Adjuvant Effects of Various Lipopeptides and Interferon-γ on the Humoral Immune Response of Chickens

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ABSTRACT The adjuvant effects of various lipopeptides and recombinant chicken interferon γ (IFN-γ) on the humoral immune response of laying hens was investigated in four immunization studies. We used the lipopeptide Pam_3Cys-Ser-$(Lys)_4$ (PCSL), the conjugate P-T_{h1} consisting of the lipopeptide P_3CS and the T-helper epitope T_{h1} (FISEAIIHVLHSRHPG), and the conjugate P-T_{h2} of the lipopeptide P_3CSS and the T-helper epitope T_{h2}, which corresponds to the peptide EWEFVNTPPLV, as adjuvants. Human serum albumin (HSA), recombinant bovine somatotropin (RBST), and human immunoglobulin G (IgG) served as antigens in the different experiments. All tested adjuvants enhanced the humoral immune response with various intensities. Chickens showed high antibody titers after the immunization with HSA even without adjuvant, but the adjuvant effects of PCSL and the combination of PCSL and recombinant chicken interferon-γ (IFN-γ) were much more pronounced using the antigens RBST and IgG. Especially after the third immunization, higher titers of antibodies were induced by the coadministration of P-T_{h1} and, to a greater extent, by the combination of PCSL and P-T_{h1} compared with the use of PCSL. Also, chickens that had received PCSL and P-T_{h2} showed the highest immune response, even after the second booster. The average concentrations of chicken immunoglobulin Y were significantly higher in 5-mo-old chickens (9.4 mg/mL serum and 10.1 mg/mL egg yolk) compared with 9-mo-old chickens (5.9 mg/mL serum and 5.1 mg/mL egg yolk). The specific serum antibody response was higher in the older chickens than in the younger chickens. Because chicken antibodies are likely to be used increasingly for diagnostic and therapy in the future, lipopeptides and recombinant chicken IFN-γ may find many applications as adjuvants, thus contributing to the welfare of experimental animals.

(*Key words*: chicken, adjuvant, egg yolk antibodies, lipopeptide, interferon-γ)

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INTRODUCTION

The use of chicken antibodies instead of antisera of mammalian origin is very attractive because these antibodies can be obtained by simple collection of eggs without taking blood (Schade et al., 1996). Large amounts of immunoglobulin Y (IgY) found in the egg yolk have led to the development of several immunological test systems (Bar-Joseph and Malkinson, 1980; Larsson et al., 1991; Ntakarutimana et al., 1992; Sturmer et al., 1992; Larsson and Lindahl, 1993). Additionally, egg powder with specific antibodies to enterotoxic *Escherichia coli* and rotavirus was used for prevention and therapy of infectious diarrhea (Ikemori et al., 1992; Yokoyama et al., 1992; Erhard et al., 1993, 1996; Kellner et al., 1994; Özpinar et al., 1996).

Vaccine improvement is frequently accompanied by the loss of epitopes and immunogenicity of the molecules used as antigens. On the other hand, for scientific purposes, antibodies against such substances are often required. Thus, there is an increasing need for effective adjuvants that are applicable together with various antigens to different animal species and that cause only minor side effects. Freund's adjuvant, which is generally accepted and the most common adjuvant used in laboratory animals, leads to severe inflammation at the injection site and sometimes to extensive systemic disorders as shown in chickens (Schmidt et al., 1996; Wanke et al., 1996).

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Abbreviation Key: G1-POD = peroxidase-conjugated mouse monoclonal anti-chicken IgY antibody; HSA = human serum albumin; IFN-γ = recombinant chicken interferon-γ; IgG = human immunoglobulin G; IgY = chicken immunoglobulin Y; PCSL = Pam_3Cys-Ser-$(Lys)_4$; P-T_{h1} = lipopeptide P_3CS with the T-helper epitope T_{h1}; P-T_{h2} = lipopeptide P_3CSS with the T helper epitope T_{h2}; RBST = recombinant bovine somatotropin.

By using the lipohexapeptide Pam_3Cys-Ser-$(Lys)_4$ (PCSL) synthesized by Wiesmüller et al. (1983), excellent adjuvant effects can be achieved in chickens (Hofmann et al., 1996; Erhard et al., 1997). Various amino acids were attached to the basic compound Pam_3Cys, which corresponds to the biologically active N-terminus of *Escherichia coli* lipoprotein. Stimulatory effects on the humoral immune response in other species have been investigated in other species with the lipopeptides P-T_{h1}, comprising the lipopeptide P_3CS and the T-helper epitope T_{h1} (FJSEAIIHVLHSRHPG), which corresponds to a segment of sperm whale myoglobin (AS 106–121), and P-T_{h2}, comprising the lipopeptide P_3CSS and the T-helper epitope T_{h2}, which corresponds to the peptide EWEFVNTPPLV (AS 413–423) of the reverse transcriptase of the *HIV*-1 strain BRU (Mittenbühler et al., 1997).

Cytokines such as interferon-γ (IFN-γ) are natural mediators of the immune system. Their immune-stimulating activities are well known, and their adjuvant effects have been shown in various animals (Health et al., 1989; Cao et al., 1992; Health and Playfair, 1992; Coobold et al., 1994). Chicken IFN-γ displays functions that are analogous to its mammalian counterpart. After cloning chicken IFN-γ (Digby and Lowenthal, 1995; Weining et al., 1996), there are many possibilities for using recombinant chicken IFN-γ. Lowenthal et al. (1998a,b) showed that chicken IFN-γ acts as an adjuvant in immunization studies with sheep red blood cells.

The purpose of this study was to examine different lipopeptides, recombinant chicken IFN-γ, and their combinations for their adjuvant effects in laying hens by using protein antigens.

MATERIALS AND METHODS

Immunization of Chickens

All experiments were conducted with commercial 5- to 9-mo-old laying hens,[2] and all antigen preparations were given as an i.m. injection in the pars thoracica profunda of the musculus pectoralis (0.5-mL injection volume per chicken).

In the first experiment (n = 4 to 5 per group; Table 1), the adjuvant effect of recombinant chicken IFN-γ, alone or in combination with the lipopeptide PCSL,[3] was investigated using the human serum albumin (HSA)[4] as antigen at a concentration of 1 mg per injection volume. The doses of IFN-γ ranged from 10^3 to 10^5 U per injection (10^3 U of the laboratory IFN-γ standard corresponded to 1.3 μg IFN-γ). Chicken IFN-γ was produced according to the method developed by Weining et al. (1996) and has been shown to be biologically active in vivo by Plachy et al. (1999). The lipopeptide PCSL was dissolved in physiological saline solution and was used at 250 μg per i.m. injection. After 4 and 8 wk, the booster injections were given in the same way.

In the second experiment (n = 6 per group; Table 1), human immunoglobulin G (IgG)[5] served as the antigen (100 μg/injection). Chickens were grouped according to immunization (i.m.) with PCSL (250 μg/injection), IFN-γ (1,000 U/injection), or with a combination of both adjuvants. The booster injections were given in the same way after 4 and 8 wk.

In the third experiment (n = 5 per group; Table 1), recombinant bovine somatotropin (RBST)[6] was used as antigen (1 mg/injection). The lipopeptides PCSL and P-T_{h2} were tested in doses of 250 μg per i.m. injection. The dose of the combination of the two lipopeptides was 125 μg each. Additionally, IFN-γ (6,000 U/injection) was used alone or in combination with PCSL. After 4 and 8 wk, the booster injections were carried out in the same way.

In the fourth experiment (n = 6 per group; Table 1), the adjuvant effects of PCSL, P-T_{h1}, and the combination of the two lipopeptides were investigated after i.m. immunization with RBST. The adjuvant doses were 250 μg, and a concentration of 1 mg per injection volume of antigen was used. The booster injections after 4 and 8 wk were given only with PCSL in all experimental groups treated with lipopeptides.

Determination of Antibody Titers by ELISA

Specific antibody titers in chicken sera for the antigens were determined by ELISA. Primary antibody response to antigens was not examined, because in previous studies (e.g., Erhard et al., 1997), only low antibody titer levels were achieved in that phase.

Microtiter plates[7] were coated with antigens (10 μg HSA/mL, 5 μg RBST/mL, or 5 μg IgG/mL; each 200 μL per well) that were dissolved in coating buffer (0.1 mol/L carbonate buffer; pH 9.6). After incubation overnight at 4 C, the plates were blocked with 4% milk powder (HSA) or 1% bovine serum albumin (RBST and IgG) in PBS (pH 7.2; 200 μL per well) for 1 h at 37 C. The serum and egg yolk samples were incubated for 1 h at 37 C in $\log_2$ series (each 100 μl per well; in PBS-Tween 20, pH 7.2) starting at a dilution of 1:100 (HSA) or 1:250 (RBST, IgG). Binding of specific antibodies to the immobilized antigen was determined with a peroxidase-conjugated mouse monoclonal anti-chicken IgY antibody [G1-POD (Erhard et al., 1992); 1:15,000, 100 μl per well]. According to Erhard et al. (1992), G1-POD showed no cross-reactivity with immunoglobulin M. The enzyme reaction was made visual with tetramethylbenzidine[4] (0.2 mg/mL in 0.1 mol/L acetate citrate buffer, pH 5, with 0.0005% H_2O_2; 100 μL per well) and was measured at 450 nm with an ELISA reader[8] after stopping the reaction with H_2SO_4 (1 mol/L, 50 μL per well, after 10 min). The antibody titers (ELISA units/mL) were calculated at an absorbance of 0.1.

[2]Lohmann Selected Leghorn, Lohmann LSL-Rhein Main, D-64809 Dieburg, Germany.
[3]Boehringer Mannheim, Mannheim D-68298, Germany.
[4]Sigma Chemicals, D-82041 Deisenhofen, Germany.
[5]Therasorb, Unterschleißheim D-85716, Germany.
[6]Monsanto, D-40470 Düsseldorf, Germany.
[7]Maxisorb; Nunc, Wiesbaden-Biebrich D-65203, Germany.
[8]Tecan, D-74564 Crailsheim, Germany.

TABLE 1. Treatment groups (n = 4 to 6 laying hens) of the four experiments using the antigens human serum albumin (HSA), human immunoglobulin G (IgG), and recombinant bovine somatotropin (RBST) and the adjuvants $Pam_3Cys\text{-}Ser\text{-}(Lys)_4$ (PCSL), P_3CS with the T-helper epitope T_{h1} ($P\text{-}T_{h1}$), P_3CSS with the T-helper epitope T_{h2} ($P\text{-}T_{h2}$), and recombinant chicken interferon-γ (IFN-γ)[1]

Experiment 1 6-mo-old chicken antigen HSA (1 mg) n = 4 to 5 per group	Experiment 2 7-mo-old chicken antigen IGG (100 μg) n = 6 per group	Experiment 3 5-mo-old chicken antigen RBST (1 mg) n = 5 per group	Experiment 4 9-mo-old chicken antigen RBST (1 mg) n = 6 per group
PBS (control)	PBS (control)	PBS (control)	PBS (control)
PCSL (250 μg)	PCSL (250 μg)	PCSL (250 μg)	PCSL (250 μg)
IFN-γ (10^3 U)	IFN-γ (10^3 U)	$P\text{-}T_{h2}$ (250 μg)	$P\text{-}T_{h1}$ (250 μg)
IFN-γ (10^4 U)	PCSL (250 μg) + IFN-γ (10^3 U)	PCSL (125 μg) + $P\text{-}T_{h2}$ (125 μg)	PCSL (250 μg) + $P\text{-}T_{h1}$ (250 μg)[2]
IFN-γ (10^5 U)		IFN-γ (6×10^3 U)	
PCSL (250 μg) + IFN-γ (10^3 U)		PCSL (250 μg) + IFN-γ (6×10^3 U)	
PCSL (250 μg) + IFN-γ (10^4 U)			
PCSL (250 μg) + IFN-γ (10^5 U)			

[1]The immunizations were given by i.m. injections (0.5 mL). The booster injections were carried out 4 and 8 wk after the first immunization.
[2]Booster injections with PCSL (250 μg) alone.

Determination of Immunoglobulin Y in the Serum and Egg Yolk

Total IgY concentrations in serum and pure egg yolk were determined using the ELISA described by Erhard et al. (1992). No additional extraction of IgY was necessary.

Histopathology and Pathology

After the immunization period (Week 11, respectively 12), all chickens were euthanized, and necropsy was performed to evaluate organ alterations. For light microscopy, samples from the pectoral muscle of selected chickens were taken at the site of injection, fixed in 7% neutral-buffered formalin, processed routinely, and embedded in paraffin. Three-micrometer sections were stained with hematoxylin and eosin.

Statistical Analysis

Each adjuvant group was compared with the corresponding control group of the experiment. Differences among serum antibody titers were analyzed by an unpaired, nonparametric test (Mann-Whitney, one-tail P value). The significance level for all analyses was $P < 0.10$ or $P < 0.05$.

RESULTS AND DISCUSSION

Adjuvant Effects on the Antibody Titers

The ability of IFN-γ to enhance the humoral immune response in chickens was shown by Lowenthal et al. (1998a) in a previous study with sheep red blood cells as antigen. The authors described one of the likely modes of adjuvant action as the ability of IFN-γ to activate macrophages by inducing the secretion of cytokines such as interleukin 1 and the secretion of nitric oxide intermediates. Additionally, T cells may enhance antibody production. Murine IFN-γ inducers immunoglobulin IgG2a production. However, in chickens no IgY subclasses are described. Therefore, induction of the synthesis of IgY subclasses cannot be measured or used to discriminate T_{h1}- and T_{h2}-type immune responses.

The mode of adjuvant action of lipopeptides in chickens is not known. In other species, the ability of lipopeptides to enhance the secretion of cytokines, nitric oxide, and prostaglandins has been described. Bessler et al. (1985), Scheuer et al. (1986), and Bessler and Jung (1992) were the first to describe lipopeptide adjuvanticity as an unspecific insertion of the adjuvant into the membranes of leukocytes. The authors suggested that an early step in leukocyte activation may involve intracellular binding of proteins or a direct interaction of the lipopeptides with nuclear components. Lipopeptides have been demonstrated to be active mitogens and polyclonal B lymphocyte activators. These functions are due to their N-terminal lipopeptide region containing three fatty acids. The lipopeptides are designated as $P\text{-}T_{h1}$ and $P\text{-}T_{h2}$ according to experiments in mammalian species. Thus as discussed with IFN-γ (see above), a T_{h1}- or T_{h2}-type immune response of chickens, resulting in a cell-mediated versus an antibody-mediated response, cannot be postulated with these lipopeptides.

In Experiment 1, the changes of HSA-specific antibody titers in the serum of immunized chickens were investigated (Figures 1 and 2). The lipopeptide PCSL or 1,000 U IFN-γ alone had no adjuvant effect with this type of antigen. The highest titers, which were mostly significantly different from the PBS control group, were achieved with the combination of PCSL and IFN-γ 1 wk after the first and second booster injections (Figure 1). A dose-dependent effect of IFN-γ could not be demonstrated (Figure 2). The antibody titers after application of 1,000, 10,000, or 100,000 U IFN-γ per injection varied only marginally. With the combination of identical doses of IFN-γ and 250 μg PCSL, no differences were achieved. The highest titers were reached 1 wk after the first and second booster injections. Therefore, the prefered dose of IFN-γ in combination with 250 μg PCSL was 1,000 U per injection.

Experiment 2 was set up to verify the results of Experiment 1 using human IgG as the antigen. Again, the combined use of 250 μg PCSL and 1,000 U IFN-γ caused significantly stronger immunostimulation than the in PBS control group. The highest titers were reached 1 wk after

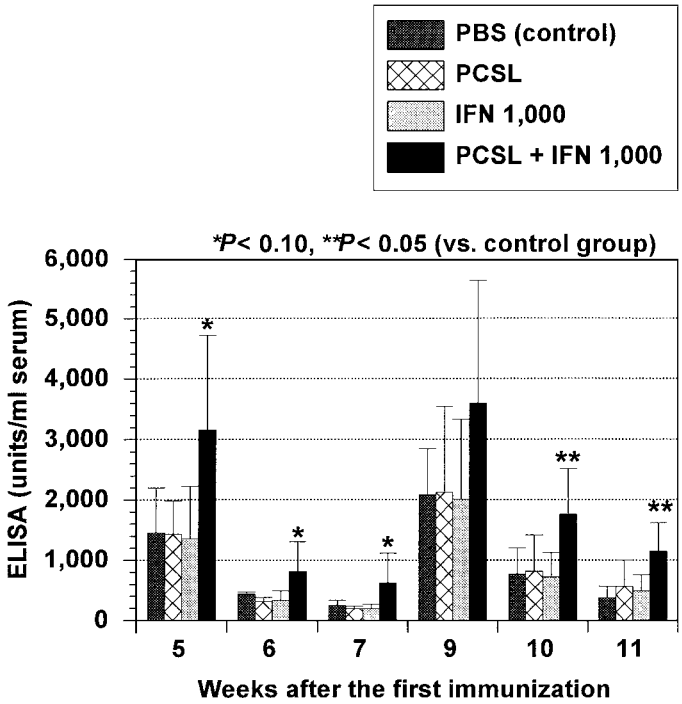

FIGURE 1. Adjuvant effects of the lipopeptide $Pam_3Cys\text{-}Ser\text{-}(Lys)_4$ (PCSL; 250 μg per injection), recombinant chicken interferon-γ (IFN; 1,000 U/injection; corresponds to 1.3 μg), and their combination on human serum albumin (HSA; 1 mg/injection)-specific IgY titer in chicken sera (n = 4 to 5 per group; mean and SD). The control group received only HSA in PBS buffer. The 6-mo-old chickens (Lohmann Selected Leghorn) were immunized i.m. in Weeks 0, 4, and 8 (Experiment 1).

the first and second booster injections (Figure 3). In contrast to the results with HSA, PCSL alone had an adjuvant effect 1 wk after the first booster. An adjuvant effect of IFN-γ could also be observed 1 wk after the first booster.

Following the results of Lowenthal et al. (1998b), the dose of IFN-γ was increased to 6,000 U per injection in Experiment 3. By using RBST as the antigen, the antibody responses confirmed the results mentioned previously. Again, the highest titers were reached 1 wk after the first and second booster injections. The combination of PCSL and 6,000 U IFN-γ induced the highest titers, which were significantly different from the PBS control group (Figure 4). Thus, it could be demonstrated that the combination of both adjuvants induced the best immunostimulation.

Two additional groups in Experiment 3 received the lipopeptide $P\text{-}T_{h2}$ with or without PCSL as adjuvant. Again, the highest titers were shown 1 wk after the first and second booster injections. Only the combination of both lipopeptides enhanced the titers significantly compared with the PBS control group (Figure 5).

In contrast to $P\text{-}T_{h2}$, the lipopeptide $P\text{-}T_{h1}$ showed a significant adjuvant effect when given alone (Experiment 4). Especially after the second booster injection, higher antibody titers were obtained with the application of $P\text{-}T_{h1}$ and, to a greater extent, from the combination of PCSL and $P\text{-}T_{h1}$ compared with the use of PCSL (Figure 6).

According to Bollen and Hau (1999), the specific antibody response differs between 5- and 18-mo-old chickens. The specific serum antibody response was consistently lower in the younger chickens than in the older chickens. As shown in Figure 7, the antibody titers of RBST were higher in the 9-mo-old chickens than in 5-mo-old chickens. This age effect was most prominent after the second booster immunization. By using PCSL as an adjuvant, age-dependent differences were not found.

According to the literature, many different types of compounds have been used as adjuvants, including oil emulsions such as Freund's adjuvant. The strong adjuvanticity of oil adjuvants is correlated to the depot effect, which results in encapsulation of the antigen by the nonbiodegradable mineral oil. Thus, the antigen is probably protected from elimination when specific circulating antibodies are present. The persistance of specific antibody titers over a long period after application of mineral oil is also attributed to the protracted local immunological stimulation and is due to permanent release of antigen from the oily material deposit and to the formation of chronic inflammation at the site of injection. Other categories of adjuvants, as investigated in the present study, do not have such a depot effect. For that reason, high but not sustained antibody responses with the different adjuvants and their combinations were achieved after one or two subsequent injections (Figures 1 to 6; primary response is not shown in figures). The highest titers were reached 1 wk after the first and second booster injections in all experiments. The

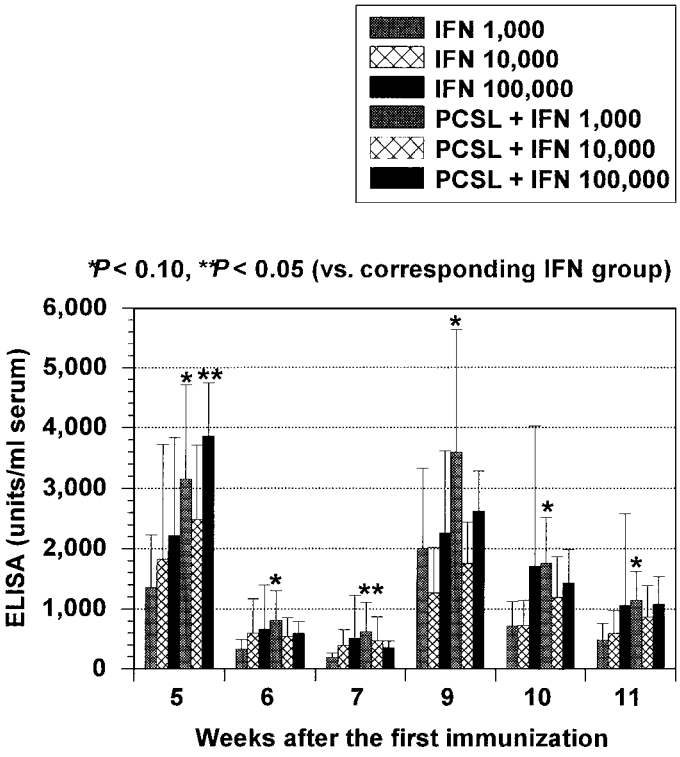

FIGURE 2. Dose-dependent adjuvant effects of recombinant chicken interferon-γ (IFN; 1,000 to 100,000 U/injection) with or without $Pam_3Cys\text{-}Ser\text{-}(Lys)_4$ (PCSL; 250 μg/injection) on human serum albumin (HSA; 1 mg/injection)-specific IgY titer in chicken sera (n = 4 to 5 per group; mean and SD). The 6-mo-old chickens (Lohmann Selected Leghorn) were immunized i.m. in Weeks 0, 4, and 8 (Experiment 1). The corresponding PBS control is shown in Figure 1.

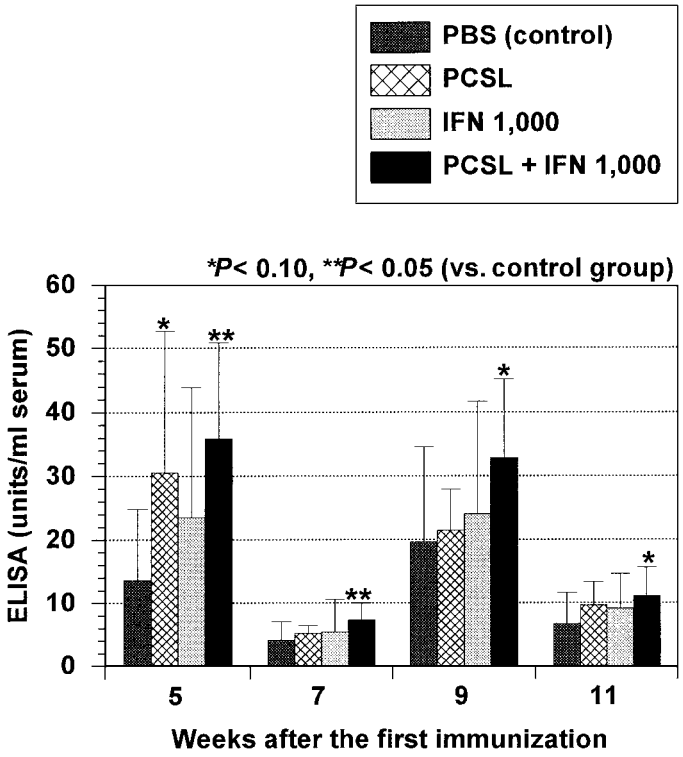

FIGURE 3. Adjuvant effects of the lipopeptide $Pam_3Cys\text{-}Ser\text{-}(Lys)_4$ (PCSL; 250 μg/injection), recombinant chicken interferon-γ (IFN; 1,000 U/injection), and their combination on human immunoglobulin G (IgG; 100 μg/injection)-specific IgY titer in chicken sera (n = 6 per group; mean and SD). The control group received only IgG in PBS buffer. The 7-mo-old chickens (Lohmann Selected Leghorn) were immunized i.m. in Weeks 0, 4, and 8 (Experiment 2).

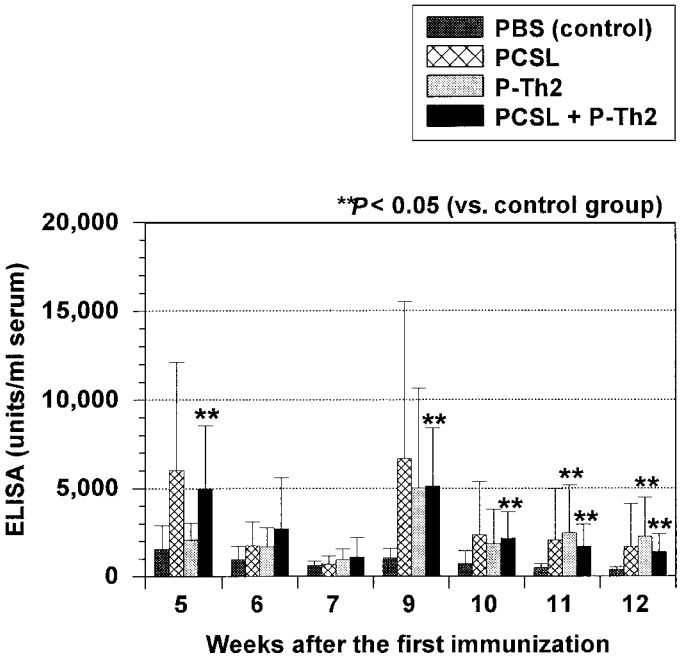

FIGURE 5. Adjuvant effects of the lipopeptides $Pam_3Cys\text{-}Ser\text{-}(Lys)_4$ (PCSL; 250 μg/injection), P-T_{h2} (T-helper epitope EWEFVNTPPLV), and their combination on recombinant bovine somatotropin (RBST; 1 mg/injection)-specific IgY titer in chicken sera (n = 5 per group; mean and SD). The control group received only RBST in PBS buffer. The 5-mo-old chickens (Lohmann Selected Leghorn) were immunized i.m. in Weeks 0, 4, and 8 (Experiment 3).

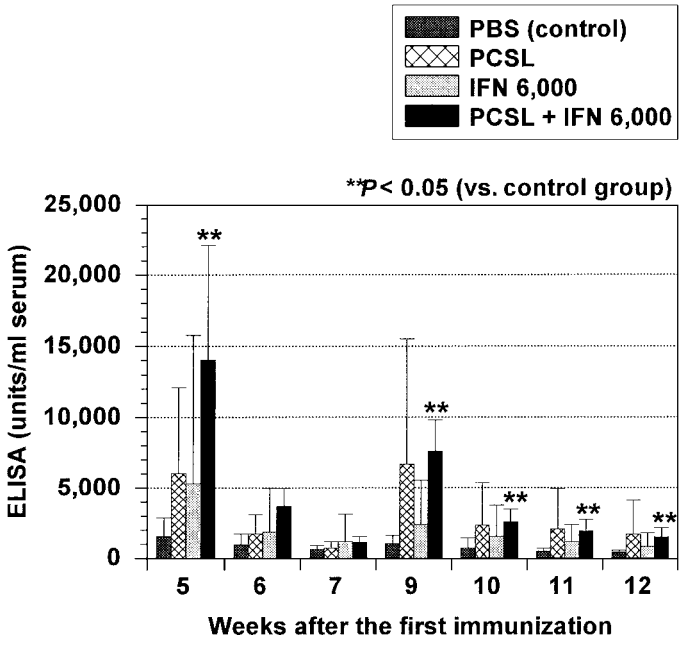

FIGURE 4. Adjuvant effects of the lipopeptide $Pam_3Cys\text{-}Ser\text{-}(Lys)_4$ (PCSL; 250 μg/injection), recombinant chicken interferon-γ (IFN; 6,000 U per injection), and their combination on recombinant bovine somatotropin (RBST; 1 mg/injection)-specific IgY titer in chicken sera (n = 5 per group; mean and SD). The control group received only RBST in PBS buffer. The 5-mo-old chickens (Lohmann Selected Leghorn) were immunized i.m. in Weeks 0, 4, and 8 (Experiment 3).

results shown in Figure 7 demonstrate the efficacy of secondary responses after booster injections, but a sustained secondary immune response was absent because of the missing depot effect of the adjuvants used.

Immunoglobulin Y

The concentrations of total IgY, which were determined during the immunization period with the antigen RBST, are given as means. Significant differences existed between the preimmune samples of the two experiments with regard to the serum concentrations and the quantitative transfer of IgY into the yolk. Before immunization the mean serum IgY values were 9.4 mg/mL in 5-mo-old chickens (Experiment 3) and 5.9 mg/mL in 9-mo-old chickens (Experiment 4). In egg yolk samples, the IgY concentrations were 10.1 mg/mL in the younger chickens and 5.1 mg/mL in the older chickens. No significant changes in the IgY serum and IgY egg yolk levels were found after immunization in any of the groups. According to Erhard et al. (1997), the influence of the immunization on the total IgY concentrations present in sera depended on the adjuvant used. Freund's complete adjuvant enhanced the IgY concentrations after immunization, resulting in a maximal IgY concentration after the second booster. In contrast, IgY concentrations in the egg yolks were similar in the PCSL groups and groups treated with Freund's complete adjuvant.

Histopathology and Pathology at the Injection Site

In all groups treated with the lipopeptide adjuvants or IFN-γ, necropsy did not reveal specific organ lesions or gross pathological alterations at the site of injection. As shown by Wiedemann et al. (1991) in mice after lipopeptide administration, minor proliferation of the local lymphoid tissue was detected by light microscopy in the chickens. In contrast, Wiedemann et al. (1991) found enhanced antibody levels using lipopeptide adjuvants comparable to these achieved using Freund's adjuvant. However, Freund's adjuvant caused pronounced alterations, as shown in previous studies (Schmidt et al., 1996; Wanke et al., 1996; Erhard et al., 1997). Multiple miliar granulomas and multiple abscesses in the pectoral muscle were found, and focal necrosis and chronical granulomatous and fibrosing myositis with pseudoeosinophilic granulocytes, lymphocytes, epitheloid cells, multinucleated giant cells of the foreign body type, and plasmocytes developed. The comparatively higher circulating IgY concentrations and the persistence of specific antibody titers over a long period after application of Freund's adjuvant are attributed to a protracted local immunological stimulation caused by permanent release of antigen from the oily depot and to formation of a chronical inflammation at the site of injection.

The present investigation clearly shows that the humoral immune response of immunized chickens varies according to the type of antigen and to the type of adjuvant used as an immune stimulant. The mechanisms involved in the biological activity of immunoadjuvants represent an integrated cascade of events depending upon a large number of factors that are often unknown. In our study, secondary humoral immune responses in chickens were enhanced with various intensities, but none reached an antibody titer as high as that sustained by oil adjuvants. The best results were achieved by combining different adjuvants, such as lipopeptides and recombinant chicken IFN-γ. Data interpretation of the presented results is somewhat limited according to the numbers of animals used per group. Nevertheless, the initial results of our study show that the ability of the tested adjuvants to enhance antibody responses makes them excellent candidates for use as adjuvants in poultry vaccines due to their excellent purity, their lack of toxicity, their stability, their easy and reproducible chemical preparation in large amounts, and the minor local irritation of tissues. Further studies comparing the adjuvants investigated here and commercial adjuvants must be performed on more animals. However, in view of the imminent need of poultry vaccines, the continued search for new adjuvants is both necessary and highly justified.

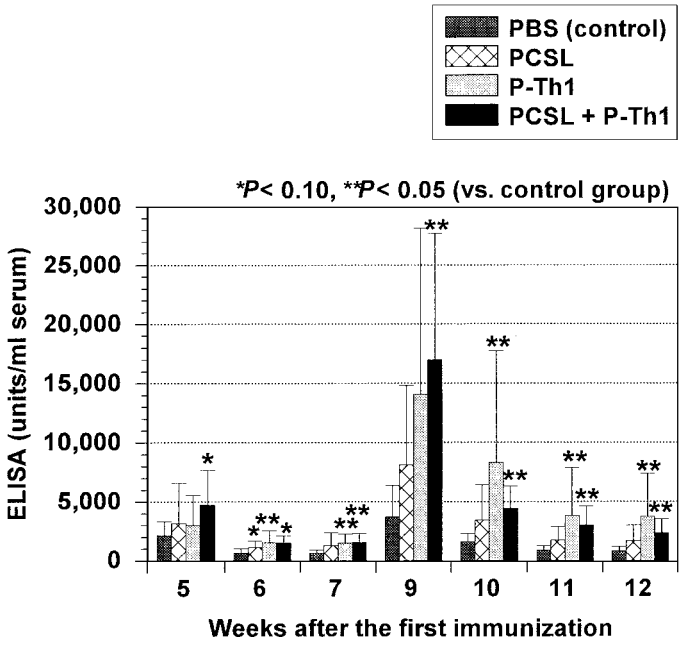

FIGURE 6. Adjuvant effects of the lipopeptides $Pam_3Cys\text{-}Ser\text{-}(Lys)_4$ (PCSL; 250 μg/injection), $P\text{-}T_{h1}$ (T-helper epitope FISEAIIHVLHSRHPG), and their combination on recombinant bovine somatotropin (RBST; 1 mg/injection)-specific IgY titer in chicken sera (n = 6 per group; mean and SD). The control group received only RBST in PBS buffer. The 9-mo-old chickens (Lohmann Selected Leghorn) were immunized i.m. in Weeks 0, 4, and 8 (Experiment 4).

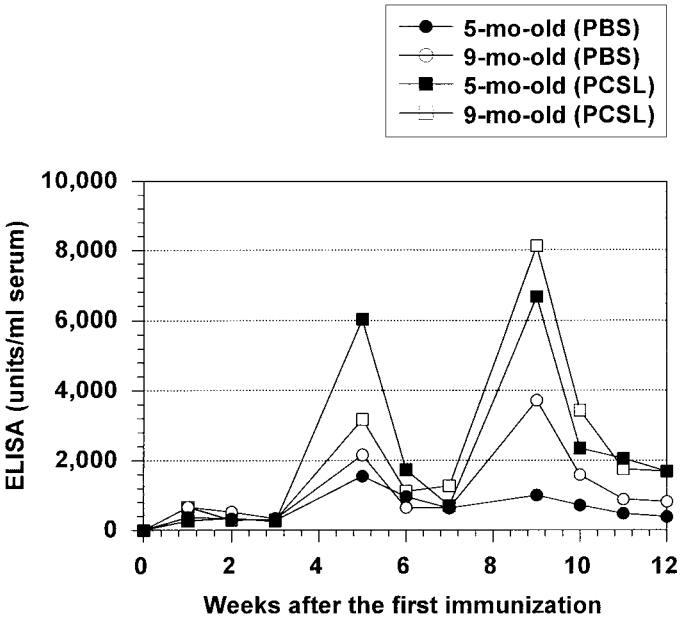

FIGURE 7. Antibody titers to recombinant bovine somatotropin (RBST; 1 mg/injection) in 5- and 9-mo-old chickens using PBS in the antigen control group or the lipopeptide $Pam_3Cys\text{-}Ser\text{-}(Lys)_4$ (PCSL; 250 μg/injection) as adjuvant (n = 6 per group; mean and SD). The chickens (Lohmann Selected Leghorn) were immunized i.m. in Weeks 0, 4, and 8 (Experiments 3 and 4).

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REFERENCES

Bar-Joseph, M., and M. Malkinson, 1980. Hen egg yolk as a source of antiviral antibodies in the enzyme-linked immunosorbent

assay (ELISA): A comparison of two plant viruses. J. Virol. Methods 1:179–183.

Bessler, W. G., M. Cox, A. Lex, B. Suhr, K.-H. Wiesmüller, and G. Jung, 1985. Synthetic lipopeptide analogs of bacterial lipoprotein are potent polyclonal activators for murine B lymphocytes. J. Immunol. 135:1900–1905.

Bessler, W. G., and G. Jung, 1992. Synthetic lipopeptides as novel adjuvants. Res. Immunol. 143:548–553.

Bollen, L. S., and J. Hau, 1999. Comparison of immunospecific antibody response in young and old chickens immunized with human IgG. Lab. Anim. 33:71–76.

Cao, M., O. Sasaki, A. Yamada, and J. Imanishi, 1992. Enhancement of the protective effect of inactivated influenza virus vaccine by cytokines. Vaccine 10:238–242.

Coobold, S., M. Holmes, and B. Willett, 1994. The immunology of companion animals: Reagents and therapeutic strategies with potential veterinary and human clinical applications. Immunol. Today 15:347–353.

Digby, M. R., and J. W. Lowenthal, 1995. Cloning and expression of chicken interferon-γ gene. J. Interferon Cytokine Res. 15:939–945.

Erhard, M. H., J. Bergmann, M. Renner, A. Hofmann, and K. Heinritzi, 1996. Prophylaktische Wirkung von spezifischen Dotterantikörpern bei Escherichia coli K88 (F4)-bedingten Durchfallerkrankungen von Absatzferkeln. (Prophylactic effect of specific egg yolk antibodies in diarrhea of weaned piglets caused by *Escherichia coli* K88 (F4)). J. Vet. Med. Ser. A 43:217–223.

Erhard, M. H., J. Kellner, J. Eichelberger, and U. Lösch, 1993. Neue Möglichkeiten in der oralen Immunprophylaxe des Kalbes—Ein Feldversuch mit spezifischen Eiantikörpern (New aspects in oral immunprophylaxis for the prevention of infectious diarrhea of newborn calves—A field study with specific antibodies). Berl. Münch. Tierärztl. Wochenschr. 106:383–387.

Erhard, M. H., P. Schmidt, A. Hofmann, J. Bergmann, P. Mittermeier, P. Kaufmann, K.-H. Wiesmüller, W. G. Bessler, and U. Lösch, 1997. The lipopeptide Pam_3Cys-Ser-$(Lys)_4$: An alternative adjuvant to Freund's adjuvant for the immunisation of chicken to produce egg yolk antibodies. Alternatives to Laboratory Animals (ATLA) 25:173–181.

Erhard, M. H., I. von Quistorp, I. Schranner, A. Jüngling, B. Kaspers, P. Schmidt, and R. Kühlmann, 1992. Development of specific enzyme-linked immunosorbent antibody systems for the detection of chicken immunoglobulins G, M, and A using monoclonal antibodies. Poultry Sci. 71:302–310.

Health, A. W., M. E. Devey, I. N. Brown, C. E. Richards, and J.H.L. Playfair, 1989. Interferon-gamma as an adjuvant in immunocompromised mice. Immunology 67:520–524.

Health, A. W., and J.H.L. Playfair, 1992. Cytokines as immunological adjuvants. Vaccine 10:427–434.

Hofmann, A., M. H. Erhard, P. Schmidt, W. Bessler, P. Zinsmeister, M. Stangassinger, and U. Lösch, 1996. Lipopeptide als Adjuvantien zur Immunisierung von Legehennen. (Lipopeptides as adjuvants for the immunization of laying hens). Alternativen Tierversuch. (ALTEX) 13:26–29.

Ikemori, Y., M. Kuroki, R. Peralta, and Y. Yokoyama, 1992. Protection of neonatal calves against fatal enteric colibacillosis by administration of egg powder from hens immunized with K99-pilated enterotoxic Escherichia coli. Am. J. Vet. Res. 53:2005–2008.

Kellner, J., M. H. Erhard, M. Renner, and U. Lösch, 1994. Therapeutischer Einsatz von spezifischen Eiantikörpern bei Saugferkeldurchfall—Ein Feldversuch. (A field trial of the treatment of diarrhea in piglets with specific antibodies). Tierärztl. Umsch. 49:31–34.

Larsson, A., A. Karlsson-Parra, and J. Sjöquist, 1991. Use of chicken antibodies in enzyme immunoassays to avoid interference by rheumatoid factors. Clin. Chem. 37:411–414.

Larsson, A., and T. Lindahl, 1993. Chicken anti-Protein G for the detection of small amounts of protein G. Hybridoma 12:143–147.

Lowenthal, J. W., T. E. O'Neil, M. Broadway, A. David, G. Strom, M. R. Digby, M. Andrew, and J. J. York, 1998a. Coadministration of IFN-γ enhances antibody responses in chickens. J. Interferon Cytokine Res. 17:617–622.

Lowenthal, J. W., J. J. York, T. E. O'Neil, R. A. Steven, D. G. Strom, and M. R. Digby, 1998b. Potential use of cytokine therapy in poultry. Vet. Immun. Immunopathol. 63:191–198.

Mittenbühler, K., M. Loleit, W. Baier, B. Fischer, E. Sedelmeier, G. Jung, G. Winkelmann, C. Jacobi, J. Weckesser, M. H. Erhard, A. Hofmann, W. G. Bessler, and P. Hofmann, 1997. Drug specific antibodies: T-cell epitope-lipopeptide conjugates are potent adjuvants for small antigens in vivo and in vitro. Int. J. Immunopharmacol. 19:277–287.

Ntakarutimana, V., P. Demedts, and M. van Sande, 1992. A simple and economical strategy for downstream processing of specific antibodies to human transferrin from egg yolk. J. Immunol. Meth. 153:133–140.

Özpinar, H., M. H. Erhard, N. Aytug, A. Özpinar, C. Baklaci, S. Karamüptüglu, A. Hofmann, and U. Lösch, 1996. Dose dependent effects of specific egg-yolk antibodies on diarrhea of newborn calves. Prev. Vet. Med. 27:67–73.

Plachy, J., K. C. Weining, E. Kremmer, F. Puehler, K. Hala, B. Kaspers, and P. Staeheli, 1999. Protective effects of type I and type II interferons toward rous sarcoma virus-induced tumors in chickens. Virology 256:85–91.

Schade, R., C. Staak, C. Hendriksen, M. H. Erhard, H. Hugl, G. Koch, A. Larsson, W. Pollmann, M. van Regenmortel, E. Rijke, H. Spielmann, H. Steinbusch, and D. Straughan, 1996. ECVAM workshop on avian antibody (egg yolk antibody, IgY). Alternatives Lab. Anim. (ATLA) 24:925–934.

Scheuer, W. V., L. Biesert, and W. G. Bessler, 1986. Binding of a synthetic analogue of mitogenic bacterial lipoprotein to murine major histocompatibility complex (MHC) gene products. Biol. Chem. Hoppe-Seyler 367:1085–1094.

Schmidt, P., M. H. Erhard, R. Wanke, A. Hofmann, and W. Schmahl, 1996. Gewebsveränderungen nach Anwendung verschiedener Adjuvantien für die Immunisierung. (Local reactions after the application of several adjuvants in the chicken). Alternativen Tierversuch. (ALTEX) 13:30–34.

Sturmer, A. M., D. P. Driscoll, and D. E. Jackson-Matthews, 1992. A quantitative immunoassay using chicken antibodies for detection of native and recombinant alpha-amidating enzyme. J. Immunol. Methods 146:105–110.

Wanke, R., P. Schmidt, M. H. Erhard, A. Sprick-Sanjose Messing, M. Stangassinger, W. Schmahl, and W. Hermanns, 1996. Freundsches komplettes Adjuvans beim Huhn: Effiziente Immunstimulation bei gravierender lokaler inflammatorischer Reaktion. (Freunds complete adjuvant in the chicken: efficient immunostimulation but severe local inflammation). J. Vet. Med. A 43:243–253.

Wiedemann, F., R. Link, K. Pumpe, U. Jacobshagen, H. E. Schaefer, K.-H. Wiesmüller, R.-P. Hummel, G. Jung, W. G. Bessler, and T. Böltz, 1991. Histopathological studies on the local reactions induced by complete Freund's adjuvant (CFA), bacterial lipopolysaccharide (LPS), and synthetic lipopeptide (P3C) conjugates. J. Pathol. 164:265–271.

Weining K. C., U. Schulz, U. Münster, B. Kaspers, and P. Staeheli, 1996. Biological properties of recombinant chicken interferon-γ. Eur. J. Immunol. 26:2440–2447.

Wiesmüller, K.-H., W. G. Bessler, and G. Jung, 1983. Synthesis of the mitogenic S-[2,3,-Bis(palmitolyloxy)propyl]-N-palmitoylpentapeptide from *Escherichia coli* lipoprotein. Physiol. Chem. Hoppe-Seyler 364:593–606.

Yokoyama, H. R., R. C. Peralta, R. Diaz, S. Sendo, Y. Ikemori, and Y. Kodama, 1992. Passive protection effect of chicken egg yolk immunoglobulins against experimental enterotoxic Escherichia coli infection in neonatal piglets. Infect. Immun. 60:998–1007.