

Proliferator Activity, Expression Interleukin-2 and Interferon- γ of Immunoglobulin Y Anti HIV in Peripheral Blood Mononuclear Cells

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Abstract: The present study was performed to investigate the effect of Immunoglobulin (Ig)Y anti HIV on PHA-activated peripheral blood mononuclear cells (PBMCs) proliferation, interleukin (IL)-2 and interferon (IFN)- γ secretion. Effects of IgY anti HIV in different concentrations of 25, 50 and 100 $\mu\text{g/ml}$ on phytohemagglutinin (PHA) activated-human peripheral blood mononuclear cells (PBMC) proliferation were determined by MTT assay. The concentration of IL-2 and IFN- γ in the PBMCs supernatant was determined using enzyme-linked immunosorbent assay (ELISA). Determination of mRNA expression of IL-2 and IFN- γ by reverse transcription-polymerase chain reaction (RT-PCR). IgY anti HIV significantly enhanced the proliferation of PBMC induced by PHA. Furthermore, IgY anti HIV dose-dependently enhanced IL-2 and IFN- γ production in PHA-activated PBMC, suggesting that pharmacological activities of Ig Y anti HIV in PHA-activated PBMC may be mediated by regulating the production of cytokines in PBMC. In the RT-PCR, production of cytokines such as IL-2 and IFN- γ in PHA-activated PBMC cultures were increased by IgY anti HIV. We concluded that the enhancements of IL-2 and IFN- γ productions in PHA-activated PBMC were related to IgY anti HIV stimulating the mRNA transcription of these cytokines. IgY anti HIV increased IL-2 and IFN- γ productions in PHA-activated PBMC cultures by modulation of their gene expression. Cell proliferation, IL-2 and IFN- γ production of PBMC play important roles against bacterial and viral infection. It suggests that IgY anti HIV may be an immunotherapy. However, its detailed mechanisms of action are subjected for further study.

Keywords: IgY anti HIV; IL-2; IFN- γ ; human PBMCs.

1. Introduction

Inovative new approaches to HIV-1 prophylaxis and therapy are desperately needed. Despite the successes of highly active antiretroviral therapy (HAART), more than 2 million people die each year and more than 33 million individuals are infected worldwide. Although HAART is typically effective, it is not without problems, including complicated drug-drug interactions, adherence issues, and a myriad of side effects. The development of potent and broadly acting biologic drugs might offer a solution to some of these problems and complement traditional HAART. Currently, no reliable and user friendly treatment can be claimed to combat

these diseases. The current anti-HIV drugs that include reverse transcriptase (RT) and protease inhibitors have experienced drug resistance with HIV strains [1]. While broadly neutralizing human immunodeficiency virus (HIV)-specific antibodies have the capacity to prevent or suppress HIV infection, they are rarely produced by infected individuals, thereby markedly compromising the ability of the humoral response to control HIV infection. The need for novel antibodies for managing HIV is indisputable. The nature of the virus to evolve faster than the antivirals being made available has made it imperative that newer approaches to combating the virus are considered.

Hens' eggs have long been known as an excellent source of nutrients for humans. They are also an important source of antibodies, the most abundant being immunoglobulin (Ig) Y. This characteristic has attracted increasing interest in recent decades [2]. The natural transfer of antibodies that occurs from hen to chick *via* the egg yolk can be exploited to produce antibodies specific to a given pathogen, simply by immunizing the laying hens with an antigen from this targeted pathogen [3]. Recently the utilization of Immunoglobulin Y (IgY) from eggs of chickens, which were immunized against certain pathogens, has been the focus of attention in immunotherapy and immunodiagnosis, since IgY antibodies are the predominant serum immunoglobulin in birds, reptiles and amphibians, and are transferred in the female from serum to egg yolk to confer passive immunity to embryos and neonates [4;5]. Therefore, research and diagnostic community constantly demand new alternatives and procedures to produce cost-effective antibodies. The use of laying hens to produce polyclonal antibodies is an alternative to the use of mammals, such as rabbits and, since more than two decades, egg yolk antibodies (IgY) are a low cost and ethical alternative [6;7;8]. Compared with the stressful bleeding of mammals to obtain serum, IgY can be easily obtained non-invasively from the egg yolk.

IgY anti HIV was successfully elicited by immunizing the hens with formalin-inactivated HIV antigen emulsified in Freund's adjuvant. The IgY concentration in egg yolk increased during the immunization period until week 6 where it began to increase dramatically at 2 weeks and it reached a plateau at 4 weeks after immunization. After week 6 the levels decreased gradually [9]. The immunization of hens with HIV virus could be a strategy to obtain at low cost a relatively high concentration of anti HIV egg yolk IgY, could be a useful tool for research, diagnosis and therapy of HIV infection.

Considering the epidemiological change in HIV disease in developing countries around the world and the rise in the morbidity of the disease, it would be of interest to develop alternative for the immunotherapy of HIV infection. In this study, we developed a new immunotherapy that can be used against HIV by utilizing IgY obtained from chickens immunised with HIV antigen.

2. Material and Methods

Preparation of human PBMCs

Ten healthy male subjects (25 to 35 yr, mean age 29 yr) were chosen for this investigation. Heparinized human peripheral blood (20 ml) were obtained from healthy donors. PBMC was isolated by the Ficoll-Hypaque gradient density method as described previously. The 20 ml peripheral blood was centrifuged at 2000 rpm, 4 °C for 10 min to remove the plasma. Blood cells were diluted with PBS buffer then centrifuged in a Ficoll-Hypaque discontinuous gradient at 1500 rpm for 30 min. The PBMC layers were collected and washed with cold distilled water and 10 x Hanks' buffer saline solution (HBSS) to remove red blood cells. The cells were resuspended to a concentration of 2×10^6 cells/ml in RPMI-1640 medium supplemented with 2% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin.

MTT Cell viability assay

The effect of the IgY anti HIV on cell viability of PHA-stimulated PBMC was first determined by using a colorimetric technique which is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, 100 µL of RPMI-1640 media with 10% of FBS was added into the all the well except row A in the 96 well plate (TPP, Switzerland). Then, 100 µL of diluted IgY anti HIV at 100 µg/ml was added into row A and row B. A series of two fold dilution of extract was carried out down from row B until row G. Row H was

left untouched and the excess solution (100 μ l) from row G was discarded. Hundred μ l of lymphocyte (from human peripheral blood) with cell concentration at 5×10^5 cells/ml was added into all wells in the 96 well plate to make up the final volume of 200 μ l and thus diluted the extract into the concentration range of 100 to 1.56 μ g/ml. All the plates were incubated in 37°C, 5% CO₂ and 90% humidity incubator for selected period (72 h). After the corresponding period 72 h, 20 μ l of MTT (Sigma, USA) at 5 mg/ml was added into each well in the 96 well plate and incubated for four hours in 37°C, 5% CO₂ and 90% humidity incubator. 170 μ L of medium with MTT was removed from every well and 100 μ L DMSO (Fisher Scientific, UK) was added to each well to IgY anti HIV and solubilize the formazan crystal by incubating for 20 min in 37°C, 5% CO₂ incubator. Finally, the plate was read at 570 nm wavelength by using μ Quant ELISA Reader (Bio-tek Instruments, USA). The results of the IgY anti HIV were compared with the result of LPS (1 μ g/ml) as control. Each IgY anti HIV and control was assayed in triplicate in three independent experiments. The percentage of proliferation was calculated by the following formula:

$$\% \text{ Proliferation} = \frac{[\text{OD sample} - \text{OD control}] \times 100}{\text{OD control}}$$

Determination of cytokine production in PBMCs

PBMCs (2×10^5 cells/well) were cultured with PHA alone or in combination with various concentrations of IgY anti HIV (25-100 μ g/ml) for 72 h. The supernatant samples were collected and stored at -20°C until use. Production of IL-2 and IFN- γ in PBMC supernatant samples was measured using a sandwich enzyme-linked immunosorbent assay (ELISA) method (Endogen, Boston, MA, USA), according to manufacturer instructions.

Extraction of total cellular RNA

Total cellular RNA was extracted from PBMCs by using a previously described method [10]. PBMCs (5×10^6 cells) were activated with or without PHA and co-cultured with various concentrations of IgY anti HIV for 18 h. After incubation, the collected cells were lysed in Tri Reagent (Molecular Research Center, Cincinnati, OH, USA) as described in the manufacturer protocol. After centrifugation, the extracted RNA was precipitated with isopropanol. The mixture was centrifuged, and the total cellular RNA pellet was washed with 75% ethanol. Diethyl pyrocarbonate (DEPC)-treated water was added to re-dissolve the RNA pellet before further processing. The concentration of the extracted RNA was measured at an optical density of 260 nm.

Determination of mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR)

Before the RT-PCR, about 1 μ g total RNA was treated with RNase-free deoxyribonuclease I (DNase I) (Fermentas, Burlington, ON, Canada), according to manufacturer instructions. First-strand cDNA was synthesized from about 800 ng DNase I-treated RNA by using the ImProm-IITM Reverse Transcription System (Promega) and oligo(dT)₁₇ primers, as per the manufacturer protocol. RT was carried out at 50°C for 65 min. After cDNA synthesis, the desired DNA fragments were amplified for 30-35 cycles by using DNA polymerase and specific primers for IL-2, IFN- γ , and β -actin transcripts. Primer sequences for the internal control, β -actin, were 5'-TAC ATG GCT GGG GTG TTG AA-3' for the downstream primer, and 5'-AAG AGA GGC ATC CTC ACC CT-3' for the upstream primer (Eurogenetech, Liege, Belgium). Primer sequences for cytokines were as follows: for IL-2, 5'-AAC TCC TGT CTT GCA TTG CAC TA-3' for the 5' primer, and 5'-TTG CTG ATT AAG TCC CTG GGTC-3' for the 3' primer (Beite Kaito); for IFN- γ , 5'-AGT TAT ATC TTG GCT TTT CA-3' for the 5' primer, and 5'-ACC GAA TAA TTA GTC AGC TT-3' for the 3' primer (Eurogenetech). The final PCR products were subjected to electrophoresis and stained with EtBr. The DNA bands corresponding to IL-2, IFN- γ , and β -actin transcripts were 229, 435, and 656 bp, respectively.

Statistical analysis

Statistical analysis was performed with SPSS version 16 (SPSS Inc, Chicago, IL, USA). Differences between means were evaluated using ANOVA test (one way) followed by Duncan test. Results are expressed as Mean \pm Standard Error (SE). $p < 0.05$ was considered as statistically significant.

3. Results

IgY anti HIV increase human PBMC proliferation induced by PHA

IgY anti HIV effect on human PBMC proliferation was studied by treating resting cells or PHA-activated cells with 25; 50 and 100 $\mu\text{g}/\text{mL}$ IgY anti HIV for 72 h. Cell proliferation was determined using non-radioactive cell proliferation assay or MTT assay. Significant effects on PHA-stimulated PBMC proliferation were noted after 72 h treatment with 50 and 100 $\mu\text{g}/\text{mL}$ IgY anti HIV but not 25 $\mu\text{g}/\text{mL}$ IgY anti HIV (Table 1). Moreover, after 72 h treatment, 50 and 100 $\mu\text{g}/\text{mL}$ IgY anti HIV increased the viability of PHA-stimulated PBMCs. IgY anti HIV induced its stimulatory effect on PHA-activated PBMC proliferation in concentration-dependent fashion.

Table 1. Proliferation effect of IgY anti HIV on PHA activated-PBMC

Group	Proliferation effect (%)
PHA	124.7 ^a \pm 12.8
PHA and IgY anti HIV 25 $\mu\text{g}/\text{mL}$	132.5 ^a \pm 14.3
PHA and IgY anti HIV 50 $\mu\text{g}/\text{mL}$	154.7 ^b \pm 12.8
PHA and IgY anti HIV 100 $\mu\text{g}/\text{mL}$	187.6 ^c \pm 15.1

The data represent the average from n = 6

Superscript within each column indicate significant difference between the means (p < 0.05).

Effects of IgY anti HIV on IL-2 and IFN- γ levels measured using ELISA

Immunotherapeutic activity of IgY anti HIV can be measured by detecting the changes in the production of immune molecules such as cytokines. To elucidate the molecular mechanisms underlying IgY anti HIV biological effect on human PBMCs, we measured the levels of IL-2 and IFN- γ by using ELISA. IL-2 and IFN- γ production were increased by 25, 50, and 100 $\mu\text{g}/\text{mL}$ IgY anti HIV in a dose-dependent manner (Table 2). IL-2 and IFN- γ production was significantly increased by 50, and 100 $\mu\text{g}/\text{mL}$ IgY anti HIV but not 25 $\mu\text{g}/\text{mL}$ IgY anti HIV.

Table 2. Effects of IgY anti HIV on IL-2 and IFN- γ levels

Group	IL-2 (pg/ml)	IFN- γ (pg/ml)
PHA	189.8 ^a \pm 17.1	2576.8 ^a \pm 116.3
PHA and IgY anti HIV 25 $\mu\text{g}/\text{mL}$	179.6 ^a \pm 21.3	2697.3 ^a \pm 151.6
PHA and IgY anti HIV 50 $\mu\text{g}/\text{mL}$	221.7 ^b \pm 16.8	3149.7 ^b \pm 165.9
PHA and IgY anti HIV 100 $\mu\text{g}/\text{mL}$	247.3 ^c \pm 19.1	3632.5 ^c \pm 173.1

The data represent the average from n = 6

Superscript within each column indicate significant difference between the means (p < 0.05).

IL-2 and IFN- γ mRNA expression of IgY anti HIV using RT-PCR

To determine whether the stimulation of cytokine production in activated PBMCs treated with 25, 50 and 100 $\mu\text{g}/\text{mL}$ IgY anti HIV was due to a transcriptional impact, we extracted total RNA from treated cells and determined the levels of IL-2 and IFN- γ mRNA by using RT-PCR. IgY anti HIV remarkable impact on the mRNA levels of the cytokines (Figure 1). Lane 1: Control (0.1% DMSO), Lane 2: PHA, Lane 3: 100 μM IgY anti HIV, Lane 4: 50 μM IgY anti HIV, Lane 5: 25 μM IgY anti HIV. The RT-PCR results proved that the levels of IL-2 and IFN- γ transcripts could be enhanced in PHA treated PBMC. In the present study, production of cytokines such as IL-2 and IFN- γ in PHA activated-PBMC cultures were increased by anti HIV. By RT-PCR, we have demonstrated that 0.1% DMSO did not affect IL-2 and IFN- γ gene expression of IgY anti HIV in PHA activated- PBMC. It suggests that the increases in IL-2 and IFN- γ transcripts in the presence of anti HIV in PHA activated- PBMC are not related to vehicle. We concluded that the enhancements of IL-2 and IFN- γ productions in PBMC were related to anti HIV stimulating the mRNA transcription of these cytokines.

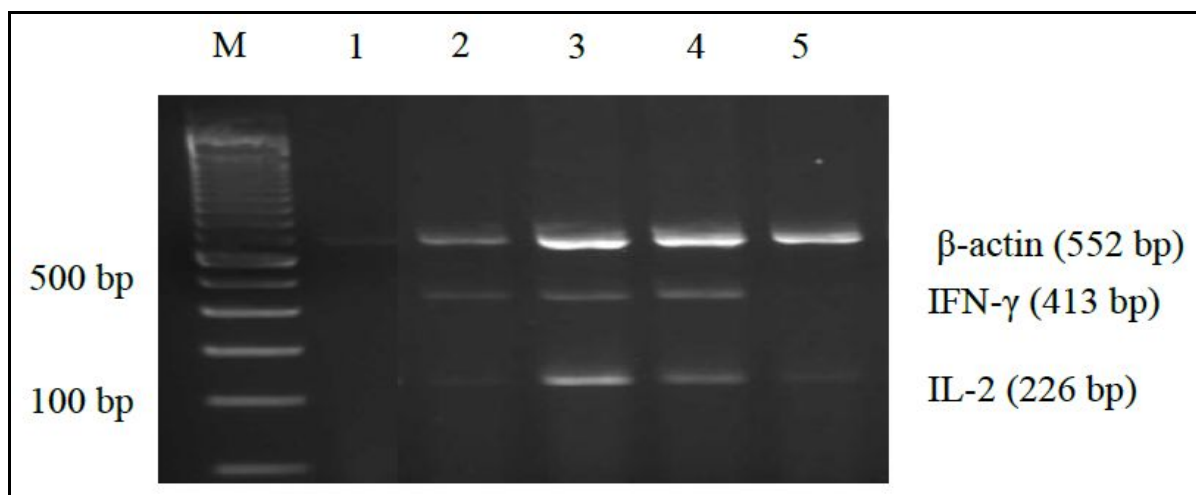


Figure 1. RT-PCR analysis of IL-2 and IFN- γ expression in phytohemagglutinin (PHA)-stimulated human PBMCs treated with IgY anti HIV (100, 50, 25, 0 $\mu\text{g/mL}$) for 18 h. Representative result is shown on 2% agarose gel electrophoresis displaying mRNA transcripts of IL-2, IFN- γ and β -actin. *Lane 1* = control (DMSO 0.1 %); *lane 2* = PHA-stimulated PBMCs; *lanes 3, 4 and 5*= IgY anti HIV (100, 50, 25 $\mu\text{g/mL}$).

4. Discussion

The IgY anti HIV were subjected to biological activity assay. We found that 50 and 100 $\mu\text{g/mL}$ IgY anti HIV significantly increased PHA-activated PBMC proliferation in a dose-dependent manner. The IL-2, IL-3, and IFN- γ production in activated PHA-activated PBMC were also increased by IgY anti HIV treatment. We predict that immunotherapy agents are present in IgY anti HIV. It has been demonstrated in previous studies with IgY anti HIV protection functions. This is the first report of immunotherapy functions on human lymphocytes identified in IgY anti HIV.

The central event in generation of immune responses is the activation and clonal expansion of T cells [11]. Interaction of T cells with antigens or phytohemagglutinin (PHA) initiates a cascade of biochemical events and gene expression that induces resting T cells to enter the cell cycle, proliferate and differentiate. It has been demonstrated in many previous studies with T cells that a series of genes such as interleukin-2 (IL-2) and interferon- γ (IFN- γ) are pivotal in the growth of T lymphocytes induced by antigens or PHA [12].

PHA is a mitogen for T lymphocytes which binds to N-acetylgalactosamine glycoproteins expressed on the surface of T cells then activates the cells to proliferate in PBMC. As we know interaction of T cells with antigens or mitogens initiates a cascade of genes expression such as IL-2 and IFN- γ mRNA that induces the resting T cells to enter the cell cycle (G0 to G1 transition) and culminates in expression of the high affinity receptor for IL-2 and secretion of IL-2 [11]. In response to IL-2, the activated T cells progress through the cell cycle, proliferation and differentiating into memory cells or effector cells. Our results indicated that the IgY anti HIV stimulated IL-2 and IFN- γ production in PBMC cultures. We hypothesize that action mechanisms of IgY anti HIV on PBMC proliferation may have involved the regulation of IL-2 and IFN- γ production in the cell cultures. The cell proliferation and IL-2 and IFN- γ production of T lymphocytes play important roles in against bacterial and viral infection [13]. In the present study, we found that IgY anti HIV enhanced PBMC proliferation and IL-2 and IFN- γ production. It suggests that immune stimulators may be included in the IgY anti HIV. Regulation of T lymphocyte activation and proliferation and cytokine production has been shown to be one of actions of immunomodulatory drugs. Many studies have indicated that the production of cytokines such as IL-2 and IFN- γ is involved in the regulation of PBMC proliferation. The agents that affect PBMC proliferation are likely to control the expression or function of IL-2 and IFN- γ [14].

The RT-PCR results proved that the levels of IL-2 and IFN- γ transcripts could be enhanced in IgY anti HIV in PHA-activated PBMC. By real-time PCR, we have demonstrated that 0.1% DMSO did not affect IL-2 and IFN- γ gene expression in PHA-activated PBMC. It suggests that the increases in IL-2 and IFN- γ transcripts in the presence of IgY anti HIV are not related to vehicle. In the present study, production of cytokines such as

IL-2 and IFN- γ in PHA-activated PBMC cultures were increased by IgY anti HIV. We concluded that the enhancements of IL-2 and IFN- γ productions in PHA-activated PBMC were related to IgY anti HIV stimulating the mRNA transcription of these cytokines. The results suggest that IgY anti HIV might be an immunotherapy source that can potentially alter cytokine secretion by human PBMCs and affect its putative pharmacological activities. Conclusions in the present study, IgY anti HIV increased IL-2 and IFN- γ productions in PHA-activated PBMC cultures by modulation of their gene expression. This is the first report of immunostimulatory functions on PBMC identified in IgY anti HIV. Cell proliferation, IL-2 and IFN- γ production of PBMC play important roles against bacterial and viral infection [14]. It suggests that IgY anti HIV may be an immunotherapy. However, its detailed mechanisms of action are subjected for further study.

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