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Mycobacterium phlei as an oral immunomodulator with Newcastle disease vaccine

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Experiments were conducted in chickens to understand the effects of oral immunomodulation. Heat inactivated *M phlei*, a commensal Mycobacterium and a non-specific immunomodulator, was administered orally prior to live Newcastle disease F (ND F) strain vaccination. In experimental birds it lead to an enhanced cell mediated Immune response (CMI) against the vaccine. There was a reduction in the Haemagglutination inhibiting (HI) antibodies. However, it did not affect the protection against a virulent challenge, as the protection percentage was more or less same in vaccinated birds irrespective of the *M.phlei* administration. *M.phlei* administration could not enhance the immune response to inactivated ND F vaccine administered orally. The results indicate that *M.phlei* favours a CMI response to orally administered live ND F vaccine. It may be of potential use in enhancing CMI against vaccines and a cheaper alternative to costlier recombinant cytokines.

Various prophylactic vaccination strategies have been practiced for controlling Newcastle disease (ND), an economically important paramyxo viral infection of poultry[1]. Oral vaccination and immunomodulation are cheaper and feasible alternatives to parenteral routes for both commercial and backyard poultry farming [2,3]. Use of adjuvants along with oral ND vaccines has received very little attention in India. Conventional ND F strain vaccine induces a short-term protective immunity. Immunomodulators such as aviridine, Quil A, muramyl dipeptide and Iscoms have been used to augment immune responses to oral vaccination with lentogenic strains[4,5]. The development of cheaper and effective alternatives for oral immunomodulation is necessary for profitable use in poultry industry.

Mycobacterium phlei, a fast growing environmental Mycobacterium, has been used experimentally to potentiate immune response[6,7]. In chickens, *M.phlei* has shown to increase non-specific effector functions of intestinal intra- epithelial lymphocytes (IELs), the major immune cells on gut mucosa[8]. The effect of *M. phlei* on specific immune response to various orally presented antigens are not known. The present study was carried out to evaluate its modulation of specific immune response against oral ND F vaccine in chicks.

Materials and Methods

Newcastle disease vaccine—Freeze-dried ND lentogenic F strain live vaccine was obtained from the division of Avian Diseases, IVRI. The vaccine was reconstituted in 5 ml of phosphate buffered saline(PBS). 0.1 ml of 10-fold dilutions of the stock was inoculated into 7-day-old embryonated chicken eggs by allantoic cavity route. Mortality observed after 24 hr of inoculation was recorded for a period of 7 days. The proportionate distance was measured using Reed and Muench formula[9] and embryo infective dose 50 (EID_{50}) was calculated. The titre was found to be 1×10^6 EID_{50} per ml.

Live ND F vaccine was inactivated using 0.2% Formalin[10]. 0.1 ml was inoculated into the allantoic cavity of 7-day-old chicken embryos. Absence of mortality confirmed inactivation.

Mycobacterium phlei—A seed culture was obtained from the division of Biological Products, IVRI. It was grown in nutrient broth (Hi-Media) containing 5% glycerol for 15-20 days. The yellow surface growth was filtered onto a sterile muslin cloth, washed with sterile PBS thrice, and heat inactivated at 100°C for 15 min. The preparation was found to be sterile on nutrient agar. It was air dried in an incubator at 40°C and used in the experiments.

Experimental design—Sixty, 2 weeks old, unvaccinated, White Leghorn chicks of either sex were used. Birds were housed in clean cages and provided with *ad libitum* feed and water. They were divided into six different groups and the following treatments were done.

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Group A: 10mg (dry weight) of inactivated *M.phlei* was suspended in 1ml of PBS and was administered orally to each bird. Two doses were given 72 hrs. apart. After 72 hr of the last dose, they were vaccinated orally with 0.5 ml of the RD F vaccine (10^6 EID_{50}, reconstituted in 10ml of sterile PBS).

Group B: Were vaccinated orally with 0.5 ml of the RD F vaccine (10^6 EID_{50} reconstituted in 10ml of sterile PBS) only.

Group C: Treatment same as group A except that formalin inactivated RD F vaccine was used for vaccination.

Group D: Received formalin inactivated RD F vaccine only.

Group E: Only inactivated *M.phlei.*

Group F: Served as controls, receiving no *M. phlei* or vaccine.

Samples of blood were collected from all the birds on 0,7,14 and 21st day post-vaccination. Blood was collected in heparin for cell mediated immune response (CMI) studies on 14th day of vaccination.

Lymphoproliferative Response—The MTT colorimetric assay for cellular proliferation [11] was followed with necessary modifications.

Peripheral blood lymphocytes (PBLs) were isolated from heparinized blood on Histopaque 1077 (Sigma). 2×10^6 PBLs/ml were used in the transformation assays. Live and inactivated ND F at an optimized dose of 6×10^4 EID_{50}/ml was used in subsequent experiments. After addition of the antigens in appropriate wells in triplicate, the volume in all the wells was made up to 100µl with RPMI 1640 containing 2m*M* L-glutamine (Sigma), 0.05m*M* β-mercaptoethanol (Sigma), 100 µg/ml streptomycin, 100 IU/ml penicillin and 10% new born calf serum. The plates were incubated at 39°C in 5% CO_2 in a CO_2 incubator for five days. 20µl of MTT (3-[4,5-dimethyl thiazol-2-yl]-2,5 diphenyl tetrazolium bromide) solution (5mg/ml) was added to all the wells and incubated further for 4hr. 150µl of dimethyl sulfoxide was added to all the wells and the contents were mixed thoroughly by pipetting. After 15 min, the OD was taken in an ELISA reader (Anthos Lab Tec.) at a test wavelength of 492 nm with a reference reduction of 650 nm. The stimulation index was calculated using the following formula:

$$SI = \frac{OD_{stimulated} - OD_{unstimulated}}{OD_{unstimulated}}$$

Haemagglutination inhibition (HI) test—HI test was done following the method previously described[12]. Proper positive and negative controls were included in all the tests. The HI titre was calculated as the reciprocal of the highest dilution of the test sera showing complete haemagglutination and was expressed as $\log_2$ values.

Challenge test—Challenge test was conducted in an isolated animal house 21 days after vaccination. Experimental and control birds were inoculated (im) with 10^4 virulent ND virus in a volume of 0.5ml. The birds were observed for a period of 7 days and the mortality was recorded to calculate the per cent protection.

Statistical analysis—For each of the trials, analysis of variance was conducted by multiple comparisons using the Tukey-Kramer Test[13].

Results

(a) *Cell mediated immune response to oral ND F vaccine.*

Live ND F vaccination—The PBLs from *M. phlei* fed birds showed an enhanced proliferative response to *in vitro* stimulation with live and inactivated antigens (Table 1). The specific stimulation against ND virus in group A was significantly higher ($P<0.01$) than group B. Birds in group E also showed an enhanced proliferative response to ND antigens compared to group F ($P<0.01$). This response can be due to a non-specific immunostimulation offered by *M.phlei*, as group E was not vaccinated previously.

Inactivated ND F vaccination—The proliferative response of PBLs in group C and D was only slightly increased compared to group F (Table 1). This can not be considered specific as the comparison between the different groups were statistically not significant ($P>0.05$)

(b) *Antibody induction to oral ND F vaccination and response to challenge*—Table 2 shows the antibody response of various groups to oral ND F vaccination. Groups A and B had a higher HI titre, which showed a progressive increase. In group B, the HI titre peaked on 14th day and showed a dip on 21st

day. In group A, however, the HI values maintained the ascending trend.

In groups C and D, where inactivated vaccine was given, there was induction of HI antibodies. But the values were low compared with the live vaccine given groups.

The protection test conducted in group A and group B gave an almost identical protection (80% and 83.3% respectively) irrespective of their HI titres. The virulent challenge of inactivated vaccine given birds indicated a low protection (33.3% in group C; 20% in group D) irrespective of the prior administration of *M.phlei.*

Discussion

Immunomodulators are agents that enhance the general immune status of the host. In the present study, the efficacy of *M. phlei* as an oral immunomodulator was evaluated.

An oral route of immunomodulation was selected based on the following considerations:- (1)Mycobacterial antigens can attach to intestinal epithelium and activate gut associated lymphoid tissue[14]. (2) *M.phlei*, on oral administration, can activate intestinal intra epithelial lymphocytes (IELs)[8]. (3) Oral vaccination and immunomodulation are easier methods for flock treatments.

MTT colorimetric assay employs the indirect measurement of viable cells by measuring the amount of succinic dehydrogenase present in cellular mitochondria. This assay has been found to be comparable with conventional ^{3}H Thymidine assay in sensitivity and reproducibility in lymphocyte transformation assays[15,16].

M.phlei primed birds were found to induce an enhanced CMI against ND F vaccine. This was indicated by a significantly higher proliferative response of PBLs against ND antigens (Table 1). Cytokines play a major role in directing the immune response to a cell mediated or humoral one[17]. An enhanced CMI results from an increase in the level of Th1 cytokines like interferon-γ and interleukin-2. Our results indicate that *M.phlei* favoured induction of Th1 cytokines. Mycobacterial antigens are known to produce Th1 response in mammalian hosts[18]. Circulation of activated lymphocytes from mucosa to distant sites has been reported[19]. To understand this enhanced CMI, analysis of the cytokine profiles of the circulating and mucosal lymphocytes is essential. In challenge studies the enhanced CMI was found to compensate for a lower antibody titre in affording protection. This confirms the previous reports[20,21] on

Table 1—Cell mediated immune response against oral live NDF and oral inactivated NDF vaccine in chicks fed with *M.phlei* : PBL proliferation 2 weeks postvaccination-MTT colorimetric assay

Gp.	Treatment	SI (mean±SE), *in vitro* antigen	
		Live NDF	Inactivated NDF
		Oral live NDF	
A	*M.phlei*+Live NDF	0.7786±0.0161*	0.744±0.0261*
B	Live NDF	0.5173±0.0308	0.4304±0.0159
C	*M.phlei* + Inactivated NDF	0.542±0.017*	0.491±0.007
D	Inactivated NDF	0.3979±0.018	0.3118±0.011
E	*M.phlei*	0.540±0.043	0.501±0.016
F	Control	0.368±0.013	0.446±0.027

$P<0.01$; *$P>0.05$; n=6

Table 2—Antibody Response induced by orally administered ND F vaccine alone and in combination with heat killed *M.phlei* in chicks

Group	HI titres (log_2 values)				Challenge	
	0	7	14	21	No.of survivors/No challenged	% Protection
A	1.0	1.41±0.16	3.01±0.23	3.52±0.45	8/10	80.0
B	0.5	2.66±0.66	5.8±0.6	5.3±0.818	5/6	83.3
C	1.0	1.25±0.56	2.16±0.97	2.49±0.13	3/9	33.3
D	1.0	1.14±0.12	1.88±0.91	2.12±0.08	3/10	30.0
E	0.5	1.01±0.08	1.51±0.47	1.41±0.01	2/6	33.3
F	0	1.25±0.52	1.31±0.19	1.58±0.09	2/10	20.0

the role of systemic CMI in protection against Newcastle Disease

As in mammals, chickens also have T helper cells with αβ and γδ T-Cell Receptor (TCR)[22]. Chickens have an abundance of γδ TCR +ve T cells in their peripheral circulation. These cells play suppressor role on activation with Th 1 cytokines such as IFN-γ and IL-2[23]. They inhibit secretion of IgG1 from B-cells[24]. Whether activation of these cells by Th1 cytokines lead to a low antibody titre needs to be assessed.

In *M.phlei* primed birds, the HI titre showed a gradual increase up to the 21 days of observation. In un-primed birds it showed the highest value on 14th day and showed a dip on the 21st day. This gives an indication that *M.phlei* may help in prolonging the HI titre albeit its low values. Further evaluation for a longer duration is necessary to understand whether immunomodulation with *M.phlei* could facilitate long term protection.

Poor immunogenicity of inactivated ND antigens has been previously reported[5]. *M.phlei* also failed to enhance this immune response.

The results from the present experiments indicate that *M.phlei* may be a beneficial immunomodulator for diseases where enhanced CMI is essential. Further work on this line will help in evolving this environmental mycobacterium as a cheaper alternative for non-specific immunomodulation. This may replace the costlier recombinant cytokines used in the form of cytokine therapy in chicken[25].

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