Effects of salinomycin on cell-mediated immunity of broiler chickens against hydropericardium syndrome and Newcastle disease viruses

K. Munir,*1 M. A. Muneer,† A. Tiwari,‡ E. Masaoud,§# and R. M. Chaudhry*

*Lahore Veterinary Diagnostic Laboratories, Shadman, Lahore-54000, Pakistan; †University of Veterinary and Animal Sciences, Lahore-54000, Pakistan; ‡Animal Health Risk Assessment Unit, Canadian Food Inspection Agency, Ottawa, Ontario, K2H 8P9, Canada; §Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PE, C1A 4P3, Canada; and #University of Seventh April, Faculty of Science, Zawia, Libya

ABSTRACT The effects of salinomycin (SAL) on protective cell-mediated immune (CMI) responses of vaccinated and challenged broiler chicks against hydropericardium syndrome virus (HPSV) and Newcastle disease (NDV) were investigated while comparing 3 reference drugs [levamisole, cyclophosphamide (CYP), and cyclosporine (CYS)]. Peripheral lymphoproliferation, skin hypersensitivity reactions, and the ability of chicks to resist virulent HPSV and virulent NDV challenges were used to determine the effects of drugs on CMI responses in chicks. Salinomycin-medicated chicks showed significant (P < 0.05) stimulation of lymphoproliferation and nonsignificant (P > 0.05) stimulation of skin hypersensitivity reactions compared with untreated control chicks. However, skin thickness and lymphoproliferation of SAL-medicated chicks were significantly greater (P < 0.05) than those of CYP- and CYS-treated chicks. The greatest survival rate was recorded in SAL-medicated chicks compared with immune-suppressed (CYP- and CYS-treated) and untreated control chicks after virulent NDV and virulent HPSV challenges. Thus, it was concluded that SAL had beneficial effects on the CMI responses of broiler chicks against HPSV and NDV.

Key words: salinomycin, cell-mediated immunity, broiler chick, Newcastle disease virus, hydropericardium syndrome virus

INTRODUCTION

Newcastle disease (ND) and hydropericardium syndrome (HPS) pose serious threats to the broiler chicken industry because they have the potential to cause severe economic losses in terms of high mortality, poor growth or loss of productivity, and extra costs of medication for secondary infections (Afzal and Ahmad, 1990; Naeem et al., 1995; Ganesh and Raghavan, 2000; Alexander, 2001; Office Internationale des Epizooties, 2003; Kumar et al., 2003; Shivachandra et al., 2003; Tsai et al., 2004). Recent reports indicate that a major way of managing infectious diseases could be through the positive manipulation of humoral and cell-mediated immune (CMI) responses with drugs that are part of routine medication programs (Pantin-Jackwood et al., 2004; Dalloul and Lillehoj, 2005; Tayade et al., 2006; Munir et al., 2007). Positive immune manipulation could lead to increased antibody production, increased CMI responses, inhibited tumor growth, improved and efficient macrophage functions, and the expression of protective cytokine profiles in the host animal or bird (Muneer et al., 1988; Pantin-Jackwood et al., 2004; Munir et al., 2007). Clinically, this is exhibited as improved growth performance, reduced mortality, optimal immune responses to antigens administered via vaccines or improved efficacy of vaccines, and enhanced capability of chickens or animals to resist field infections (Muneer et al., 1988). In contrast, negative immune manipulation with immunosuppressive drugs may result in impairment of the functional activity of T and B lymphocytes, phagocytes, or other cells of the intrinsic or adaptive immune system (Muneer et al., 1988; Chaudhry, 1991; Sharma et al., 2000; Pantin-Jackwood et al., 2004; Munir et al., 2007). These effects may transform clinically into poor growth performance, increased mortality, and suboptimal immune responses to vaccination or increased vulnerability to field infections (Muneer et al., 1988; Chaudhry, 1991; Sharma et al., 2000; Pantin-Jackwood et al., 2004). Depending on the virus type, humoral or CMI responses or both may be important for protection against viral infections (Rajs et al., 2000; Shivachandra et al., 2003; Pantin-Jackwood...
Immunization against ND and HPS, the 2 economically important viral diseases of broiler chickens, is a common practice (Kumar et al., 2003; Balamurugan and Kataria, 2006; Munir et al., 2007). In the literature, many chemicals, vitamins, and drugs used for growth, therapeutic, or prophylactic purposes have been reported to influence humoral and CMI responses in chickens (Muneer et al., 1988; Kim et al., 2004; Tayade et al., 2006). These drugs may affect the efficacy of vaccines either positively or negatively (Muneer et al., 1988; Munir et al., 2007). Thus, the use of immunosuppressive drugs may have clinical consequences for the chickens and may result in economic losses for the farmers. The combined use of immunostimulating drugs and vaccines could improve the efficacy of vaccines (Muneer et al., 1988; Tayade et al., 2006; Munir et al., 2007; Yin et al., 2007). Therefore, it is important to investigate the effects of drugs on the protective immune responses of broiler chickens to prevent or control infectious diseases.

We previously reported that salinomycin (SAL) enhances the protective antibody responses of broiler chickens against ND virus (NDV) and HPS virus (HPSV) (Chaudhry, 1991; Munir et al., 2007). However, the effects of this drug on CMI responses in chickens are still largely unknown. It would be more beneficial to include a drug in the poultry feed that could serve as a coccidiostat as well as an immunomodulator that could enhance protection against pathogens by stimulating both the humoral and CMI responses of chickens toward antigens included in vaccination programs.

This work was undertaken to investigate the effects of SAL medication on the CMI responses of broiler chickens to HPSV and NDV. Three reference drugs, levamisole (LEV), an anthelmintic drug with known immunostimulating effects, and cyclophosphamide (CYP) and cyclosporine (CYS), known immunosuppressive chemicals, were included in the study to compare the effects of SAL on CMI responses in chickens. To our knowledge, this is the first report to evaluate SAL as a modulator of CMI responses to NDV and HPSV.

**MATERIALS AND METHODS**

Immediately after arrival, a total of 200 one-day-old broiler chicks (Hubbard × Hubbard) were color-marked and randomly divided into 4 groups (A to D), each with an equal number of chicks. Chicks in group A received SAL at 0.0066% in poultry feed throughout the experimental period, whereas those in groups B and C received LEV and the immunosuppressive agents (CYP and CYS), respectively. Chicks in group B received LEV orally at a daily dose of 15 mg/kg of BW for a period of 1 wk, beginning at 5 d of age and ending at 12 d of age. Chicks in group C were injected daily in the abdominal region with a CYP solution containing 3 mg of CYP, 12.5 IU of penicillin, and 0.0125 mg of streptomycin/mL during the first 4 consecutive days of life. Chicks in group C were also injected intramuscularly with CYS at a dose of 100 mg/kg of BW every 3 d for the first 5 wk. Chicks in group D did not receive any medication and served as unchallenged controls. Chicks in all groups received a live NDV (LaSota strain) vaccine via eye drop (ED) on d 5 of age and a killed bivalent infectious bursal disease and ND vaccine as well as an inactivated oil-emulsion HPS vaccine subcutaneously in the neck region at 12 d of age. The HPS vaccine was prepared as described previously (Munir et al., 2007).

Three parameters, peripheral lymphocyte proliferation (PLP), skin contact sensitivity, and the ability of chicks to resist virulent HPSV (vHPSV) and virulent NDV (vNDV) challenges, were used to determine the effects of these drugs on the CMI responses of the chicks. Blood samples (5 mL) were collected from each bird on d 21, 28, 35, 42, and 49 of age directly from the heart of 5 chicks in each group into sodium heparin-containing sterile tubes for PLP assay by the 4,5-dimethyl-thiazole-2,5-diphenyl tetrazolium bromide method (Sun et al., 2006) with minor modifications. Briefly, blood samples, after mixing with Hanks solution, were layered on lymphocyte separation medium. After centrifugation at 1,000 × g for 20 min at 20°C, lymphocytes were collected and washed 2 times with RPMI 1640 medium without any fetal bovine serum (FBS) supplement, and cells were adjusted to 4 × 10^6 per mL in RPMI 1640 medium containing 10% FBS and antibiotics. Each sample was seeded into 8 wells of a 96-well culture plate, with each well receiving 80 μL of cell suspension. Twenty microliters of phytohemagglutinin, a T-cell mitogen, was added to each of the first 4 wells, whereas each of the remaining 4 wells received 20 μL of RPMI 1640 medium supplemented with antibiotics and FBS medium. After 2 d of incubation at 38°C in a carbon dioxide chamber, 20 μL of 4,5-dimethyl-thiazole-2,5-diphenyl tetrazolium bromide per well was added, and plates were reincubated for 4 h. After incubation, plates were centrifuged and the supernatant was discarded. Dimethylsulfoxide (100 μL) was added to each well to extract the formazan. The optical density (OD) of cells in each well was measured at a wavelength of 570 nm. The skin contact sensitivity method was performed according to a procedure reported previously (Tiwary and Goel, 1985; Shivachandra et al., 2003), with minor modifications. Briefly, 5 chicks from each group were color-marked at 28 d of age and sensitized with 0.25 mL of 2,4-dinitrochlorobenzene (DNCB) solution (10 mg/mL). After 14 d, these sensitized chicks were challenged with 0.25 mL of DNCB (1.5 mg/mL) and their contact sensitivity was assessed at zero (defined as immediately after DNCB challenge), 24, 48, 72, and 96 h post-DNCB challenge by measuring skin thickness with electronic calipers. Skin sections from 4 chicks at 48 h post-DNCB challenge were also processed for histological examination.
Virulent NDV and vHPSV, isolated from field outbreaks, were characterized (Munir et al., 2007) and used to infect chicks in this study. The virulence of the NDV strain was assessed by standard in vivo pathogenicity tests (Office Internationale des Epizooties, 2003). Intracerebral pathogenicity index, mean death time, and intravenous pathogenicity index values of 1.88, 53 h, and 2.60 suggested this isolate to be a velogenic viscerotopic NDV isolate (Munir et al., 2007). The vHPSV for challenge studies was prepared as follows. Infected livers from a broiler chicken flock showing high HPS-associated mortality were collected and a 30% liver homogenate was prepared, centrifuged, and filtered. Antibiotics were then added, and the filtrate was inoculated into the primary chicken embryo liver cells. Cell rounding, degeneration, and the presence of basophilic intranuclear inclusions characterized HPSV-associated cytopathic effects. The identity of the HPSV isolate was also confirmed by an agar gel precipitation test using the reference serum, as described elsewhere (Naeem et al., 1995). This HPSV isolate was shown to cause up to 90% mortality in unvaccinated chicks in a previous challenge study (Munir et al., 2007). The infectivity titer (50% tissue culture infective dose) was calculated according to the method of Reed and Muench (1938).

For the challenge study, 10 randomly selected 28-d-old chicks from each group were transferred to a separate room and each chick was challenged intramuscularly, with each chick receiving 0.5 mL of vHPSV suspension having a $10^{5.60}$ 50% tissue culture infective dose, as described previously (Munir et al., 2007). Similarly, 10 randomly selected 36-d-old chicks from each group were challenged via ED, intranasal, and intramuscular routes, with each chick receiving 1.0 mL ($10^{5.25}$ 50% egg infective dose) of vNDV preparation (Munir et al., 2007). Chicks were observed daily for clinical signs and mortality up to 14 d after vNDV or vHPSV challenge. Necropsy examination of dead chicks was performed to record gross lesions.

Statistically significant differences among mean OD values of the 4 different groups were analyzed by using the GLM (Christensen, 1998). Time was considered as a block factor (Christensen, 1998). The GLM for time and treatment groups as well as for the interaction between groups and time was used to analyze the OD values. Statistically significant differences among mean skin thicknesses of the 4 different groups were determined by the linear mixed model (Dohoo et al., 2003). The associations between repeated measurements were explored by using the random intercept model with heterogeneous variance and a first-order autoregressive correlation structure. The Bonferroni procedure for multiple group comparisons was used (Christensen, 1998). Results were considered statistically significant at $P < 0.05$. Minitab software (Minitab Inc., 2006) and SAS/STAT software (SAS Institute, 2003) were used to perform the above-mentioned statistical analyses.

**RESULTS**

Temporal changes in mean OD values of the different groups are depicted in Figure 1a. The mean OD values of the SAL-treated group and of the LEV-treated group were significantly greater ($P < 0.05$) than those of the untreated control group on all days except d 49. There were significant differences ($P < 0.05$) in mean OD values between the SAL-treated or LEV-treated group and the CYP- and CYS-treated group on d 21, 28, 35, 42, and 49. The difference in OD values between SAL-treated and LEV-treated chicks was NS ($P > 0.05$) on all days. The difference in OD values between the CYP- and CYS-treated group and the untreated control group was significant ($P < 0.05$) on all days. Overall, there were significant effects of treatment groups and times as well as an interaction between treatment groups and time on the mean OD values of groups. Results indicated that SAL medication had significant effects on lymphocyte proliferation (OD values) in the peripheral blood of chickens. This model explained 97.8% of the variability in OD values, with SD equal to 0.0012. The normality assumptions of the residual were tested by using the Anderson-Darling test.

In the linear mixed model, the variance-covariance matrices reflected the variation in skin thickness. Temporal changes in skin thicknesses of the different groups are depicted in Figure 1b. The mean skin thickness of groups was dependent on time. In general, there was a significant difference ($P < 0.05$) in the mean skin thickness among groups. The difference in mean skin thickness between the SAL-treated and LEV-treated groups was NS ($P > 0.05$). Differences in mean skin thickness between the SAL-treated and the CYP- and CYS-treated chicks, as well as between the SAL-treated chicks and the untreated control chicks were significant ($P < 0.05$). Similarly, differences in mean skin thickness between the LEV-treated and the CYP- and CYS-treated groups, as well as between the LEV-treated and untreated control groups were also significant ($P < 0.05$). The mean skin thickness of the CYP- and CYS-treated chicks was significantly lower ($P < 0.05$) than that of untreated control chicks. There was a statistically NS difference ($P < 0.05$) in the mean skin thickness across groups at time zero. The difference in mean skin thickness between the SAL-treated and LEV-treated chicks was also NS ($P > 0.05$) at all other times tested. The difference in mean skin thickness between the SAL-treated or LEV-treated group and the CYP- and CYS-treated group was significant ($P < 0.05$) at 24, 48, 72, and 96 h. However, the difference in mean skin thickness between the SAL-treated chicks and untreated control chicks was NS ($P > 0.05$) at all times tested, with a marginal difference ($P = 0.07$) at 48 h. The difference in mean skin thickness between the LEV-treated group and the untreated control group was not significantly ($P > 0.05$) large at any time ex-
cept at 48 h. There was a significant difference ($P < 0.05$) in mean skin thickness between the CYP- and CYS-treated group and the LEV-treated group at 24, 48, and 72 h. Skin thickness increased slowly, reaching its peak around 48 h after DNCB challenge and attenuating subsequently. Edema and congestion, with the presence of mononuclear cells and lymphocytes, were observed on histological examination of skin sections collected 48 h after DNCB challenge (data not shown). In general, there was a significant difference ($P < 0.05$) in mean skin thicknesses among groups. One chick in the CYP- and CYS-treated group died on the 46th day of age. This chick probably died as a result of stress inflicted when it was being challenged with DNCB, because we did not observe any significant lesions on necropsy examination of this chick.

Vaccine reactions, as demonstrated by mild conjunctivitis and sneezing, were recorded in a few chicks within 48 to 72 h of live LaSota strain-vaccine administration via ED on d 5 of age. Survival rates for groups after vHPSV and vNDV infection are shown in Table 1. The greatest survival rate(s) (100%) were recorded in the SAL-treated and LEV-treated groups, whereas the lowest survival rates (20 and 30% after vNDV and vHPSV, respectively) were observed in the CYP- and CYS-treated group. Survival rates of 90 and 100% post-vNDV and post-vHPSV challenge, respectively, were recorded in the untreated control group. Mortality

Figure 1. Cell-mediated immune responses of broiler chicks. Panel a: Mean (optical density, OD) values of groups on different days. Panel b: Mean skin thickness of groups at different hours post-dinitrochlorobenzene challenge. Chicks in group A received salinomycin, those in group B received levamisole medication, and those in group C received a cyclophosphamide and cyclosporine injection. Chicks in group D served as untreated control birds. The error bars indicate SE. The mean OD or skin thickness values for each group are representative of 5 data points or chicks. See text for details.
peaked within 4 to 7 d of NDV challenge in the CYP- and CYS-treated group. Mortality in this group began 3 d post-vHPSV challenge. The CYP- and CYS-treated chicks that received the vNDV challenge showed clinical signs that included nasal discharge, gasping, coughing, dyspnea, cyanosis of combs, and yellowish-green diarrhea. Gross lesions observed were congested lungs; mucous in the trachea, bronchi, and nasal sinuses; hemorrhages in the proventriculus; and hemorrhagic enteritis. One chick from the untreated control group that died after vNDV challenge also showed signs and lesions similar to those observed in CYP- and CYS-treated chicks. Chicks that survived the vNDV challenge up to 14 d showed no significant lesions in their organs. The CYP- and CYS-treated chicks that received the vHPSV infection showed depression, with ruffled feathers. The gross lesions observed in these chicks were lung congestion, the presence of straw-colored fluid in the pericardial sac, nephritis, and hepatitis.

**DISCUSSION**

The results of this study suggest that SAL medication enhanced the CMI responses of chicks, as manifested by increased skin thickness after challenge with DNCB, greater OD values indicative of lymphoproliferation, and enhanced resistance of chicks to NDV infections (Figure 1 and Table 1). It is difficult to explain the results that skin thickness of the SAL-treated or LEV-treated group was not significantly greater and that OD values were significantly greater than those of the untreated control group on most of the times tested (Figure 1). Previous workers have also reported inconsistency between information provided by the PLP assay and DNCB-induced skin hypersensitivity reactions (Tiwary and Goel, 1985). The differences in cytokine expression might explain these slightly inconsistent results between the 2 methods. The results that HPSV- and ND-vaccinated chicks that received the SAL or LEV treatment did not develop disease because of the infecting dose of vHPSV and NDV are consistent with previous reports of similar observations (Munir et al., 2007; Yin et al., 2007). Yin et al. (2007) reported improved protection of chicks against velogenic NDV after using interferon-γ and LEV as an adjuvant in an NDV DNA vaccine. They observed increased T-cell proliferation and enhanced expression of cytokines such as interleukin (IL)-2, IL-4, IL-12, and IL-13. It would be interesting to investigate the effects of SAL on specific lymphocyte populations and cytokine expression further. The result that there was a 100% survival rate in the SAL-mediated, LEV-treated, and untreated control groups after vHPSV challenge (Table 1) is also difficult to explain. It has been suggested that the presence of immunosuppressive agent(s) can act as a triggering factor for the development of HPSV-specific disease and probably greater mortality (Balamurugan and Kataria, 2006). This fact was also supported by the poor survival rate of CYP- and CYS-treated chicks after vHPSV infection in the present study. In chicks, CYP- and CYS-induced immunosuppression was manifested by the least OD and by a depressed skin hypersensitivity reaction, as well as by poor survival rates, after vHPSV and vNDV infections (Figure 1 and Table 1). These results are consistent with our previous observations (Munir et al., 2007) as well as those of Pantin-Jackwood et al. (2004), who reported interference with humoral and CMI responses caused by administration of CYP and CYS to birds. The clinical signs and necropsy lesions recorded after vNDV and vHPSV infections are also similar to those reported previously (Office Internationale des Epizooties, 2003; Munir et al., 2007).

In conclusion, data suggest that SAL appeared to exert beneficial effects on CMI responses of broiler chicks against HPSV and NDV. However, more work is required to confirm the protective enhancement of CMI responses in chicks induced by SAL medication against HPSV and NDV.

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### Table 1. Survival percentage in experimental groups after challenge with virulent Newcastle disease virus (vNDV) and virulent hydropericardium syndrome virus (vHPSV)

<table>
<thead>
<tr>
<th>Group</th>
<th>Decration</th>
<th>Survival post-vNDV challenge (%)</th>
<th>Survival post-vHPSV challenge (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Salinomycin medicated</td>
<td>100 (10/10) ^1</td>
<td>100 (10/10)</td>
</tr>
<tr>
<td>B</td>
<td>Levamisole treated</td>
<td>100 (10/10) ^1</td>
<td>100 (10/10)</td>
</tr>
<tr>
<td>C</td>
<td>Cyclophosphamide and cyclosporine treated</td>
<td>20 (2/10)</td>
<td>30 (3/10)</td>
</tr>
<tr>
<td>D</td>
<td>Untreated control</td>
<td>90 (9/10)</td>
<td>100 (10/10)</td>
</tr>
</tbody>
</table>

^1Ten randomly selected 36-d-old chicks from each group were challenged via eye drop, intranasal, and intramuscular routes; each chick received 1.0 mL (10^6.25 50% egg infective dose) of vNDV suspension prepared as described previously (Munir et al., 2007).

^2Ten randomly selected 28-d-old chicks from each group were challenged intramuscularly; each chick received 0.5 mL of vHPSV suspension having 10^7 TCID50 tissue culture infective dose as described previously (Munir et al., 2007).

^3Numbers in parentheses represent chicks that survived of the challenged chicks.
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REFERENCES


