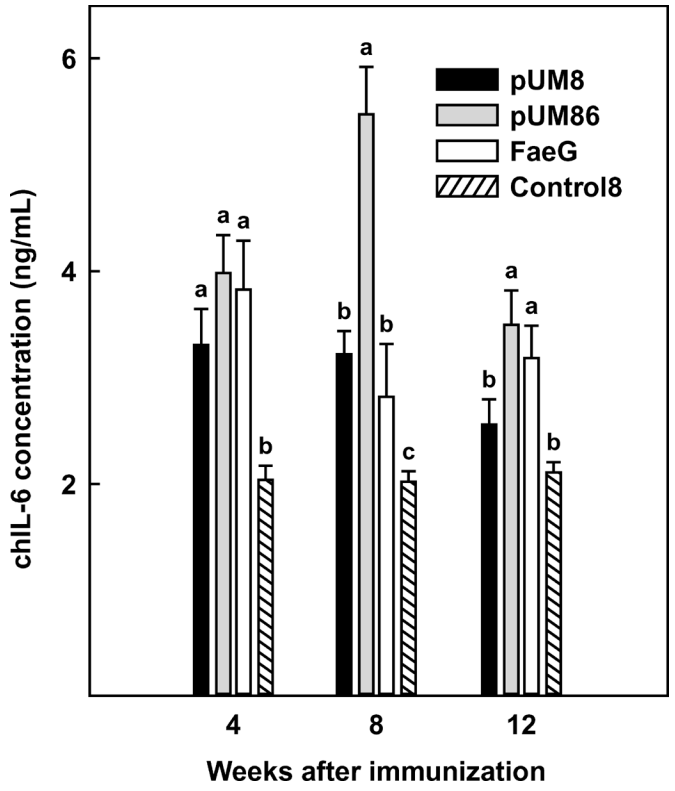

FIGURE 3. The level of chicken interleukin-6 (chIL-6) in the serum of laying hens immunized on wk 0 and 6 are indicated for pUM8 (black), pUM86 (gray), K88 fimbrial protein (white), and saline or pSLIAgDs as control (hatched). Values represent mean ± SE of 6 replicates and values for each treatment group for each week having different letters (a, b, c) are different ($P < 0.05$).

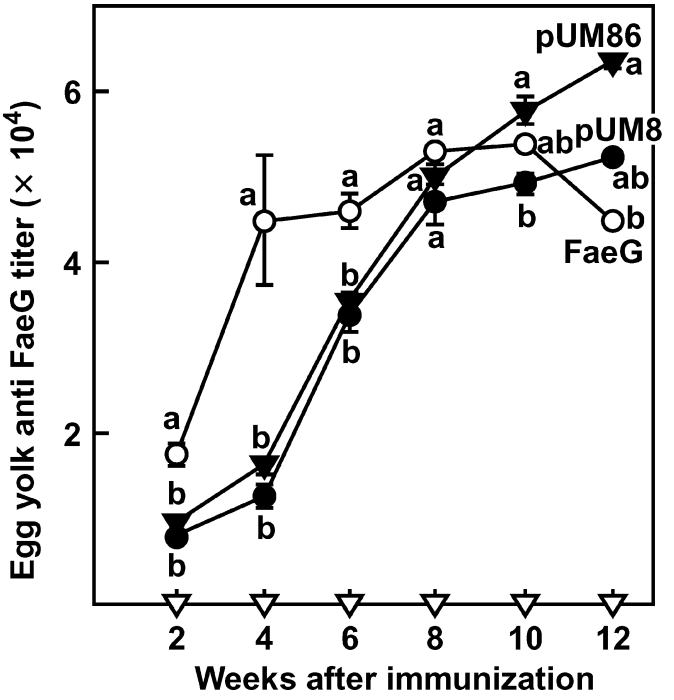

FIGURE 4. Relative antibody concentration in the yolks of eggs from hens immunized on weeks 0 and 6 with pUM8 (●), *p*UM86 (▲), K88 fimbriae protein (○), and saline or pSLIAgDs (control, ▽). Values represent mean ± SE of 8 replicates. Error bars smaller than symbols are not shown. Values for each treatment group for each week having different letters (a, b, c) are different ($P < 0.05$).

< 0.05) than those from protein immunization. Chickens injected with either saline or the pSLIAgDs, lacking the K88 *fae*G gene, did not produce antiK88 antibody.

DISCUSSION

Plasmids encoding fimbrial protein from enterotoxigenic bacteria are effective agents for the production of antifimbrial protein antibodies in chickens and their eggs. The technology offers many advantages compared with protein immunization, including the avoidance of the time-consuming and potentially dangerous task of preparing large amounts of the fimbrial protein from pathogens, the elimination of adjuvants such as Freund's, and the longer response time. The benefits of using antibody-containing eggs for oral immunization of pigs is well established, and DNA immunization will enhance the technique immensely, making possible the production of many different types of antibodies in egg yolk. The pattern of antibody induction by the plasmid, lower relative to protein immunization initially but higher later on, is probably a result of more protein antigen being initially available to stimulate immunity when the antigen is directly injected rather than expressed from plasmid. However, protein continues to be expressed from the DNA plasmid for a longer period providing for longer-term stimulation of antibody production. Optimization of dosages and injection times remain to be determined.

This study provides the first report of an enhancement of an immune response in the chicken using an avian cytokine. In addition to the stimulation of antibody production in egg yolks, this demonstration suggests the potential for combating avian diseases through cytokine treatment. The use of cytokines as agents to enhance humoral and cell-mediated responses has been effective in mammals (Raz et al., 1993; Chen et al., 1994; Harvill et al., 1996; Chow et al., 1997; 1998; Steidler et al., 1998; Scheerlinck et al., 2001). Furthermore, fusion of the antigen with IL-2 enhanced antiantigen antibody levels by 300% (Harvill et al., 1996), offering 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). The fact that the fimbrial antigen was not fused to chIL-6 may explain the lower response in this study compared with mammalian systems and suggests one approach to enhancing the response in the chicken system. Alternatively, chIL-6 may simply be less efficient at stimulating antibody production than IL-4 in mammalian systems. Not withstanding the ineffectiveness of mammalian IL-2 in avian systems and the fact that IL-2 is mainly associated with cellular immune responses, it may be appropriate to investigate the effectiveness of avian IL-2 in chickens.

The fact that the levels of chIL-6 in the serum are increased after immunization with a plasmid encoding the cytokine demonstrates clearly that it is expressed, but

also raises the expectation that it may be influencing the entire immune system. For example, plasmid-encoded IL-6 stimulates mucosal T and B cell growth and induces specific serum IgG and IgA production (Larsen et al., 1998). Whether plasmid-encoded chIL-6 has such a general effect in chickens remains to be determined, but the influence on antifimbrial antibody suggests this may be the case.

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