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Enhancement of monoclonal antibody production after single and combination treatment of the hybridoma cells with all-trans retinoic acid and docosahexaenoic acid: An *in vitro* and *in vivo* study

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ABSTRACT

Murine hybridoma cells can produce monoclonal antibody (MAb) and the production of these antibodies in culture and peritoneum can be affected by different factors, including stimulants, inhibitors and supplements. Among these factors, the impact of micronutrients on the production of MAbs by mouse hybridoma cells has not fully been explored. In this study the murine hybridoma cells, M3C5, were cultured and treated with different concentrations of ATRA and DHA, alone, in combinations, and at different time of exposure. Then, changes in the production of MAb in culture medium were evaluated using ELISA. The hybridoma cells after single and combined treatment with ATRA, DHA and vehicles were IP injected to Balb/c mice and the changes in production of MAb in ascites were determined by ELISA. The results showed that single and combined treatment of ATRA and DHA elevated the production of MAb by hybridoma cells in both *in vivo* and *in vitro*. The production of MAb following *in vitro* single treatment with 1 μM of ATRA and 10 μM of DHA for 2 days was significantly increased. The *in vitro* effects of ATRA on increase of MAb production was obtained more than DHA. The MAb productions in combined treatment with 0.5 μM of ATRA plus 5 μM of DHA were significantly increased in *in vivo* and *in vitro*. However, the effect of DHA was obtained more significant in *in vivo* conditions. The results of this study showed for the first time that *in vitro* and *in vivo* treatments of ATRA and DHA could increase the production of MAb in mouse M3C5 hybridoma cells.

1. Introduction

Monoclonal antibodies (MAbs) have been widely used in immunological assays, diagnosis, and treatment of a variety of diseases. Because of the significance of these molecules, scientists are trying to find more novel techniques like “phage display” and to use transgenic mice for their production. Different studies lead to the development of MAbs in various therapeutic and pharmaceutical purposes as well. Numerous researchers have focused on improving the quality and quantity production of MAbs in a more cost-effective way [1]. In addition, a variety of methods have been successfully used to enhance the *in vitro* production of MAbs such as genetic engineering, clonal selection, and cell culture optimization [2]. It has recently been shown that the treatment of hybridoma cells with reagents such as coenzyme Q_{10}, biurate, rapamycin, spermine, valproic acid, and vitamin A enhancer resulted in a highly effective antibody production that have been more studied [3]. Previous studies showed that in the presence of all trans-retinoic acid (ATRA), the active metabolite of vitamin A, and docosahexaenoic acid (DHA), the structural part of omega3, the production of MAbs from both human hybridomas [4,5] and plasma cells [7] substantially increased [6,7]. Expression of the receptors in B cells is highly regulated by ATRA and DHA [7–10]. Omega3 and retinoids are signaling molecules which their nuclear receptors act as transcription factors in different immune cells [11]. The omega3 receptor, peroxisome proliferator activated receptor (PPAR), presents in the nucleus of some immune cells. This receptor has three subgroups including α, β/δ, and γ [10]. Retinoic acid receptor (RAR) and retinoid X receptor (RXR), are also nuclear receptors for retinoic acid (RA) which each having three α, β, and γ subgroups [12]. To run the cell signaling by RARs and RXRs, these nuclear receptors, should form homodimers or

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heterodimers which allow them to play their role in regulating the expression of genes *via* binding to retinoic acid response elements (RAREs) [11]. Similarly, RXR is an obligate partner of PPARγ and they can bind to the peroxisome proliferator response element (PPRE), induce the target transcription factors [13], and regulate the expression of target genes. It has been shown that RA plays an important role in the proliferation, differentiation, morphological characteristics of cells and has a regulatory role in the *in vivo* and *in vitro* antibody-mediated responses [4,15]. In a previous study, administrations of RA and DHA to animal model and in cell culture caused an improvement in immunoglobulin (Ig) production [7]. Some evidence revealed that RA is capable of up-regulating the expression of light and heavy chains of Ig, especially those of IgG. RA can also mediate a greater rise in the secretion of Ig from the membrane [5]. DHA, an important derivative of omega3, improves B cells function and enhances their differentiation into plasma cells by elevating the expression levels of B lymphocyte-induced maturation protein-1 (Blimp-1) and X-box binding protein-1 (Xbp-1). Blimp-1 can inhibit the function of paired box 5 (Pax-5), an important factor in immature B cells that can down-regulate the expression of Xbp-1, a factor with the capability of elevating the levels of antibody secretion by facilitating their proper folding [6,14]. The combinatory effects of 9-cis-RA and prostaglandins can lead to the activation of PPARγ/RXRα heterodimer signaling pathways and increase differentiation of B cells into the plasma cells which subsequently lead to an elevated antibody production [13]. In this study, the effect of ATRA alone and in combination with DHA on both *in vivo* and *in vitro* production of monoclonal antibody in murine M3C5 hybridoma cells was explored.

2. Materials and methods

2.1. Hybridoma cells

The mouse hybridoma cells, M3C5, have been produced in Department of Immunology, Tehran University of Medical Sciences (TUMS). These hybridoma cells produce monoclonal antibody (IgG2a) against human chorionic gonadotropin hormone.

2.2. Chemicals, reagents, and antibodies

Stock solutions of 0.01 M of ATRA (R2625, Sigma, USA) and 60 mM of DHA (D2534; Sigma-Aldrich) were prepared in dimethyl sulfoxide (DMSO; D2650, Sigma-Aldrich) at darkness and under argon gas. The stock solutions were aliquoted, covered by aluminum foils, and stored in −70 °C. Different concentrations of ATRA and DHA were prepared with Dulbecco's modified eagle's medium (DMEM; D5671, Sigma-Aldrich) containing 10% fetal bovine serum (FBS; F0804, Sigma-Aldrich) and used for the treatment of hybridoma cells in culture. The DMEM culture medium with and without DMSO was used as vehicle control and control, respectively, for all experiments in this study.

2.3. Cytotoxicity effects of ATRA and DHA on M3C5 cells

The M3C5 cells were seeded at density of 1×10^4 cells per well in a 96-well plate; cultured in DMEM, and incubated in a CO_2 incubator at 37 °C, 5% CO_2 under saturating humidity. The cells were then treated with different concentrations of ATRA and DHA (10^{-3}–10^{-8} M) for 72 h. The cytotoxicity of these drugs was assessed using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; M5655, Sigma-Aldrich) assay [16].

2.4. Effects of ATRA and DHA at different doses and times of exposure on the production of antibodies

Hybridoma cells, M3C5, were cultured in 24-well plate at 3×10^4 cells per well, in DMEM containing 10% FBS, in CO_2 incubator (37 °C, 5% CO_2 and under saturating humidity) for 12 h; then cells were treated with different concentrations of ATRA and DHA (0.1, 1, and 10 μM) and incubated for 3 different times (24, 48, and 72 h).

2.5. Single and combined effects of ATRA and DHA on the production of antibodies

Similarly, M3C5 cells were seeded at a density of 3×10^4 cells per well in a 24-well plate for 12 h and under the above-mentioned conditions. The cells then were treated with single doses of 0.1 and 1 μM ATRA and 10 μM DHA. The combined treatments were as follows: 0.1 μM ATRA plus 10 μM DHA, 1 μM ATRA plus 10 μM DHA, 0.05 μM ATRA plus 5 μM DHA, and 0.5 μM ATRA plus 5 μM DHA. The exposure time for all treatments was 48 h.

2.6. The effect of ATRA and DHA on hybridoma cloning efficiency

The cells were suspended and seeded in 96-well plates in three groups, including 10, 2, and 1 cell per well in 100 μl of DMEM containing 10% FBS [17] and then cultured for 1–2 weeks at the conditions mentioned above. Subsequently, the cells were treated with 1 μM of ATRA and 10 μM of DHA in each group and the number of colonies in the wells was studied.

2.7. Measurement of secreted antibodies and cell numbers

The concentration of secreted antibodies in cell culture was measured using sandwich enzyme-linked immunosorbent assay (ELISA) [4,5]. The goat-anti-mouse IgG (M8642; Sigma-Aldrich) and the HRP-rabbit anti-mouse IgG (A9044; Sigma-Aldrich) were used as the capture and detector antibodies, respectively. Cell count and viability were performed using Neubauer chamber and Trypan blue staining.

2.8. Animals

A total of 32 male Balb/c mice (aged 8–10 weeks, weighting 18 ± 2 g) were purchased from the Pasteur Institute, Tehran, Iran. The mice were kept in the animal house at the Tehran University of Medical Sciences (TUMS) in standard conditions and 12/12 (day/night) light cycle. Animals had free access to food and water. This study protocol has been approved by animal care and the ethics committee of TUMS.

2.9. Hybridoma cell culture in peritoneal fluid

The mice were randomly allocated into 4 groups, with each group including 8 mice. 400 μl of Pristane (P2870; Sigma-Aldrich) was intra peritoneal (IP) injected into each mouse. After 7 days, each group was IP injected with 3×10^6 hybridoma cells suspended in 500 μl of serum free media. The injected cells have previously been cultured in 4-wells plates and treated with 1 μM of ATRA (Group A), 10 μM of DHA (Group B), a combination of 0.5 μM of ATRA plus 5 μM of DHA (Group C), and also with vehicle (Group D or Control).

2.10. Collection of mice ascites fluid

After 8 days, mice were anesthetized by IP injection of 100 mg/kg ketamine and 10 mg/kg xylazine, then in the sterile conditions, a small cross slit was prepared in the belly of mice and the ascites fluids were collected using Pasteur pipette and transferred to Falcon tubes and the volume of ascites was measured. Ascites fluid was separated by centrifugation and the precipitated cells were collected and stored in liquid nitrogen.

2.11. Measurement of the concentration of MAbs in the ascetic fluids

The antibody concentration was measured using sandwich ELISA