ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

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International Journal of Poultry Science 8 (2): 122-127, 2009
ISSN 1682-8356

Immunomodulatory Effect of Recombinant Chicken Interferon-gamma (rchIFN-γ) on Specific and Non-specific Immune Responses in Chicken Vaccinated Against Newcastle Disease Virus (NDV)

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Abstract: Present study was undertaken to evaluate the immunomodulatory effect of recombinant chicken interferon -gamma (rchIFN-γ) on specific and non-specific immune responses in chicken vaccinated against Newcastle Disease Virus (NDV). A total of 180 day old layer chicks (BV-300) were divided into three groups. Test Group I (TG-I) (64 chicks) was injected with 5 microgram/chick subcutaneously of rchIFN-γ along with ND vaccinations (LaSota and R_2B strains), Test Group II (TG-II) (64 chicks) was injected with same dose and route of rchIFN-γ 6 h after ND vaccinations on both occasions, whereas, third group remained as vaccinated control group (52 chicks). Haemagglutination Inhibition (HI) test and Migration Inhibition (MI) in percentages estimated by Leukocyte Migration Inhibition Test (LMIT) were conducted to evaluate humoral and cell mediated immune responses respectively. Nitroblue Tetrazolium (NBT) reduction assay was conducted to evaluate the non-specific immune response by estimating the Phagocytic Index (PI) in percentages. The test results revealed that, significantly ($p \leq 0.05$) increased specific (humoral and cell mediated) and non-specific immune responses were recorded in the groups treated with rchIFN-γ i.e., higher results when given along with the vaccine and highest results when given 6 h after vaccinations. The results advocated that, rchIFN-γ may be used as an immunopotentiator in chicken vaccinated against NDV.

Key words: rchIFN-γ , HI test, LMIT , NBT reduction assay, NDV

INTRODUCTION

Among the most common and highly pathogenic viral diseases of domestic birds, Newcastle Disease (ND) is one; causing serious economic losses in terms of mortality and drastic reduction in production, still exist as an enzootic in different parts of the world. In spite of regular vaccinations, severe disease outbreaks occur in intensive farming units, which are reasoned mainly due to breakdown in immune status, which is prompting researchers, especially immunologists to work on variety of immunomodulators to improve the immune status of birds to encounter sudden invasion by viruses. Interferon-gamma (IFN-γ) is an important cytokine produced mainly by T-cells and Natural Killer (NK) cells following antigenic or mitogenic stimulation of these cells, which has got a range of immunomodulatory effects on humoral, cell mediated and non-specific immune responses such as, expression of Major Histocompatibility (MHC) antigens on Antigen Presenting Cells (APCs), augmentation of isotype specific immunoglobulin (Ig G2a and Ig G3) synthesis, influencing production of other cytokines which in turn stimulate activation of various cells of immune system and increase in phagocytosis by mononuclear cells, apart from its antitumor and antiviral activities. Since its discovery by Isaacs and Lindenmann in 1957, the central obligatory role played by IFN-γ in the regulation of immune response has been well documented. Thus, it is an integral part of the complex cytokine network.

Keeping in view the above mentioned immuno-modulatory role of rchIFN-γ and deadly nature of Newcastle disease, the present study was undertaken to evaluate the immunomodulatory effect of rchIFN-γ on specific and non-specific immunity in chicken vaccinated against NDV.

MATERIALS AND METHODS

Source of chickens and vaccines: A total of 180 day old layer chicks (BV-300) were obtained from M/s. Venkateshwara Hatcheries Pvt. Ltd. (VHL), Hyderabad, India. They were maintained on a balanced feed and water *ad libidum*. The lentogenic (LaSota) and mesogenic (R_2B) strains of NDV vaccines were procured from M/s. Ventri Biologicals, VHL, Hyderabad.

Extraction and purification of rchIFN-γ protein: Extraction and purification of rchIFN-γ protein was done as per the procedure of Sreekumar (2004). The protein was extracted from $BL_{21}DE_3$ strain of *E. coli* which was expressed with rchIFN-γ gene by induction with Isopropyl Thio Galactoside (IPTG), purified by Ni-NTA Affinity chromatography. Imidazole and endotoxins were removed by Sephadex G-25 gel filtration chromatography and Polymyxin-B sepharose chromatography respectively. Sterilization of the resultant protein was done by membrane filtration and stored at -20°C.

Experimental design: A total of 180 day old layer chicks (BV-300) were divided into three groups, TG I and II

containing 64 chicks each and control group which was vaccinated containing 52 chicks. All the groups were vaccinated with NDV (LaSota strain) intra-ocularly (I/o), on 7th day and NDV (R_2B strain) during 9 th week subcutaneously (S/c) respectively. The schedule was partly in accordance with Verma *et al.* (1985). rchIFN-γ was injected at the rate of 5 µgm/chick by S/c route in the TG-I along with NDV vaccines and in TG-II was injected with same dose and route 6 h after each vaccination. The schedule of administration of rchIFN-γ was in accordance with Mifune *et al.* (1987) and Kaur *et al.* (1998).

Sampling: Whole blood without anticoagulant for serum separation was collected as well as heparinized blood (20-50 IU of Heparin per mL of blood) was collected from wing vein from sample birds (20 birds from each group) on the '0' day, 14th , 21st and 28th day post immunization.

Haemagglutination Inhibition (HI) test : The HI assay is based on the binding of antibody to the viral haemagglutinin glycoprotein with a resultant inhibition of viral haemagglutination, resulting in virus identification as well as qualification of antibodies to a specific viral antigen.

Leukocyte migration inhibition test (LMIT): Migration Inhibition (MI) in percentages was assessed by LMIT. Leukocytes were separated from the blood collected in Heparin (20-50 IU/mL of blood) as per the method described by Naylor and little (1975) and the migration inhibition was evaluated. Higher MI values of leucocytes indicated higher CMI response to a particular antigen mediated mainly by T-lymphocytes.

Nitroblue Tetrazolium (NBT) reduction assay: Phagocytic Index (PI) in percentages was assessed by NBT reduction assay as per the method described by Park *et al.* (1968) with slight modifications. A reaction mixture consisting of leukocytes suspension (0.4 mL), activated plasma (0.1 mL) and 5% NBT solution (0.8 mL) was incubated at 37°C in water bath with shaker for 30 min and reaction was stopped with cold PBS (pH 7.2). Then it was centrifuged at 1000 RPM for 5 min. After discarding supernatant, a drop of PBS was added and gently the cells were resuspended. A drop of this reaction mixture was spread on a clean slide, dried and fixed in methanol for two minutes, stained with 0.8% aqueous Safranin for 2 min. The smear was washed, dried and mounted. NBT positive cells (dark blue colored) were counted (under 100X).

Statistical analysis: The data were expressed as the mean (± SE) and comparison among the groups were made by one way ANOVA.

RESULTS

Haemagglutination Inhibition (HI) test : The results of HI are presented in Table 1. The highest HI mean log titres after primary ND (LaSota) vaccination in the test groups, 2.28 (0.12) and 2.83 (0.08) in TG-I and TG-II respectively, were higher when compared to control group with 1.85 (0.09) on 21st day post immunization. After booster vaccination with ND (R_2B) vaccination TG II recorded highest titres of 2.86 (0.09) on 21st day and TG-I recorded 2.38 (0.09) on 28th day post immunization respectively, whereas, control group recorded 2.00 (0.09) on 21st day post immunization. Among the test groups, TG-II which received rchIFN-γ 6 h after NDV vaccinations recorded significantly ($p \leq 0.05$) increased results over the TG-I.

Leukocyte Migration Inhibition Test (LMIT): The results are presented in Table 2 and Plate 1. On majority of the post immunization sample screenings for evaluating cell mediated immune response, there were considerable and statistically significant ($p \leq 0.05$) increased results were recorded in test groups over the control group. With ND (LaSota) vaccination TG-II recorded highest mean MI (in percentages) of 45.50 (2.29), TG-I recorded 39.33 (1.52) on 28th day post immunization. Whereas, control group recorded 24.33 (1.40) on 21st day post immunization. With ND (R_2B) vaccination TG II recorded highest mean MI of 66.00 (1.82) on 28th day post immunization, whereas, TG-I recorded 51.50 (2.34) and control group recorded 41.67 (1.45) on 21st day post immunization. Results in control group were lower when compared to either of the test groups.

Nitroblue Tetrazolium (NBT) reduction assay: In this investigation, there were considerable and statistically significant ($p \leq 0.05$) increased results (Table 3) were recorded in test groups over the control group. With ND (LaSota) vaccination TG-II recorded highest mean PI (in percentages) of 69.00 (1.29), TG-I recorded 57.17 (2.76), whereas, control group recorded 50.33 (2.47) on 21st day post immunization. With ND (R_2B) vaccination TG II recorded highest mean PI of 79.17 (2.89) on 28th day post immunization followed by TG I with 70.17 (1.19) on 21st day post immunization with this assay and control group recorded highest mean PI with 49.81 (3.26) on 14th day post immunization, lower as compared to either of the test groups. Above all results indicated that, administration of rchIFN-γ had immunostimulatory effect on specific and non specific immune responses. Further, among the test groups, TG-II recorded higher and sometimes significantly ($p \leq 0.05$) increased values over the TG-I test group I, indicating that rchIFN-γ administered 6 h after vaccination had better effect than that administered along with vaccination.

Table 1: HI antibody titre values after NDV vaccinations

	ND (LaSota) Vaccination			ND (R_2B) vaccination		
DPI Sampling	Control	TG-I	TG-II	Control	TG-I	TG-II
'0' Day	1.28±0.08	1.27 ±0.08	1.10± 0.09	0.90^{a}±0.05	1.18^{ab}±0.11	1.35^{b}±0.08
14th Day	1.56^{a}±0.11	1.88^{ab}±0.09	2.23^{b}±0.11	1.35^{a}±0.09	2.00^{b}±0.09	2.31^{b}±0.13
21st Day	1.85^{a}±0.09	2.28^{b}±0.12	2.83^{c}±0.08	2.00±0.09	2.28±0.12	2.86±0.09
28th Day	1.55^{a}±0.11	1.88^{a}±0.12	2.38^{b}±0.11	1.55^{a}±0.11	2.38^{b}±0.09	2.76^{c}±0.12

Table 2: LMIT results after NDV vaccinations

	ND (LaSota) Vaccination			ND (R_2B) vaccination		
DPI Sampling	Control	TG-I	TG-II	Control	TG-I	TG-II
'0' Day	12±1.15	12.67±1.02	12.83±1.24	26.33^{a}±1.49	29.83^{ab}±1.64	32.83^{b}±1.44
14th Day	17.83^{a}±0.94	24.17^{b}±1.70	35.17^{c}±2.15	34.33^{a}±2.65	40.17^{ab}±2.44	44.33^{b}±2.12
21st Day	24.33^{a}±1.4	33.67^{b}±2.12	39.33^{c}±1.22	41.67^{a}±1.45	51.50^{b}±2.34	52.83^{b}±1.32
28th Day	21.33^{a}±1.05	39.33^{b}±1.52	45.50^{c}±2.29	21.00^{a}±1.73	35.00^{b}±1.46	66.00^{c}±1.82

Table 3: NBT reduction assay results after NDV vaccinations

	ND (LaSota) Vaccination			ND (R_2B) vaccination		
DPI Sampling	Control	TG-I	TG-II	Control	TG-I	TG-II
'0' Day	9.67±0.88	10.08±1.54	8.80±1.13	30.67^{a}±0.88	39.33^{b}±2.43	34.33^{ab}±2.15
14th Day	24.50^{a}±1.40	32.50^{b}±2.44	49.00^{c}±2.62	49.83^{a}±3.26	59.33^{b}±2.21	66.17^{b}±2.35
21st Day	50.33^{a}±2.47	57.17^{b}±2.76	69.00^{c}±1.29	41.17^{a}±1.30	70.17^{b}±1.19	72.67^{b}±3.48
28th Day	31.17^{a}±1.49	50.50^{b}±2.06	66.67^{c}±2.59	35.17^{a}±2.27	50.83^{b}±1.90	79.17^{c}±2.89

Note: 1) Values are mean ± SE, 2) Those with same superscripts do not differ significantly at $p \leq 0.05$, 3) DPI_Day of post immunization

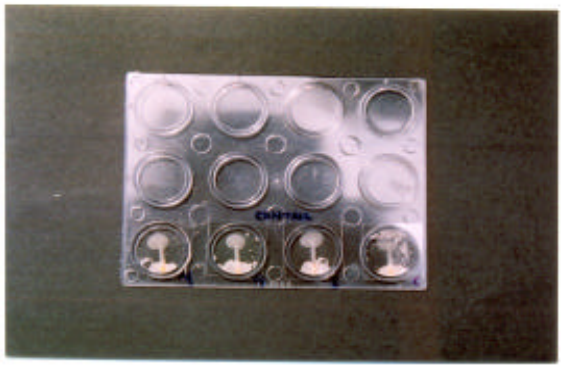
DISCUSSION

IFN-γ has shown promising results in immunomodulation and growth promotion in poultry production (Lowenthal *et al*., 1998). IFN-γ modulates antibody mediated and cellular immune response in animals that when administered with bacterial, viral, protozoal, or other antigens or vaccines (Mifune *et al*., 1987; Semnani *et al*., 1993; Erhard *et al*., 2000; Farnell *et al*., 2001 and Jia-LiJun *et al*., 2004). The findings suggested that cytokines and particularly IFN-γ had the potential as adjuvant in humans, animals and avian species. Avian IFN-γ was extracted and purified in the present study to use as an immunomodulator. The present study was aimed at immunological evaluation of rchIFN-γ as an immunomodulator.

The HI titre results were highly significant ($p \leq 0.05$) in test groups when compared to control group indicating rchIFN-γ had profound immunomodulatory effect on the humoral immunity. There was no literature available on evaluation of effect of rchIFN-γ on humoral immunity estimated by HI test, however, this humoral immunopotentiation could be due to isotype specific enhancement of immunoglobulins (Rabin *et al*., 1986; Snapper and Paul, 1987), synergistic action with IL-2 to enhance immunoglobulin light chain synthesis (Sidman *et al*., 1984), adjuvant effect when given along with antigen (Anderson *et al*., 1988) and its action on B-cells to promote switching to Ig G2a and Ig G3 subclasses and antibody dependent cell mediated cytotoxicity (Hari Babu, 2004).

On interaction with a specific antigen sensitized T-cells mediate the CMI by producing a number of molecular mediators collectively known as Lymphokines. The extent of migration inhibition in percentages was dependent on lymphocyte sensitivity to the specific antigen and the response of leucocytes and macrophages to the resulting lymphokines (Hari Babu, 2004). There was no literature available on immunomodulatory effect rchIFN-γ on CMI response evaluated by LMIT. Both test groups recorded significantly ($p \leq 0.05$) increased MI than the control group. Significantly increased CMI response in test groups could be due to, increased expression of MHC-class II antigens on Antigen Presenting Cells (APCs) (Frasca *et al*., 1985), induced non-MHC restricted cytotoxicity of virus infected cells in peripheral blood leucocytes (Campos *et al*., 1989), induced the up-regulation of γδT-cells and decreased the number of Ig M^{+} lymphocytes (Murrakami *et al*., 2004), increased transcription of pro-inflammatory cytokines (IL-1β, IL-6 and IL-8) induced by phagocytic antagonists but also up-regulated the expression of Th1 cytokines (IL-18 and IFN-γ) (Kogut *et al*., 2005).

The PI was assessed by NBT reduction assay showed non-specific immune response to the ND vaccinations. Stimulated peripheral blood mononuclear cells ingest the NBT dye into phagocytic vacuoles and convert it into an insoluble precipitate (Formasan) by highly reactive oxygen components which imparted them blue/purple color whereas, non reactive cells which were not activated in the immune response took Safranin stain and appeared as red/pink cells. NBT is reduced mainly



T1 T2 C1 C2

Plate 1: Leukocyte migration inhibition test (LMIT) results, Chambers T1 and T2 → Test samples Chambers C1 and C2 → Control samples

by superoxide anion, therefore NBT reduction assay used as marker of intracellular bactericidal activity of neutrophils and mononuclear cells.

There was scanty information available on the evaluation of immunomodulatory effect of rchIFN-γ on non-specific immune response estimated by NBT reduction assay in chicken *in vivo,* but the results were in congruence with findings of Semnani *et al.* (1993), who showed that, *in vitro* pre-treatment of neutrophils with rchIFN-γ increased phagocytic and NBT reduction activities. However, there were reports which have indicated the effect of NBT reduction assay as a measure of non-specific immunity, Park *et al.* (1968) stated that intensity of NBT reduction roughly correlated with bactericidal activity, Nagahata *et al.* (1986) assessed bactericidal activity of bovine neutrophils by an improved quantitative NBT reduction assay, Nakagava *et al.* (1993) found that, non-specific host defense mechanism against bacterial infection in mice and intracellular killing of *Staphylococcus aureus* by NBT reduction. This significant increase in non-specific immune response could be due to, increased release of Hydrogen peroxide (H_2O_2) by neutrophils (Bielefeldt-Ohmann and Babiuk, 1985), stimulation of F_cR, expression of Mac-1 which is a receptor for C_3b, decreased affinity for C_3b (Wright *et al.*, 1986 and Murrey, 1988), increased production of Tumor Necrosis Factor (TNF) by macrophages (Murrey, 1988), increased Antibody Depended Cell Mediated Cytotoxicity (ADCC) in neutrophils (Roth and Frank, 1989; Brown *et al.*, 1991 and Chiang *et al.*, 1991), induced nitrite secretion and enhanced MHC class II expression on macrophages (Kaspers *et al.*, 1994 and Lowenthal *et al.*, 1998) and induced Nitric Oxide (NO) production, upregulated Ia expression on cell surfaces, inhibited replication of NDV in chicken macrophage cell lines (Hung-Yueh Yeh *et al.*, 1999).

Overall evaluation of the immunomodulatory effect of rchIFN-γ on specific and non-specific immune responses in chicken vaccinated against NDV suggested that, there was considerable and on majority of the sample screening days post immunization, statistically significant ($p \leq 0.05$) increase in specific and non-specific immune responses in the test groups compared to control group. The results were principally in accordance with findings of Anderson *et al.* (1988) who stated that IFN-γ could stimulate the production of antibodies if given at the same time along with the antigen and findings of the present investigation were more specifically in accordance with observations of Chester *et al.* (1973) who stated that interferons (not specifically IFN-γ) administered prior to the antigen tended to be immunosuppressive, but when given after immunization they were enhancing. But, the results were contradictory to the findings of Mifune *et al.* (1987) who stated that intrferons (not specifically IFN-γ) had no effect when given later during the vaccination, as they could not notice any enhancement in antibody response in mice vaccinated against Rabies when administered IFNs along with or 6 h after vaccination. It was also imperative to note that two key factors of dose and timing were important factors attributed to the adjuvant effect of IFN-γ as well as to the other interferons as stated by Anderson *et al.* (1988). Observations of the present study might through light on the possibility of the type of antigen or vaccine along with dose and timing might also influence the immunomodulatory or adjuvant effect of rchIFN-γ.

The present investigation has formed as a unique application part of recombinant chicken IFN-γ for assessing the immunomodulatory effect in chicken vaccinated against NDV and its treatment has resulted in consistent immunopotentiation of the humoral, cell mediated and non-specific immune responses. The results obtained in the present study have remained unique in absence of sufficient specific relevant studies that were available in the literature. In conclusion, there was overall marked increase in humoral, cell mediated and non-specific immune responses in the experimental birds administered with rchIFN-γ.

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