Adjuvant Effect of Chicken Interferon- γ for Inactivated *Salmonella* Enteritidis Antigen

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(Received 28 February 2003/Accepted 20 August 2003)

ABSTRACT. The adjuvant effect of chicken interferon- γ (ChIFN- γ) was examined for protecting chickens against intestinal colonization of *Salmonella* Enteritidis (SE) following oral exposure. Ten 7-week-old chickens per group were immunized with inactivated SE twice with or without co-administration of ChIFN- γ intramuscularly, and all chickens were challenged with SE. Sera collected from immunized groups with or without ChIFN- γ , and from unimmunized group were measured for SE antibody by agglutination test. The levels of antibodies were raised by 1 week post-immunization and did not show any difference between groups with and without ChIFN- γ . No antibodies were detected in unimmunized group before challenge. Fecal samples from each group were cultured at 1, 4, 7, and 13 days post-challenge to determine the incidence of intestinal colonization and the numbers of SE shed into the environment. Co-administration of ChIFN- γ significantly reduced the incidence of intestinal colonization ($P < 0.05$). At 13 days post-challenge, the bacterial counts of SE in organs were also reduced in ChIFN- γ administered group. These data suggest co-administration of ChIFN- γ with SE antigen enhances protection against SE challenge without acceleration of antibody production.

KEY WORDS: adjuvant, avian, interferon- γ , recombinant, *Salmonella*.

J. Vet. Med. Sci. 65(12): 1337–1341, 2003

Cytokines, as the natural modulators of the immune response, are obvious candidates for vaccine adjuvants. Interleukin 12 (IL-12) and interferon gamma (IFN- γ) are strong adjuvant candidates for both general enhancement of the immune response induced by a vaccine antigen and alteration of the type of immune response generated. These responses lead to the establishment of more desirable effector mechanisms and greater protection against disease [1, 2, 4, 14, 15, 22].

Since the gene coding a chicken IFN- γ (ChIFN- γ) was isolated and sequenced by Digby and Lowenthal [3], many reports have been published to produce recombinant ChIFN- γ s [3, 8, 20, 21, 24]. Lowenthal *et al.* [11] showed enhancement of antibody responses in chickens co-administered of ChIFN- γ with sheep red blood cells. Lowenthal *et al.* [12] also reported *in vivo* effects of ChIFN- γ administration during infection with *Eimeria*. So far most reports on ChIFN- γ and disease deal with coccidiosis [9, 10, 25]. In mammalian systems, IFN- γ has been shown to be important for *Salmonella* infection [16, 19]. Kaiser *et al.* [7] investigated the changes in cytokine expressions after *in vitro* invasion of primary chicken kidney cell cultures by *Salmonella* Typhimurium, *Salmonella* Enteritidis (SE) and *Salmonella* Gallinarum. They showed the invasion had little effect on the production of IFN- γ , but caused increase in production of IL-6.

Quite recently we produced and purified a bioactive recombinant ChIFN- γ using a baculovirus expression system. Here, we describe co-administration of the recombinant ChIFN- γ with *Salmonella* antigen in chickens.

MATERIALS AND METHODS

Recombinant ChIFN- γ : A recombinant ChIFN- γ that has the 12 C-terminal amino acid cleaved and bears poly-His tag at C-terminal (ChIFN- γ THis) was expressed using a baculovirus system [21].

***Salmonella* antigen preparation:** HY-1 strain of SE phage type 4 [17] was cultured with heart infusion broth (Eiken, Tokyo, Japan) for 3 hr. The bacterial growth was harvested in phosphate buffered saline (PBS) and adjusted to 10^9 colony-forming units (CFU)/ml and inactivated with 0.5% formalin at 37°C for 72 hr, then stored at 4°C. Just before use, 0.5 ml of the inactivated bacteria was spun down, rinsed with PBS, and resuspended in 4 ml of PBS and used for SE antigen.

Experimental design: For co-administration of antigen with IFN- γ , 1 ml of the purified ChIFN- γ THis at 10^6 arbitrary unit (AU)/ml [21] was mixed with 4 ml of SE antigen. For immunization with antigen alone, 1 ml of PBS was mixed with 4 ml of SE antigen. Thirty layer chickens at the age of 7 weeks were divided into 3 groups (10 birds/group). Group 1 (cytokine group) was immunized intramuscularly with 0.5 ml of the mixture of antigen and ChIFN- γ THis, thus corresponding with 5×10^7 CFU antigen and 10^5 AU IFN- γ were administered per bird. Group 2 (antigen group) was immunized intramuscularly with 0.5 ml of SE antigen containing 5×10^7 CFU. Group 3 (control group) was injected with PBS only. One week later, all groups were immunized again at the same dose and the same route as primary immunization. At 1 week after 2nd-immunization, all birds were orally challenged with 10^8 CFU of live rifampicin-resistant mutant of SE HY-1 [17] and cecal droppings

were collected for SE culture at 1, 4, 7, 10, and 13 days post-challenge. At 13 days post-challenge, all birds were euthanized by cervical dislocation, and samples of the liver, spleen, and cecal contents were collected.

Bacterial count: Bacterial counts in each sample were carried out as previously described [17]. Briefly, all samples including cecal droppings were diluted 1:10 with Hajna tetrathionate broth (Eiken) and homogenized. The mix was serially diluted 10-fold in PBS, and 25 μ l of each dilution was spread onto desoxycholate-hydrogen sulfide-lactose agar (Eiken) plates containing 100 μ g rifampicin/ml (DHL-rif). Samples, both plates and tetrathionate enrichments, were incubated at 37°C for 24 hr, and bacterial counts were made. The log number of viable counts per gram of samples and standard deviation were calculated. The sensitivity of detection was 400 cells per gram of the samples. For the direct enrichment culture (one-day-enrichment), the broth was incubated at 41°C for 24 hr. The broth was then held at 25°C for 5 days for delayed secondary enrichment (DSE). One ml of the broth was added to a fresh tube containing 9 ml of Hajna tetrathionate broth and incubated at 37°C overnight. One loopful of each broth was then streaked onto DHL-rif and incubated at 37°C. The identity of prospective SE colonies was confirmed biochemically and serologically. When SE colonies were detected from one-day-enrichment or DSE, the SE counts in the original samples were calculated as 100 CFU/g or 10 CFU/g, respectively.

Testing for specific antibodies against SE: Serum samples collected every week were assayed for the presence

of specific O antibodies in a microagglutination test by using Pullorum Disease diagnostic antigen (PDA) purchased from Chiba Serum Institute (Chiba, Japan). Antibody titers were determined by serial twofold dilution of an initial 1:5 dilution of each serum sample.

Statistical analysis: Significant differences between treatment groups were determined by analysis of variance using ANOVA with Bonferroni for bacterial counts from samples.

RESULTS

Serum antibody titer: The O antibody titers of the immunized hens rose quickly by 1 week post-immunization, reaching peak levels at 2 weeks post immunization. Little difference between the groups 1 and 2 (the cytokine group and the antigen group) could be observed (Fig. 1). No antibody titers were detectable in unimmunized control hens. After challenge exposure, antibody titers of immunized hens were decreased slightly, while those of control hens rose, reaching the same level as the immunized groups by the day of sacrifice.

Recovery of SE from cecal samples: Figure 2 shows frequency of recovery of SE from cecal droppings. These data are based on the results obtained in direct counts, one-day-enrichment, and DSE cultures. The bacterial counts were significantly reduced in cecal samples from the group 1 (the cytokine group) at 1, 7, 10, and 13 days post-challenge ($P < 0.05$ or $P < 0.01$) compared to those of the group 3 (the control group). At 1 and 7 days post-challenge, the counts in samples from group 1 were significantly lower ($P < 0.05$)

Fig. 1. Serum antibody titers of chickens immunized with *Salmonella* Enteritidis bacterins. Sera collected weekly following immunization were tested for antibody titers via a microagglutination test using Pullorum Disease diagnostic antigen. Mean titers of 10 chickens are shown. Immunization and challenge schedule are also shown.

Fig. 2. Recovery of *Salmonella* Enteritidis from cecal droppings post-challenge. Results represent mean log₁₀CFU/g. Significant differences ($P < 0.05$) between the groups 1 and 2, 1 and 3 or 2 and 3 were shown as 1*2, 1*3 or 2*3, respectively. Significant difference ($P < 0.01$) between the groups 1 and 3 was shown as 1**3.

Fig. 3. Recovery of *Salmonella* Enteritidis from organs and cecal contents. Organ samples or cecal contents were taken at 13 days post-challenge and bacterial counts in each sample were performed as described in the text. Results represent mean $\pm$ SE log₁₀CFU/g. Asterisks indicate significant differences between the groups (* $P < 0.05$).

than those from the group 2 (the antigen group).

Recovery of SE from organs: At 13 days post-challenge, all birds were sacrificed and samples of liver, spleen, cecal contents were obtained and bacterial counts in each sample were determined. As shown in Fig. 3, no bacteria were detected in livers from any chickens. The SE challenge

organism was also not detected in spleens from the group 1 (the cytokine group) but was detected in spleens from the group 2 (the antigen group) and the group 3 (the control group) ($P < 0.05$). In the cecal contents, there were no significant differences between the groups, however, the number of SE counts was the smallest in the group 1 and the highest

in the group 3.

DISCUSSION

We have previously shown that a recombinant ChIFN- γ that has the 12 C-terminal amino acid cleaved and bears poly-His tag at C-terminal (ChIFN- γ THis) could be purified and that the addition of His-tag to the truncated C-terminal seemed not to affect the biological activity of the ChIFN- γ *in vitro* [21]. In the present study, we showed the adjuvant activity of ChIFN- γ THis against SE antigen *in vivo*. Farnell *et al.* [5] reported that a recombinant-turkey IFN- γ administered to newly hatched chicks reduced SE organ invasion and suggested a potential acute immunostimulatory activity of IFN- γ . However, this protection was efficient only at 24 hr post-challenge. They did not check the adjuvant activity of IFN- γ since these newly hatched chicks seemed to possess an immature immune response, and adjuvant activity would not be likely in these chicks. In the present study, we used 7-week-old chickens that possess a more mature immune system and therefore be more immuno-reactive. Immunization of chickens with inactivated SE antigen induced humoral immunity in the chickens and co-administration of ChIFN- γ with SE antigen did not appear to increase antibody levels over those obtained by antigen alone (Fig. 1). Lowenthal *et al.* [11] showed ChIFN- γ adjuvant activity for sheep red blood cells to increase the humoral immune response especially after secondary immunization. In our study, we immunized chickens on two consecutive weeks but the antibody titer was not significantly different between the group 1 (the cytokine group) and the group 2 (the antigen group). However, SE counts in cecal droppings of the group 1 (the cytokine group) was significantly reduced compared to the group 3 (the control group) at 1, 7, 10, and 13 days post-challenge, and also with the group 2 (the antigen group) at 1 and 7 days post-challenge (Fig. 2). SE counts in spleen were also significantly reduced in the group 1 compared with the group 3 and SE counts in cecal contents were numerically lower in the group 1 versus the group 2 (Fig. 3). IFN- γ has been demonstrated to possess potent stimulatory effects on cell-mediated immunity in chickens [24]. Protection of newly hatched chickens against infection by invasive salmonellae has been associated with enhanced cell-mediated indices of immunity [6]. In accordance with these studies, Farnell *et al.* [5] showed a potential acute immunostimulatory activity of ChIFN- γ to *Salmonella*. However, their IFN- γ activity seems to exist for a very short time. Our present data suggested that immunization with SE provides chickens a certain amount of protection against SE challenge. In addition, co-administration of ChIFN- γ enhanced the further protection in chickens although the mechanisms of protection are not clear. No discernible differences could be detected between antibody titers in groups receiving SE alone versus SE plus ChIFN- γ , indicating protection may be mediated via enhanced cell-mediated immunity. This possibility remains to be investigated.

Lowenthal *et al.* [11] showed that co-administration of IFN- γ enhances antibody responses in chickens against sheep red blood cells. In our previous study, we reported that a recombinant bovine IL-12 could modulate the T helper 1 type (Th1) of immune response of calves against *Salmonella* antigen [22]. Depending on the combination of cytokines produced, a protective immune response can be generated as either an antibody-mediated (Th2) response or a cell-mediated (Th1) response [15]. In avian immune systems, dichotomy of Th1 and Th2 had not yet been established [13], but Vandaveer *et al.* [23] suggested the existence of dichotomy within the T helper cell compartment of birds. Therefore, ChIFN- γ probably work for Th1 immune response as reported in mammalian model [4, 15]. In chickens, IL-12 has not yet been cloned, but the cloning of chicken IL-18, which will work synergistically with IL-12, has recently been described [18]. If IL-12 of chicken is cloned in future, it seems that co-administration of IL-12 and IL-18 will increase IFN- γ production and work for Th1 responses showing a strong adjuvant activity even in chickens.

ACKNOWLEDGMENTS. This work was supported in part by Grant-in-Aid of Recombinant Cytokine's Project provided by the Ministry of Agriculture, Forestry and Fisheries, Japan (RCP-2002-1130), in part by the Grant of the 21st Century COE Program, Ministry of Education, Culture, Sports, Science and Technology (MEXT) and in part by the grant for Scientific Research (No. 33) from Kitasato University, School of Veterinary Medicine and Animal Sciences.

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