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Presence of Interferon-Gamma and IL-2 in Supernatants of *Salmonella enteritidis*-Immune Lymphokines

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Abstract: Investigations in our laboratories have indicated that an increased resistance to *Salmonella enteritidis* SE organ infectivity in chickens was conferred by the immunoprophylactic administration of SE-immune lymphokines (SE-ILK). Resistance has been associated with an enhanced heterophilic accumulation within 4 h of ILK injection. Further studies demonstrated that CxCLi2 is produced locally by the host in response to both the SE infection and the SE-ILK suggesting that IL-8 is a major chemotactic factor produced by the host, which aids in mediating the SE-ILK induced recruitment of heterophils to the site of SE invasion. In the present study, two bio-assays confirmed the presence of IFN and IL-2 functions in SE-ILK that may also participate in the establishment of the reported protection against *Salmonella* infections. The IL-2 and IFN effects detected in this work suggest that bird's immune response develops similarly to that of mammals, with some features corresponding to the species.

Key words: *Salmonella*, cytokines, inflammation, chickens

INTRODUCTION

Increased resistance to *Salmonella sp.* organ infectivity in neonatal chickens and turkeys can be conferred by the prophylactic administration of *Salmonella enteritidis*-immune lymphokines (SE-ILK) (Genovese *et al.*, 1999; Kogut *et al.*, 1996; Kogut *et al.*, 1997a; Lowry *et al.*, 1999; McGruder *et al.*, 1993; Tellez *et al.*, 1993; Ziprin and Kogut, 1997). This resistance against systemic SE infections, is related with an enhanced heterophilic accumulation and enhanced heterophil function within 4 h of SE-ILK injection (Genovese *et al.*, 1999; Kogut *et al.*, 1993). Investigations in our laboratories have indicated that the heterophil is extremely important in controlling both initial SE organ invasion and subsequent disease pathogenesis in chickens (Kogut *et al.*, 1994; Swaggerty *et al.*, 2005). Furthermore, published data has indicated the presence of important cytokines in SE-ILK such as: Hematopoietic Colony Stimulating Factors (CSF) (Kogut *et al.*, 1997a,b); IL-8 (Kogut, 2002a) and P33 (Crippen *et al.*, 2003; Bischoff *et al.*, 2001). Considering that interleukins have the same biological activities both in birds as in mammals, it is important to distinguish the presence of other lymphokines from SE-ILK chicken spleen cells. In the present study, two bio-assays were

conducted to evaluate the presence of IFN and IL-2 in SE-ILK that may also contribute in the establishment of the observed protection against *Salmonella* infections previously reported.

MATERIALS AND METHODS

Preparation of lymphokines: SE-ILK production was performed as previously described by (Tellez *et al.*, 1993).

Preparation of Splenocytes: Chicken splenocytes were obtained for: a) IL-2 quantification assay; b) preparation of Primary Mix Culture (PMC) and c) preparation of the secondary mixed culture (SMC) according to standard protocols (Coligan *et al.*, 1992). The SMC was used as positive control, since it has been previously described that allogeneically stimulated cell cultures release great amounts of IFN (Coligan *et al.*, 1992).

Bio-assays for IL-2 Identification: Identification of IL-2 was performed on the proliferation of the CTLL2 mouse cellular line, depending on IL-2 for its proliferation (Methyl-³H Dupont, Boston), according to standard protocols (Coligan *et al.*, 1992). The C63 cell line

supernatant was used as IL-2r source. The CTLL2 cells were also incubated with double serial dilutions of SE-ILK or Phosphate Basic Solution (PBS) solution as negative control.

Bio-assay for INF-Gamma Identification: Chicken splenocytes were incubated during 24, 48, 72 and 120 h with the SE-ILK or SMC in 10% and 20% concentrations at 37°C in 5.0% of CO₂. At the end of the incubation time, the expression of Major Histocompatibility Complex (MHC) class I and class II molecules were quantized with fluorescent isotiocinate according to standard protocols (Coligan *et al.*, 1992). Fluorescence intensity was quantized in flow cytometry (FacSort) and data was analyzed using the LYSIS II software (Becton Dickinson).

Statistical analysis: Differences in the expression of class I or class II molecules of MHC were analyzed by analysis of variance using the General Linear Models (GLM) procedure. Significant differences (p<0.05) were further separated using Duncan's multiple range test and commercial statistical analysis software (SAS Institute Inc., 2002). Analysis of correlation was performed to evaluate SE-ILK supernatant dilution incubated with CTLL2 mouse cellular line, depending on IL-2 for its proliferation (Zar, 1984).

RESULTS AND DISCUSSION

A significant negative correlation (Y = 11.73 + [0.001] Xi; r = -0.64; p<0.05) was observed in the CTLL2 mouse cellular line, depending on IL-2 for its proliferation incubated with serial dilutions of SE-ILK. Table 1 shows the Counts per Minute (CPM) of the CTLL2 mouse cellular line, depending on IL-2 for its proliferation

Table 1: Presence of IL-2 activity on the SE-ILK

Treatments	Counts per minute
IL-2r + CTLL2	63,000
CTLL2 + SE-ILK	20,000
CTLL2 + PBS	3,100

Identification of IL-2 was performed on the ability of SE-ILK to support CTLL2 ³H - Thymidine incorporation. The C63 cell line supernatant was used as IL-2r source. The CTLL2 cells were also incubated with SE-ILK or Phosphate Basic Solution (PBS) solution as negative control

incubated with either IL-2r, SE-ILK, or saline. SE-ILK induced a 645.16% increase proliferation when compared with the saline negative control treatment. IFN activity in SE-ILK was indirectly detected by the increase in the expression of MHC class I and II molecules. Table 2 shows the expression of MHC class I molecules induction with two concentrations of SE-ILK or SMC. Splenocytes incubated with SE-ILK presented a maximum expression of MHC class I at 72 h, increasing 3,549.79% (with SE-ILK 10%) or 4,146.46% (with SE-ILK 20%) times with respect to the basal value. This activity slowly decreased by 120 h in all groups evaluated (p<0.05). Table 3 shows the expression of MHC class II molecules induction with two concentrations of SE-ILK or SMC. Splenocytes incubated with SE-ILK presented a maximum expression of MHC class I at 48 h, increasing 771.27% (with SE-ILK 10%) or 777.67% (with SE-ILK 20%) times with respect to the basal value. This activity slowly decreased by 120 h in all groups evaluated (p<0.05).

Responsiveness to invasive pathogens, clearance via the inflammatory response and activation of appropriate acquired responses are all coordinated by innate host defenses. Polymorphonuclear leukocytes (PMNs) are cellular components of innate response, with the

Table 2: Increase in the expression of class I molecules of the Major Histocompatibility Complex by SE-ILK

Basal Value	Time (h)	Treatments			
		SE-ILK 10 (%)	SMC 10 (%)	SE-ILK 20 (%)	SMC 20 (%)
12.03±0.05 ^f					
	24	373.83±0.18 ^e	321.19±0.40 ^f	504.31± 0.32 ^a	373.26±0.48 ^d
	48	358.33±0.40 ^f	443.05±0.07 ^e	408.21±0.26 ^d	454.34±0.41 ^b
	72	427.04±2.63 ^c	414.93±0.17 ^c	498.82±0.31 ^b	586.67±0.53 ^a
	120	126.94±0.09 ^h	181.97±0.08 ^h	152.96±0.19 ^g	317.58±0.35 ^g

Fluorescence intensity was evaluated by flow cytometry, splenocytes were incubated with SE-ILK and SMC at different times. Values represent means±SD. Significant differences in the expression of Class I MHC are marked with literal (p<0.05)

Table 3: Increase in the expression of class II molecules of the Major Histocompatibility Complex by SE-ILK

Basal Value	Time (h)	Treatments			
		SE-ILK 10 (%)	SMC 10 (%)	SE-ILK 20 (%)	SMC 20 (%)
116.56±0.06 ^f					
	24	208.86±0.86 ^g	267.03±0.35 ^f	205.05±0.73 ^g	286.25±0.28 ^d
	48	899.00±1.03 ^b	948.00±0.15 ^b	906.40±1.62 ^a	1024.00±0.53 ^a
	72	487.36±6.82 ^d	586.67±0.53 ^d	523.96±0.07 ^c	609.36±0.95 ^c
	120	312.96±0.19 ^f	101.48±0.61 ^h	349.43±1.04 ^e	241.22±0.52 ^g

Fluorescence intensity was evaluated by flow cytometry, splenocytes were incubated with SE-ILK and SMC at different times. Values represent means±SD. Significant differences in the expression of Class II MHC are marked with literal (p<0.05)

primary PMN in poultry being the heterophil (Swaggerty *et al.*, 2005). Heterophils mediate acute protection against *Salmonella* in young poultry (Genovese *et al.*, 1999; Kogut *et al.*, 1993; Kogut *et al.*, 1994; Swaggerty *et al.*, 2005) and in previous work we have shown that recombinant chicken IL-2 can directly activate chicken heterophils to exert effector functions and that heterophil activation by rChIL-2 is also an age-dependent event (Kogut *et al.*, 2002b). Similar studies have also indicated that neonatal avian heterophils can respond with enhanced functional competence to recombinant chicken interferon-gamma during the first week of life (Kogut *et al.*, 2001; Kogut *et al.*, 2005). The two bioassays conducted in the present study, suggest the presence of IFN and IL-2 functions in SE-ILK that may also participate in the establishment of the reported protection against *Salmonella* infections and propose that birds immune response develops similarly to that of mammals, with some features corresponding to the species.

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