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PROLIFERATIVE RESPONSES BY CHICKEN LYMPHOCYTES FOLLOWING VACCINATIONS WITH NEWCASTLE DISEASE, INFECTIOUS BURSAL DISEASE AND FOWLPOX VIRUS ANTIGENS

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SUMMARY

The *in vitro* proliferative responses of lymphocytes from spleen of chickens vaccinated with either Newcastle disease virus (NDV), infectious bursal disease virus (IBDV) and fowlpox virus (FPV) antigens were determined using the tetrazolium salt (MTS) [3- (4,5 dimethyl thiazol -2-yl)-5- (3carboxymethoxyphenol) -2- (4- sulphophenyl)-2 H tetrazolium inert salt] as substrate. After five days of incubation, the chicken lymphocyte proliferations were measured using a spectrophotometer. The proliferative activities were indicated by the differences in optical density between the non-stimulated and the stimulated cultures. The NDV-antigen was the most powerful stimulant compared to the other two antigens, except when IBDV and FPV antigens were used to stimulate the lymphocytes of chicken vaccinated with their respective vaccines. The results suggest the strong ability of NDV to stimulate lymphocyte proliferation without interference of the immune system compared to the IBDV and FPV.

Keywords: Lymphocyte, proliferation, Newcastle disease, infectious bursal disease, fowlpox

INTRODUCTION

The *in vitro* proliferative response of lymphocytes to specific antigen has been used to predict the *in vivo* response of cell-mediated immunity (Valentine, 1971). A number of rapid colorimetric assays have been used to evaluate chicken lymphocyte proliferation (Maheswaran and Thies, 1975; Tapani *et al.*, 1978). Recent work by Bounous *et al.* (1992) proved the suitability of the tetrazolium salt (MTS) to measure the blastogenic response of chicken lymphocvtes compared to the conventional tritiated thymidine deoxyribose (^{3}H-TdR) method used by earlier workers (Knudsen *et al.*, 1974; Holt and Latimer, 1989).

Many other methods are available for evaluation of lymphocyte blastogenic responses. These include the glucose consumption test (de Cock *et al.*, 1980; Hussain *et al.*, 1981; Ishikawa and Shirahata, 1985; Nakanishi *et al.*, 1986), the fluorometric DNA synthesis assay (Itoh *et al.*, 1983; Nagahata *et al.*, 1986), and the ethidium bromide fluorescence assay (Itoh *et al.*, 1983; Nagahata *et al.*, 1986; Inoue *et al.*, 1987).

The present study determines the ability of Newcastle disease virus, infectious bursal disease virus and fowlpox virus to stimulate chicken lymphocytes *in vitro*.

MATERIALS AND METHODS

Chicken

Sixty-four day-old specific-pathogen-free (SPF) Lohmann chicks obtained from the African Poultry Company (Khartoum, Sudan) were used in the experiment. The chicks were divided into four equal groups. Groups 1, 2 and 3 were vaccinated with Newcastle disease, infectious bursal disease and fowlpox vaccine, respectively. Chicks in group 4 were kept without vaccination as a control group.

Vaccination programme

Vaccination with the live Newcastle disease virus (NDV) vaccine, which contained the mesogenic chick-embryo propagated, Komarov strain of NDV, was carried out at three weeks of age. The vaccine was administered via the intranasal route.

Vaccination with live infectious bursal disease vaccine was carried out at two weeks old. The vaccine, which contained the chick-embryo propagated D-78 strain, was administered in drinking water.

The fowlpox vaccination was carried out at 45 days old, using a live chick-embryo propagated vaccine. The vaccine contained the Beaudette strain of FPV, and was administered using the wing web method. Following the vaccinations, all birds were kept for 15 days before being slaughtered.

Antigens for the lymphocyte proliferative assay

The ND, IBD and FP vaccines, which contained the respective viruses were reconstituted according to the manufacturer's instructions and heat-inactivated at 56°C for one hour before being used as antigens in the test since the live virus may infect the cells and arrest their growth.

Culture medium

One litre of the Dulbeco Modified Eagles Medium (DMEM) (Sigma; St Louis, USA), supplemented with 10mL penicillin (100,000 I.U.; Sigma), 0.8mL gentamycin (10 mg/mL; Sigma) and 5mL mercaptoethanol alcohol (0.01mM; Sigma) was used to disperse cells and to inhibit the growth of scavenger cells. The same medium, supplemented with 10%(v/v) sterilised, heat-inactivated fetal bovine serum (Gibco BRL, Life Technology, Scotland) was used to maintain the lymphocyte culture.

The colour substrate

The colour substrate used in the test was a mixture containing 6 mM [3-(4,5 dimethyl thiazol-2- yl)-5-(3-carboxy methoxy phenyl)-2-(4-sulphophenyl) 2H tetrazolium inert salt] solution at the concentration of 1 mg/mL (Aldrich; Wisconsin, USA) and 0.33 mM phenazine methosulfate (PMS) (Aldrich; Wisconsin, USA). The mixture was sterilized using a 0.22 µm filter unit and was used either at once or stored at 4°C in the dark for several weeks.

Cells collection and preparation

The chicks from each group were slaughtered at 15 days post-vaccination. The spleens were removed aseptically, collected individually into sterile PBS and maintained on ice. To harvest the splenic cells, the spleen was crushed and submerged into 10mL DMEM culture medium before it was thoroughly homogenised. The cells were then aspirated and placed into a 10mL centrifuge tube. The cell suspension was maintained on ice for 10-15 minutes to allow cellular debris to settle down before the supernatant was transferred to a new tube and centrifuged at 2000xg for 10 minutes. The supernatant was discarded and the pellets were washed twice in 10mL of culture medium by centrifugation before the pellets were re-suspended in 2mL of complete culture medium.

Lymphocyte culture

The lymphocyte concentration was determined using the 0.2% trypan blue exclusion staining method before the concentration was re-adjusted to $0.5x10^7$ cells/mL. The 96-well microtitre plate was used for lymphocyte culture, in which 100µL of the lymphocyte suspension containing $0.5x10^6$ cells was placed into each well. The first well contained the medium without cells, the second well contained cells without antigenic stimulation while the 3rd, 4th and 5th wells contained cells that were stimulated with 20µL of ND, IBD and FP antigen respectively. Each treatment was carried out in triplicate. The whole plate was then incubated at 37°C in 5% $C0_2$ using the gas pack $C0_2$ system. Forty-eight hours after incubation, 15µL of the MTS colour substrate was added into each well followed by 72 hours incubation. The proliferative responses of the lymphocytes were measured using a spectrophotometer (Dynetech II; Virginia, USA) at 450 nm wavelength.

Statistical analysis

The data were statistically analysed using the two-tailed students unpaired t-test.

RESULTS

Table 1 summarises the lymphocyte proliferative responses following stimulation by various antigens. There were significantly ($p<0.05$) high rates of lymphocyte proliferation observed in chicks vaccinated with NDV following stimulation by all antigens. Significantly ($p<0.05$) high rates of lymphocyte proliferation were observed in IBD-vaccinated and FP-vaccinated chicks following stimulation by their respective antigen. The proliferation rates, however, were generally lower in chicks vaccinated with either IBDV or FPV.

DISCUSSION

In this study, tetrazolium salt was used to determine the blastogenic responses by chick lymphocytes. The measurement was based on the reduction of MTS into formazan salt by mitochondrial dehydrogenase of viable cells. This reduction was found to be closely related with the proliferative activity of the cells (Gerlier and Thomasset, 1986).

The *in vitro* proliferative response of lymphocytes to specific antigen has been shown to be closely correlated to the cell-mediated immunity (Valentine, 1971) even though some investigations revealed that viral antigens were able to stimulate both B and T lymphocytes.

Table 1. Summary for the proliferative response of lymphocytes from vaccinated chickens

Vaccination	Stimulation			
	W/0	ND	IBD	FP
Non-vaccinated	$0.39^{a} \pm 0.02$*	$0.47^{b} \pm 0.05$	$0.41^{a} \pm 0.02$	$0.40^{a} \pm 0.05$
ND-vaccinated	$0.45^{a} \pm 0.02$	$0.71^{c} \pm 0.06$	$0.66^{c} \pm 0.02$	$0.55^{b} \pm 0.03$
IBD-vaccinated	$0.41^{a} \pm 0.04$	$0.46^{a} \pm 0.03$	$0.50^{b} + 0.05$	$0.44^{a} \pm 0.05$
FP-vaccinated	$0.48^{a} \pm 0.16$	$0.50^{a} \pm 0.02$	$0.52^{a} \pm 0.01$	$0.55^{b} \pm 0.05$

*O.D value at 450 nm (mean$\pm$s.d)
[a,b,c]Means with different superscripts within a row were significantly different at $p < 0.05$.
W/0 = Without stimulation, ND = Newcastle disease, IBD = Infectious bursal disease, FP = Fowlpox.

In this study, the lymphocytes from ND-vaccinated chicks showed high proliferative responses to all stimulants, suggesting that NDV antigen is a strong immune stimulant, safer and not interfering with the immune response of chicks compared to the IBD and FP viral antigens. The IBDV has been shown to produce adverse affects on the immune system of chicken (Allan *et al.*, 1974; Rao and Rao, 1992), while no such effects were reported with the NDV (Westbury, 1984). However, the chicks vaccinated with IBD vaccine in this study still showed significant lymphocyte proliferation since IBDV is known to infect and destroy mainly the B-lymphocytes within the bursa of Fabricus, spleen and caeca-tonsils, while the T-lymphocytes are only slightly affected (Hirai *et al.*, 1974).

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RINGKASAN

GERAK BALAS PROLIFERATIF OLEH LIMFOSIT AYAM BERIKUTAN PEMVAKSINAN DENGAN ANTIGEN VIRUS PENYAKIT NEWCASTLE, PENYAKIT BURSA BERJANGKIT DAN PURU AYAM

Gerak balas proliferatif in vitro *oleh limfosit daripada limpa anak ayam tervaksin terhadap antigen virus penyakit Newcastle (ND), virus penyakit bursa berjangkit (IBDV) dan virus puru ayam (FPD) telah diasai mengguna garam tetrazolium (MTS) [garam lengai 3-(3,5 dimetil tiazol-2-yl)-5-(3 karboksimetoksifenol)-2-(4-sulfofenil)-2H tetrazolium] sebagai substrat. Selepas 5 hari penginkubatan, pemproliferatan limfosit ayam disukat mengguna spektrofotometer dan indeks perangsangan dikira. Perbezaan ketumpatan optik di antara kultur bukan terangsang dengan terangsang (indeks perangsangan > 1) menunjukkan aktiviti proliferatif limfosit. Di antara tiga perangsang yang diguna, antigen NDV merupakan perangsang paling kuat berbanding dengan dua antigen lain, kecuali apabila IBDV dan FPV diguna untuk merangsang limfosit ayam yang divaksinkan dengan vaksin masing-masing. Hasil kajian menyarankan kebolehan kuat NDV merangsang pemproliferatan limfosit tanpa gangguan ke atas sistem imun berbanding IBDV dan FPV.*