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ROLE OF CORYNEBACTERIUM CUTIS AS AN IMMUNE STIMULANT ON THE IMMUNE RESPONSE OF CHICKENS AGAIST FOWL POX VIRUS

By

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SUMMARY

Corynebacterium was used as an immune stimulant to evaluate the immune response of chickens against fowl pox virus and fowl pox vaccine. The immune response of chicken was judged by measurement of cell mediated immunity which included Lymphocyte transformation activity, by using glucose consumption assay, macrophage activity percentage and index against *Candida albicans* cells. On the other hand, the humoral immunity was evaluated by determination of total serum portein, passive haemoagglutination (PHA) and neutralizing antibodies. The effect of *C. cutis* extract on the resistance of chicken against challenge with virulent fowl pox virus gave protection (8%) and gave significant increase in both cell mediated and humoral immunity in comparison with control chicks. Birds treatd with *C. cutis* extracts 3 days before vaccination against fowl pox virus, gave significant increase in lymphocyte transformation, macrophage activity, total serum protein, PHA and neutralizing antibodies. The protection percentage of vaccinated and treated groups agaisnt challenge with virulent fowl pox was 100 % as compared with 88% in vaccinatd birds without treatment with *C. cutis*.

INTRODUCTION

Several immune-suppressive factors cause severe economic losses to poultry industry as a result of exposure of birds to several environmental, factors and infectious diseases (Pier et al., 1980 and Camphell et al., 1983). The application of non specific immunostimulants not only rise the resistance of birds but also improve their immune response to vaccines.

These immunostimulants may be imunomodulators or immunopotentiators . The immunomodulators are substances which act on the immune system and have the capacity for positive and negative action, these included viruses, bacteria and their products as well as interfer on and its inducers (Mulcahy and Quinan, 1986). Immunopotentiators act in combination with antigenic stimulation and augment antigenic response, as levamisole with acts also as immunomodulator.

The non specific immunostimulants may be biological substance such as *Mycobacteria* (BCG), *Corynebacteria* and Vitamin A, or chemically defined bacterial or fungal products, as lipopolysaccharide, or biological products of immune system as interferon, or synthetic

biological analogues as synthetic muramyldipeptid (MDP) or chemical preparation as levamisole.

Barakat et al., (1986) reported that *C. ovis* has immunopotentiating, also Soliman et al., (1981) studied the immunostimulating effect of complete lysate of *C. cutis* on chicken immune response to New castle disease (ND) virus vaccine.

This investigation was planned to study the possible effect of *C. cutis* on the immune response of chicken after vaccination and challenge with fowl pox. The immune response was measured by various parameters which are cell mediated immune response by glucose consumption assay, macrophage activity by yeast ingestion and humoral immune response parameters as determination of total serum proteins and antibody titers against fowl pox using neutraliztion and PHA tests and protection after challenge with virulent fowl pox.

MATERIAL AND METHODS

* Embryonated Chicken Eggs (ECE) and Chicks:

ECE and 43 days old chickens were used for titration of viral and vaccinal strains of fowl pox. Day-old chicks (Procured from General Poultry Company) were used for conducting the experiments.

* Fowl Pox Virus strains and Antigens:

1- Virulent strain:

Local virulent fowl pox virus strain was isolated and identifled by Sabban (1954) and was used for challenge test.

2- Veccinal strain:

Egg adapted Beaudette strain of fowl pox vaccine, produced by Serum and Vaccine Research Institute, Abbasia, Cairo, was used for vaciantion of chickens.

3- Fowl pox antigen:

Beaudette strain of fowl pox virus was adapted on chorioallantoic membrane (CAM) of 9 days old ECE.

4- Antigen for phagocytosis (C. albicans):

C. albicans was cultivated on Sabouraud's agar and collected in PBS, followed by boiling for half an hour and filtered. The filtrate was centrifuged, resuspended in PBS, the yeast cells adjusted to $3x10^7$ cell / ml and stored at -20 °C until use.

* Immunostimulants:

- C. cutis: C. cutis lysate was kindly received from Virbac Lab. (BN; 143).
- Concanvalline A (Con-A): Con-A was used as non-specific mitogen in lymphocyte transformation test, Flow Lab., Cat No. 16.938-62.

* Media:

- RPMI 1640 medium: (Flow Lab.).
- Lymphocyte separation medium (Ficoll hypaque) : (Sigma) :
  This medium was used for separation of mononuclear leucocytes from peripheral blood.

2-Kits: Kits for glucose consuption test, Bochringer Mannheim # 630939.

2-Kits for determiantion of total serum protein, Bio-Adwi, # 80505.

*** Blood samples:**

- Samples with anticoagulant:

5 ml of chicken blood were collected in sterile centrifuge tube containing 25 IU/ml heparin for separation of mononuclear cells.

- **Samples without anticoagulant** for serum separation.

Determination of virus infective dose 50 (ID_{50}):

Titration of both vaccinal and virulent strains of fowl pox was carried out on CAM of 11 day ECE according to **Dhillon et al., (1968)**. ID_{50} of the virulent strain determined in 45 days old chicks, and calculatd according to **Reed and Muench (1938)**.

*** Passive haemaglutination test (PHA):**

Horse RBCs were treated with tannic acid followed by virus sensitization according to **Tripathy et al., (1970)**.

*** Neutralization test:**

The neutralization test was carried out by the method adapted for vaccinia virus after **Boulter (1957)** on CAM of 11 days ECE.

*** Determination of total serum protein:**

Total serum protein was determined according to the method of **Weichselbaum (1946)**.

*** Lymphocyte transformation test:**

A modified method of **Lucy (1977 and 1984)** was carried out, in which the monomorphnuclear cells were separated from heparinized blood by density gradient centrifugation using Ficoll-hypaque as a lymphocytic separation medium according to **Roland (1984)**. The mitogenic stimlation of these cells was examined against Con. A mitogen in a concentration of 20 μg/ml using glucose consumption assay of **Decock et al. (1980)**. The assay was tested at 490 nm using an ELISA reader according to the following formula :-

Glucose consumption of tested sample = (C)

$$C = \frac{\text{OD of the medium}}{\text{OD of the standard}} \times 100 = \text{mg / 100 ml}$$

C_1 = Conc. of glucose in the medium.

C_2 = Conc. of glucose in the medium with control cells only.

C_3 = Conc. of glucose in the medium with con. A.

* Glucose consumed by control cells (K_1) = C_1 - C_2.

* Glucose consumed by stimulated cells (K_2)= C_1 - C_3.

$$\text{*Glucose stimulation index by Con. A (SI)} = \frac{K2}{K1}$$

*** Determination of phagocytic activity of chicken monocytes:**

The separated mononuclear phagocytic cells were cultivated in cell culture staining chambers (CCSC) according to Antley and Hazen (1982). The phagocytic activity of the adhered phagocytic cells was estimated by using Candida albicans as a phagocytosed cells. The percentage of hyagocytosis and phagocytic index were calculated according to Richardson and Smith (1981) as follows :-

* Percentage of phagocytosis =

$$\frac{\text{No. of phagocytic cells with ingestd C. albicans}}{\text{Total number of phagocytic cells}}$$

$$\text{* Phagocytic index} = \frac{\text{Total No. of ingested yeast cells}}{\text{Total No. of engulfed phagocytes}}$$

Experimental design:

One hundred and forty Lohmann chicks of 43 days old were tested serologically for detection of antibodies against fowl pox and proved to be negative. These birds were divided into 4 groups each of 35 chicks. The first group (G. 1) served as a control (negative control without any treatment), the second group (G. 2) was injected I/M with complete lysate of C. cutis extract, the third group (G. 3) was vaccinated at 46 days old with fowl pox vaccine and the fourth group (G. 4) was treated with C. cutis extract by I/M injection of 40 µl/bird 3 days before vacciantion with fowl pox vaccine. Blood samples were collected at 0, 3, 10, 17 and 24 days post treatment and vaccination. All birds were subjected to challenge with a virulent fowl pox virus at 42 days post vacination using the wing web method and kept under observation for a period of 2 weeks for calculation of protection percentage.

RESULTS

The effect of Corynebacterium on the im response of chickens vaccinated with fow and challenged with virulent strain of fow virus was examined and evaluated thr different immunological techniques which in lymphocyte transformation, phagocytic ac tests in addition to total serum protein protection percentage.

Table (1) shows the lymphocyte stimulation i (SI) of all groups; it was clear that there significant increase in the SI in the treatd vaccinated groups in comparison with co group specially at 10 and 17 days post treat while there was suppression of SI of vacciinated groups specially at 24 days treatment.

Table (1): Effect of Corynebacterium on lymphocyte transformation activity of vaccinated and non vaccinated chicks .

Time of testing	Average *Lymphocyte stimulation index*			
	G.1	G.2	G.3	G.4
43 days old (Before treatment)	1.40	1.40	1.40	1.40
46 days old (3 d.p.t.)	1.40	1.58	1.40	1.58
53 days old (10 d.p.t.)	1.60	2.10	2.17	2.45
60 days old (17 d.p.t.)	1.60	2.60	2.30	2.50
67 days old (24 d.p.t.)	1.04	2.20	1.20	1.30

G.1 = Control group (non vaccinated and non treated) .

G.2 = Corynebacterium treated group .

G.3 = Vaccinated non- C. cutis treated group .

G.4 = C. cutis treated and vaccinated group . d.p.t. = days post treatment .

Table (2) : Effect of Corynebacterium on macrophages activity of vaccinated and non vaccinated chicks .

Time of testing	*Macrophages activity*							
	G.1		G.2		G.3		G.4	
	%	Index	%	Index	%	Index	%	Index
43 days old (Before TT.)	47.1	1.30	47.1	1.30	47.1	1.30	47.1	1.30
46 days old (3 d.p.t.)	24.2	1.60	28.5	1.7	24.9	1.60	27.0	1.70
53 days old (10 d.p.t.)	42.0	1.30	74.5	1.75	45.0	1.40	78.0	1.50
60 days old (17 d.p.t.)	60.0	1.05	78.0	1.40	67.0	1.10	69.0	1.40
67 days old (24 d.p.t.)	53.5	1.07	80.0	1.30	53.0	1.20	73.0	1.30

G.1 = Control group (non vaccinated and non treated) .
G.2 = Corynebacterium treated group .
G.3 = Vaccinated non- C. cutis treated group .
G.4 = C. cutis treated and vaccinated group . d.p.t. = days post treatment .

Table (3): Total serum proteins of C. cutis treated and fowl pox vaccinated chickens .

Time of testing	Total serum proteins (gm %)			
	G.1	G.2	G.3	G.4
43 days old (Before treatment)	4.5	4.5	4.5	4.5
46 days old (3 d.p.t.)	3.9	5.2	4.5	6.2
53 days old (10 d.p.t.)	3.5	5.2	3.9	8.3
60 days old (17 d.p.t.)	3.14	7.01	3.6	11.0
67 days old (24 d.p.t.)	3.7	6.8	3.4	9.1

G. 1= Control group (non vaccinated and non treated).
G. 2= Corynebacterium treated group
G. 3 = Vaccinated non-C. cutis treated group.
G. 4= C. cutis treated and vaccinated group. d. p. t.= days post treatment.

Table (2) and Fig. (1) reveal the macrophages activity of treated chicks with C. cutis and vaccinated with fowl pox vaccine. There was a clear increase in the percentages of phagocytosis specially at 10 days post treatment in all treated and vaccinated groups in realtion to the control group.

The results in Table (3) shows the total seru protein in (gm %) of treated and vaccinate groups. It was clear that total serum protein (TSI was highly increased in C. cutis treated groups ((2 and G. 4) while there was no increase in TSP i fowl pox vaccianted and non-C. cutis treate group (C.3).

Table (4) : Effect of corynebacterium on neutralizing antibodies against fowl pox vaccine .

Time of testing	Arthim. mean of neutralizing antibodies		
	G.1	G.2	G.3
46 days old (Before vaccinat.)	0.0	0.0	0.0
53 days old (7 d.p.v.)	0.0	2.7	3.3
60 days old (14 d.p.v.)	0.0	5.9	6.7
67 days old (21 d.p.v.)	0.0	3.9	6.8
74 days old (28 d.p.v)	0.0	4.8	4.9
81 days old (35 d.p.v.)	0.0	4.9	4.5

G.1 = Control group (non vaccinated and non treated) .
G.2= Vaccinated non- C. cutis treated group
G.3 = C. cutis treated and vaccinated group ..
d.p.v. = days post treatment .

Table (5): Passive haemagglutination (PHA) of chickens treated with corynebacteria and vaccinated with fowl pox virus .

Time of testing	Arthim. mean of PHA antibody titers		
	G.1	G.2	G.3
46 days old (Before vaccinat.)	0.0	0.0	0.0
53 days old (7 d.p.v.)	0.0	5.50	4.5
60 days old (14 d.p.v.)	0.0	5.50	6.30
67 days old (21 d.p.v.)	0.0	5.25	6.10
74 days old (28 d.p.v)	0.0	5.75	6.88
81 days old (35 d.p.v.)	0.0	6.88	7.10

G.1 = Control group (non vaccinated and non treated) .
G.2= Vaccinated non- C. cutis treated group
G.3 = C. cutis treated and vaccinated group . d.p.t. = days post treatment

The neutralizing antibody titers were carried out on CAM of 11 days old ECE and the results revealed that there is a significant increase in the neutralizing titers between G.2 and G.3 in comparison to the control (G.1) as shown in table (4).

Table (5) shows the passive haemgglutonating antibodies of different groups; it was clear that there is a significant difference and increase between the vaccinated and corynebacteria treatd group (G.3) specially at 60 days old (14 d. p. v.).

Table (6): Protection percentages of *C. cutis* treated and vaccinated chickens against challenge with fowl pox virulent virus .

Groups	No.of challenged birds	No. of birds showing pox lesion	Protection percentage
G.1	25	25	0
G.2	25	0	8
G.3	25	3	88
G.4	25	0	100

G. 1 : Control group (non-vaccinated and non treated)
G. 2 = Vaccinated non- C. cutis treated group
G. 3 = C. cutis treated and vaccinated group.

Fig. (1): Phagocytic activity of chicken macrophages to *C. albicans* after *C. cutis* treatment.

The protection percentages of C. cutis treated and fowl pox vaccinated groups against challenge with virulent fowl pox virus were tabulated in Table (6). The results showed that 100% protection in treated and vaccianted group (G. 4) and 88% of vaccinated non treatd group (G. 3) while they were 0 and 8% in control (G. 1) and C. cutis treated group (G. 2).

DISCUSSION

Immunostimulators are substances that act either on the hapten or antigen enhancing its antigenic properties or on the cells involved in the immune response. Immunostimulators have the ability to enhance host resistance and immune response to viral, bacterial and fungal infection. It may act at the level of antigen by: (1) modification of antigen by confirmation changes or by altering the net electrical charge of its molecules (Jolles and Paraf, 1973); (2) Transformation of a non-immunogenic hapten into an immunogenic (3) or by denaturation of some antigens by emulsification onto other particles to facilitate the presentation of antigen to lymphocytes (Allison, 1973) or it may act at level of the host by delaying its release at its site of deposition (Glenny et al., 1931) or may cause the sequestration of lymphocytes in lymphoid organs-lymphocyte trapping (Frost and Lance. 1973).

In this work the non-specific immunostimulator, Corynebacterium was used to study its influence on the imune response of chickens against challege with fowl pox virus in vaccinated and non-vaccianted birds.

The parameters used for measuring the effect of *Corynebacterium* on cell mediated immunity were lymphocyte transformation and macrophages activity. while the humoral immunity parameters were measured by determination of total serum proteins. passive haemagglutinins. neutralizing antibodies and protection percentage of birds against challenge with virulent fowl pox virus.

Corynebacterium cutis 40 µl/ birds were injected I/M and was used as nonspecific immunostimulant, the obtained results in table (1) revealed that there was a significant increase in the stimulation index of peripheral blood lymphocyte to concanavalin A in treatd birds on the 10th , 17th and 24th day post treatment, these results support the previous findings reported by Archambault et al., (1989) who proved that the blastogenesis of peripheral blood lymphocytes to Con. A in treated calves with *Corynebacterium parvum* started in the first 3 days after treatment. Jan (1981) also reported that the blastogenic response of birds lymphocytes to PHA and Con. A enhanced after 24 hours of levamisole treatment and persist until 5th day.

The phagocytic activity of macrophages increased post *C. cutis* treatment form the 10th to 24th days post treatment (table 2), Frost and Lance (1973) reported that *Corynebacterium* is an activator of macrophages, while Malhorta et al., (1984) observed an increase in phagocytic activity of monocytes of healthy calves 2 weeks after treatment, this finding agree with the present data in table (2).

Soliman et al., (1991) reported a higher phagocytic activity in *C. cutis* treated chickens compared with non-treated ones, 2 weeks post vaccination with ND vaccine (Lasota strain), while, Corrier and Ziprin (1989) found that killed *C. parvum* suppressed the cell mediated immune response to *S. typhimurium*.

Total serum protein was significantly increased in treated groups form 3rd day to 24th post treatment and vaccination (table 3). This result confirmed by the data obtained by Ishikawa et al., (1982) and

Krasnikov et al., (1986) who found that levamisole and *corynebacterium* increase total serum proteins.furthermore, Giurgea and Coprean (1985) indicated that killed Corynebacterium suspension increased the serum gamma globuline content of treated chicks .

Serological examination revealed a significant high level of passive haemagglutinins and neutralizing antibodies from 14th days post treatment and vaccination (Table 4 and 5). Neutralizing antibodies against fowl pox virus significantly increased from 7th day post vaccination, these results agreed with those obtained by White et al., (1975) and Padany et al., (1980) who found that inactive *Corynebacterium* suspension given to mice immunized against. *E rhusiopathea* had increased the vaccine potency 1.35-2.15 times, also Soliman et al., (1991) found an increase in antibody titer against ND virus when *C. cutis* was given 3 days before vacciantion.

Protection against challenge with virulent fowl pox virus was maximum in treated and vaccinated birds (G. 4) (100 %), while non-treated and vaccianted ones (G. 3) was (88 %). Table (6); These results agree with those obtained by Gelencser et al., (1980) who observed active protection in mice immunized with tetanus toxoid and treated with *Corynebacterium.* Corrier and Wagner (1984) also found complete protection of mice treated I/P with *Corynebacterium* and challengd 3 days later with *Babesia rodhain* which caused death of all non-treated group, also Soliman et al., (1991) observed a protection degree against virulent ND virus in treated chickens with *C. cutis* and vaccianted with Lasota strain vaccine. Disagree with results of Donahoe et al., (1978) who found that non specific stimulation of *Corynebacterium* in Marek's disease vaccinated birds and resulted in higher tumors than control.

In conclusion, it is clear that *Corynebacterium* cutis proved to be effective and has an immunostimulating activity in raising the imune response of chickens when vaccinated with fowl pox vaccine and gave more protection and high imune responsein either in cell mediated or humoral immunity.

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