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缓激肽受体介导的 v-Ki-*ras* 转化成纤维细胞内钙离子振荡的调节[1]

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摘要 由单个细胞内钙离子的分析显示：缓激肽 B_2受体拮抗剂，抑制了缓激肽所诱导的在 v-Ki-*ras* 转化成纤维细胞内的钙离子振荡，B_1受体拮抗剂无此作用．降低胞外钙离子浓度以及用 thapsigargin 处理细胞均阻止了钙离子振荡的发生．这些结果提示，持续地激活 B_2受体，导致钙离子内流及钙离子从内源性贮存部位释放的波动，从而引起钙离子的振荡．

关键词 缓激肽；钙；癌基因；成纤维细胞

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Enhancement of interleukin-2 production and its mRNA expression by dihydroartemisinin[1]

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ABSTRACT Immunoregulatory properties of a novel antimalarial drug dihydroartemisinin (DHA) were investigated *in vitro*. DHA 0.5-5 $\mu mol \cdot L^{-1}$ enhanced the lymphocyte proliferation induced by Con A. Interleukin 2 (IL-2) production and its mRNA expression by both Con A-stimulated mouse splenocytes and a T cell line LBRM-33-1A5 were also augmented by DHA. In contrast, DHA 0.5-5 $\mu mol \cdot L^{-1}$ did not show any effect on the lipopolysaccharides (LPS)-induced lymphocyte proliferation and the spontaneous and mitogen-induced proliferation of transformed T cells. These results indicated that DHA might regulate lymphocyte responses through

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the induction of IL-2 production and that the enhanced T cell proliferation and IL-2 production might be mediated through different pathways.

KEY WORDS dihydroartemisinin; interleukin-2; messenger RNA; T-lymphocytes; lymphocyte transformation; cultured cells

The antimalarials, dihydroartemisinin (DHA), artemisinin (Art), and artesunate were first developed in China in 1970s. These drugs can also regulate immune responses. SRBC-driven plaque forming cell (PFC) formation, lymphocyte proliferation and delayed hypersensitivity (DH) were inhibited by Art 100—300 mg·kg^{-1} and DHA 100 mg·kg^{-1} [1-3]. In contrast, Art (50—100 mg·kg^{-1}) strengthened the phagocytosis of peritoneal macrophages and the production of interferon in serum, and to enhance the DH responses and acid phosphatase of macrophages[4-6]. When cultured with mouse splenocytes *in vitro* Art promoted the lymphocyte transformation[4]. Sodium artesunate, a water soluble derivative of Art, markedly enhanced anti-SRBC PFC formation in mice[7] at a daily concentration of 50 mg·kg^{-1} for 5 d. Art and its 2 derivatives DHA and arteether exhibited marked suppression of humoral responses at concentrations of 400—600 mg·kg^{-1}, as measured by the hemolytic plaque assay[8]. But these 3 agents did not alter DH response to SRBC[8]. Our experiments showed that Art and its derivatives stimulated immune responses in normal mice and accelerated immune reconstitution of mice after syngeneic bone marrow transplantation (SBMT) at lower concentrations (<50 μmol·L^{-1}), but exhibited moderate suppressing effect on immune responses at higher concentrations (>1000 μmol·L^{-1})[9].

In this report, we mainly studied the effect of DHA on T lymphocyte proliferation, IL-2 production and its mRNA expression, attempting to elucidate its immune modulating mechanisms.

MATERIALS AND METHODS

Reagents DHA was prepared by Dr TU You-You (Institute of Traditional Chinese Herbs, Chinese Academy of Traditional Chinese Medicine, Beijing). Con A and LPS were from Sigma. Recombinant human IL-2 (rh-IL-2) was produced in *E coli* in our Department. PhotoGen™ Nucleic Acid Detection System was from Gibco/BRL Life Technologies, Inc (Gaithersburg, USA).

Mice BALB c mice, ♀, 3 months old (Experimental Animal Center, Beijing Medical University), were group-housed for at least 1 wk before use. Food and water were given *ad lib*.

Cell lines A type-2 T helper (HT-2) cell clone and a T cell line LBRM-33-1A5 (LBRM) were maintained in our laboratory. HT-2 clone was grown in RPMI 1640 medium containing rh-IL-2 20—30 U·ml^{-1} and cultures were split 1:5 into fresh IL-2 containing medium twice a week. LBRM was passaged as HT-2 cells, but without adding IL-2 into the medium.

Splenocyte preparation and *in vitro* lymphocyte proliferation assay Spleen single cell suspensions were prepared in complete RPMI 1640[10]. To measure the effect of DHA on mitogen-induced proliferation, triplicate cultures were established containing 50 μl of splenocytes (4×10^5 cell/well) with 50 μl of sub-optimal concentration of Con A (5 μg·ml^{-1}) and 100 μl of serial tenfold dilutions of DHA. Cultures were incubated for 72 h in a humidified chamber at 37 ℃/5 % CO_2 in air, pulsed with [^{3}H]TdR 14.8 MBq/well in the final 12-h incubating period and harvested onto glass fiber filters. Cell-associated radioactivity was measured using a scintillation counter (efficiency=45 %). The results are expressed as disintegrations per minute (dpm).

IL-2 generation by splenic cells and LBRM cells Splenocytes (2×10^6) or LBRM cells (1×10^5) suspended in complete RPMI 1640 medium were incubated in 24-well tissue culture plates in 1 ml RPMI 1640 final volume containing varying concentrations of DHA

in the presence of Con A 5 μg·ml^{-1}. After cultured for 12, 24, and 48 h, the supernatants were collected for assaying the IL-2 levels.

IL-2 bioassay A modification of the bioassay established by Gillis *et al*[11] was used. Twofold dilutions of supernatants (0.1 ml) were added to 96-well culture plates containing 0.1 ml IL-2 sensitive HT-2 indicator cells (1×10^5 cells/ml). These plates were incubated for 18 h, pulsed for 6 h with [^{3}H]TdR 14.8 MBq / well and processed as described above for the determination of cell-associated radioactivity. Standard rh-IL-2 and Con A concentration response curves with HT-2 cells were always included to ensure the integrity of the indicator cells.

Preparation of RNA and dot blot analysis Each assay used 2×10^7 splenocytes or 5×10^6 LBRM cells cultured in a 15-cm plastic culture dish. At indicated times, total RNA was extracted using the AGPC method[12]. Equal amounts of RNA (5 μg) was spotted on nylon membrane[13]. The membranes were prehybridized and hybridized as described previously[13]. Quantitation of mRNA was performed by scanning the blot using a densitometer.

Statistics analysis Statistical significance was determined by 2-tail *t* test.

RESULTS

Effect of DHA on mouse lymphocyte proliferation The influence of DHA on the mitogenic responses of splenic T- and B-cells was determined at first. Con A and LPS were selected as mitogens for T- and B-cells, respectively, because both are effective polyclonal activators for T- and B-cells. Normal mouse splenocytes were incubated for 72-h in varying concentrations of DHA. DHA enhanced Con A induced T-lymphocyte proliferation in a concentration-dependent manner, but did not show any effect on LPS-induced B cell proliferation at the same concentrations. Unstimulated splenocytes, however, were not affected by DHA (Fig 1).

When the concentrations of DHA were > 500 μmol·L^{-1}, a moderate suppression of lymphocyte proliferation induced by both Con A and LPS was observed (data not shown). The inhibitory effect of DHA (>50 μmol·L^{-1}) on lymphocyte proliferation may be due to its direct cytotoxicity, because the cell viability in DHA (1 mmol·L^{-1})-treated group was weakened *vs* control (Tab 1). So, the concentrations of DHA <500 μmol·L^{-1} were used in our experiments.

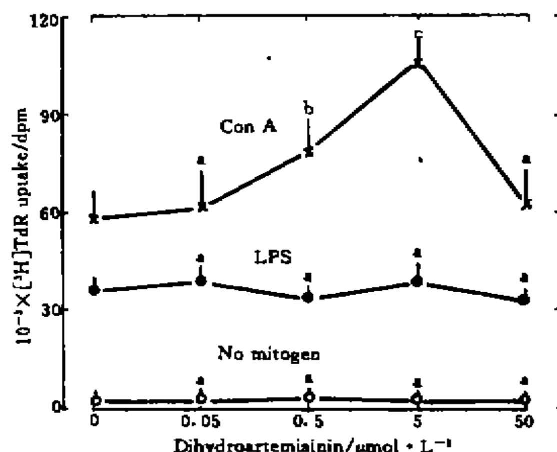

Fig 1. Effect of DHA on lymphocyte proliferation in the presence and absence of Con A (5 μg·ml^{-1}) or lipopolysaccharides (LPS, 10 μg·ml^{-1}). n=4 culture wells. $\bar{x}\pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* control.

Tab 1. Viability of Splenocytes cultured for 3 d with dihydroartemisinin in the presence or absence of Con A. n=3. $^aP>0.05$, $^bP<0.05$ *vs* control.

DHA/ μmol·L^{-1}	Cell viability/%	
	Without Con A	With Con A 5 μg·L^{-1}
0	87±9	86±12
5	88±15^a	85±14^a
50	84±11^a	87±16^a
500	87±10^a	80±10^a
1000	82±7^a	67±8^b

Kinetics of DHA enhancement on T lymphocyte proliferation The stimulating effect of DHA on Con A-induced T cell proliferation

was dependent on the time at which the compound was added to the cultures. The addition of DHA (5 μmol·L^{-1}) at 0 or 6 h resulted in maximal enhancement. A moderate augmentation of lymphoproliferation was seen when DHA was added at 12 h. There was no action on T cell proliferation when DHA was added later than 24 h (Fig 2).

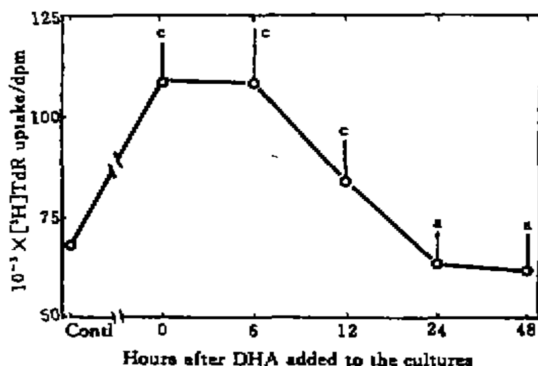

Fig 2. Kinetics of DHA on Con A (5 μg·ml^{-1}) induced lymphocyte proliferation. $n=3$ experiments. $\bar{x}\pm s$. $^aP>0.05$, $^cP<0.01$ vs control.

Effect of DHA on IL-2 production by mouse splenocytes and mouse T-cell line LBRM DHA enhanced IL-2 production by mouse splenocytes in the presence of Con A (Fig 3). However, DHA did not increase IL-2 production from unstimulated lymphocytes (data are not shown here). In order to test whether DHA had any direct stimulating effect on IL-2 production, LBRM cells, a T-cell line, were incubated in DHA and Con A. DHA strengthened the production of IL-2 by LBRM cells in the same way as that of mouse splenocytes (Fig 3). But DHA had no effect on either spontaneous or Con A-induced LBRM cell proliferation (data not shown).

Effect of DHA on IL-2 mRNA expression by mouse splenocytes and LBRM cells Splenic cells and LBRM cells were assessed for their capacity to produce IL-2 mRNA upon

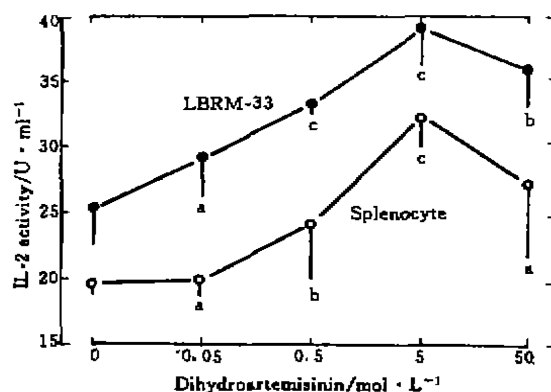

Fig 3. Effect of DHA on IL-2 production from Con A (5 μg · ml^{-1}) induced splenocytes and T-cell line LBRM-33-1A5. $n=3$ experiments. $\bar{x}\pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ vs control.

stimulation by Con A in the presence of DHA. DHA stimulated IL-2 mRNA expression over a wide range of concentrations (0.5—5 μmol·L^{-1}) and the stimulation of IL-2 mRNA expression appeared in a concentration-dependent fashion (Fig 4).

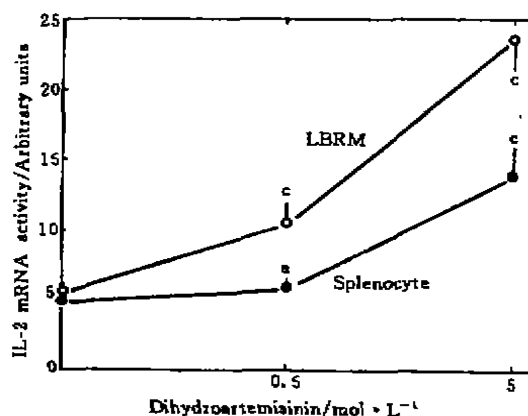

Fig 4. Effect of DHA on IL-2 mRNA accumulation in Con A-stimulated LBRM-33-1A5 and splenocytes (Spl). $n=3$ experiments. $\bar{x}\pm s$. $^aP>0.05$, $^cP<0.01$ vs control.

Kinetics of IL-2 production and IL-2 mRNA expression by DHA Kinetics of IL-2 production by DHA was determined by assessing the supernatants of splenocytes stimulated

with Con A for their capacity to support HT-2 cell growth in the presence of DHA 5 μmol · L^{-1}. The IL-2 production was enhanced by DHA at 24, 48 and 72 h culture periods. The maximal production of IL-2 was not only enhanced in its height but also broadened in its peak. In addition, the production of IL-2 decreased with lengthening of culture time to a lesser degree in DHA treated groups. IL-2 mRNA expression was augmented by DHA, especially during the 48 h incubating time (Fig 5).

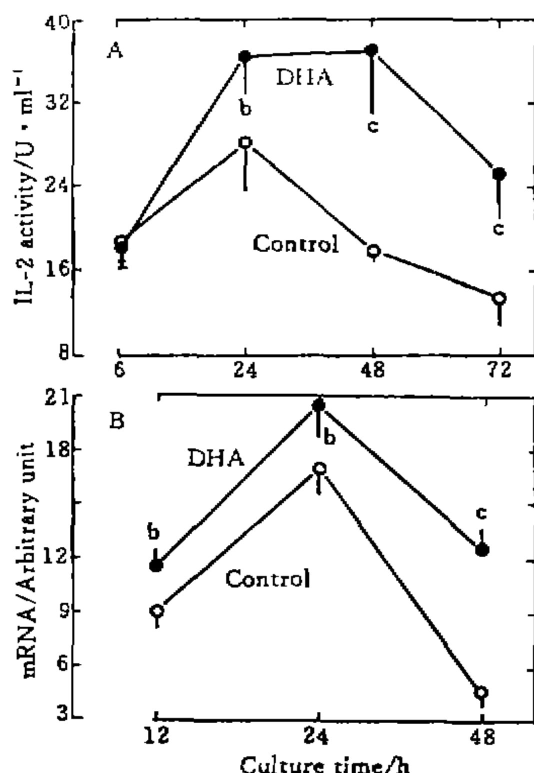

Fig 5. Kinetics of IL-2 production and mRNA accumulation in Con A-induced splenocytes by dihydroartemisinin (5 μmol·L^{-1}). IL-2 activity (A) and its mRNA (B). $n=3$ experiments. $\bar{x}\pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* control.

DISCUSSION

The present experiments showed that DHA in a concentration range of 0.05 — 5 μmol · L^{-1} consistently augmented the T-cell proliferation, while B-cell proliferation was not affected. These results confirmed our previous reports[9] and that of Qian *et al*[4]. However, our results did not conform to those of Shen *et al*[2] and Sun *et al*[3] who demonstrated that Art markedly inhibited lymphocyte proliferation induced by Con A. The apparent discrepancy may be explained by differences in experiment conditions, the compounds used and their purity. In fact, DHA was also found to inhibit lymphocyte proliferation at higher concentrations (>50 μmol ·L^{-1})[9]. The suppressed lymphocyte proliferation may be due to the direct cytotoxicity to some degrees, because the cell viability in DHA (1 mmol·L^{-1}) treated group (cultured for 3 d in the presence of Con A) was slightly decreased as assessed by the technique of trypan-blue exclusion.

While it might be argued that lymphocyte response to mitogen and specific antigens were dependent upon the production of IL-2. This was not true for the spontaneous and Con A induced proliferation of transformed T-cell lines LBRM, and it was not true for the proliferation of splenocytes previously activated by Con A, either. The kinetics studies of Con A induced mouse splenocyte proliferation by DHA demonstrated that T-lymphocyte proliferation was enhanced only when DHA was added to the culture in the continuous presence of Con A during the first 12 h culture period. That is, totally activated T-cells did not respond to DHA. Incubation of LBRM cells in the presence of DHA and mitogenic lectin Con A, increased the IL-2 production, but not the cell proliferation, which were very similar to those of Stanley *et al*[14] and Mercep *et al*[15]. Both reported that culture of T-cell lines with mitogen increased the IL-2 secretion while decreased the lymphocyte proliferation. These results suggest that IL-2 production

and T-cell growth may be mediated through different pathways.

The enhanced IL-2 production and the retarded decrease of IL-2 activity in the supernatants of T-cell cultures demonstrated a closer parallel to IL-2 mRNA levels. These may indicate that the increased IL-2 production by DHA is due to the enhanced IL-2 mRNA levels. At present, we cannot distinguish whether the increased IL-2 mRNA level was resulted from the enhanced mRNA transcription and/or increased stability of the mRNA. These and the obligatory event in the transmembrane signaling processes that lead to IL-2 production and T-cell activation needs further exploration.

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二氢青蒿素促进 IL-2产生及其 mRNA 表达

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摘要　应用细胞培养及分子杂交技术研究了二氢青蒿素(DHA)的免疫调节作用. 结果发现，DHA 明显促进 Con A 诱导的脾细胞增殖，增强小鼠脾细胞及 T 细胞株 LBRM 产生 IL-2 及其 mRNA 表达. 而在相同浓度范围内对转化的 T 细胞及 LPS 诱导的淋巴细胞增殖无明显影响. 结果提示，DHA 的促 IL-2产生及促淋巴细胞增殖作用可能由不同途径介导.

关键词　二氢青蒿素；白细胞介素-2；信使核糖核酸；T 淋巴细胞；淋巴细胞转化；培养的细胞