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Effects of serum on lymphokines producing capabilities of CD8$^+$ T cells

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Serum이 CD8$^+$ T cell의 lymphokine 생산양상의 변화에 미치는 영향

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초록 : serum factor가 CD8$^+$ T cell의 lymphokine 생산양상에 미치는 영향을 알아보기 위하여 13-20주령의 BAL B/C 마우스로 부터 CD8$^+$ T cell를 분리한 후 serum-containing medium과 serum-free medium을 사용하여 배양하였다. serum-free medium에서 배양한 CD8$^+$ T cell이 분비하는 lymphokine의 양은 serum-containg medium에서의 결과와는 달리 IL-2의 생산양은 낮았으나 IFNr의 생산양은 상당히 높았다. 이와같은 결과로 미루어 serum-derived factor가 CD8$^+$ T cell의 lymphokine 생산에 영향을 미친다고 생각된다.

Key words : CD8$^+$ T cell, IL-2, IL-4, IFNr

Introduction

The cells and organs that compose the mammalian immune system are constantly bathed by bodily fluids. So the lymphocytes recirculate and constantly exchange between the circulation and tissues. The dynamic phenomenon of the reciculation of lymphocytes is a fundamental requirement for specific immune defense. The move of lymphocytes allows efficient celluar communication between the blood, lymph and peripheral sites where antigens can be properly presented. During these processes lymphocytes are subjected to change influences to a variety of distinct microenvironment. These include steroid and polypeptide hormones and other substances within serum[1-7].

T cells are responsible for cell-mediated immune reactions as well as for coordinating the functional activation of various other cell types. Mature T cells can be divided into two subsets based on their expression of the CD4 or CD8 cell surface glycoproteins. CD4 expressing T cells generally recognize class II-restricted antigen and act as helper cells, and CD8 expressing cells recognize class I-restricted antigen and act as cytotoxic or suppressor cells[8-10].

The whole T cells isolated from lymphoid organ or

T-cell hybridomas were found to response quite differently *in vitro*, when stimulated under serum-free versus serum-containing conditions. T cells stimulated in the presences of serum were quite restricted in the types of lymphokines they produced. However T cells stimulated under serum-free conditions produced numerous types of lymphokines, including IL-2, IL-4, IL-5 and IFN*r*[6].

Based on these results, therefore, it is not unreasonable to assume that T-cell subsets response elicited under serum-free and serum-containing conditions would be quite different. In this experiment, in order to estabilish T cell subset responsiveness to regulatory influences, isolated murine $CD8^+$ T cells culture in serum-containing or serum-free medium. Lymphocytes activate by the addition of anti-CD3ε. The changes of the patterns of lymphokines produced by activated $CD8^+$ T-cell is discussed.

Materials and Methods

Mice : BALB/C strain of male mice were used. Mature adult mice ranged in age from 13-20 weeks. In any single experiment, pools of 7-8 mice were used. The age of animals within each experiment were matched. The experiment has been repeated several times.

Antibodies : The hybridoma clones secreting rat anti-murine IL-2(S4B6) and IFN*r*(XMG1.2) were obtained from DNAX(Palo Alto, CA). The hybridoma clones producing antibody specific for murine IL-4(11B11) and murine IFN*r*(R46A2) were purchased from the ATCC. Polyclonal rabbit anti-murine IL-2 was purchased from Collaborative Biomedical(A subsidiary of Becton-Dickinson, MA) and peroxidase labled goat anti-rabbit Ig and goat anti-rat Ig were purchased from Sigma(St Louis, MO). Purified, biotinylated rat anti-murine IL-4 antibody was purchased from PharMingen(San Diego, CA). Hybridomas producing antibody specific for anti-CD4(GK1.5) was purchased from the ATCC.

Cytokine standards : Murine recombinant IL-2 and IL-4 were obtained from Collaborative Research (Bedford, MA). Murine recombinant IFN*r* was obtained from Genetech(San Francisco, CA). These reagents were used as reference standards in the capture ELISA assays.

T-cell subset enrichment : Single cell suspensions of spleen were prepared in balanced salt solution, washed 2 times, counted and resuspended in 1 ml of a 1:50 dilution of Ig-enriched anti-CD4(GK1.5) containing culture supernatant. Following a 45 minute incubation on ice with occasional swirling, the cells were washed 2 times in cold balanced salt solution and then resuspended $1\text{-}1.5\times10^7$ anti-CD4-treated cells/ml were added to a plate precoated with goat anti-rat Ig. After incubation for 40 minutes at 0℃, nonadherent cells were poured onto another anti-rat Ig plate and incubated for another 40 minutes. The nonadherent cells collected from the anti-CD4 treatment were 90% $CD8^+$ cells by FACS analysis.

Culture conditions : Routinely, single cell suspensions were prepared, washed twice in sterile balanced salt solution and resuspended to a density of 1×10^7 cells/ml/well in a 24-well Cluster culture plate (Costar, Cambridge, MA), and then incubated for 24 hours at 37℃ in a 10% CO_2 humidified incubator to elicit lymphokine secretion. Culture medium consisted of RPMI 1640 supplemented with 1% Nutridoma-NS(Boehringer-Mannheim) or 10% fetal calf serum (Hyclone Laboratories, Logan, UT), antibiotics, 200mM L-glutamine and 5 X 10^5 M 2-mercaptoethanol. Lymphocytes were activated by the addition of 1μg/ml anti-CD3ε, T-cell specific mitogen. The culture period, cell concentrations, and culture medium were all carefully evaluated to determine the optimal conditions for stimulating production of the lymphokines under evaluation. The hybridoma clone producing hamster anti-murine CD3ε monoclonal antibody, 1452C-11.2, was obtained from J Bluestone (University of Chicago). Culture supernatants were collected, clarified by centrifugation and then stored at 4℃ until assayed for specific lymphokine content.

Lymphokine assays : The amount of cytokine in test supernatants was quantitated by a capture ELISA, adapted from the method of Schumacher et al[11]. Briefly, 100μl of 2μg/ml capture antibody in 0.05M Tris-HCl(pH 9.6) was adsorbed to the wells of a 96-well microtest plate, washed, and blocked with PBS/1%

BSA. Test supernatants and 2-fold serial dilutions of the appropriate reference cytokine(100μl/well) were dispensed, and after sufficent incubation and washing, 100μl of biotinylated-detection antibody, 1μg/ml, was dispensed into each well. In case of IL-2, polyclonal antibody and peroxidase labelled goat anti-rabbit immunoglobulin were dispensed orderly. The ELISA was developed using avidine-HRP and ABTS-substrate. Spectrophotometric readings were recorded at 405 nM. The limit of detection for most of these cytokines is 15-30pg/ml.

The mean and SEM were calculated and the comparisions made by student's t-test. Significance was considered at $p<0.01$ or $p<0.05$.

Results

Subpopulations of murine T lymphocytes are depleted based on their expression of cell differentiation markers CD4 and CD8. In these studies, $CD4^+$ T cell depletion was accomplished through the procedure known as panning.

Unfractionated splenocytes and $CD8^+$ T cell subpopulation from normal mice were cultured under serum-free conditions and were either stimulated with an optimum amount of anti-CD3 to induce lymphokine secretion or left unstimulated for any spontaneous lymphokine production. Culture supernatants were collected at 24 hours and analyzed for the level of IL-2, IL-4 and IFNr.

As shown in Fig 1, we observed that *in vitro* activation of $CD8^+$ T cells which cultured in serum-free conditions produce small amounts of IL-2, but significant amounts of IFNr following activation with T cell mitogens.

Some of recent results indicate that the pattern of lymphokine production by unfractionated normal lymph node T cells were affected by the serum components[2,6]. But there is uncertain that what kinds of lymphokines ard affected in $CD8^+$ T cells by the serum components. Unfractionated splenocytes and $CD8^+$ T cells were cultured under serum-free and serum-containing conditions(10% FCS), and stimulated with anti-CD3. Culture supernatants were collected and analyzed for the indicated lymphokines. The results are presented in Fig 2. A comparision of the pattern of lymphokines production by $CD8^+$ T cells under serum-free and serum-containing conditions indicates that serum components reduced the ability of $CD8^+$ T cells to secrete IFNr($p<0.01$), while enchanced the IL-2 production($p<0.05$). The results of unfractionated cells paralleled with these data.

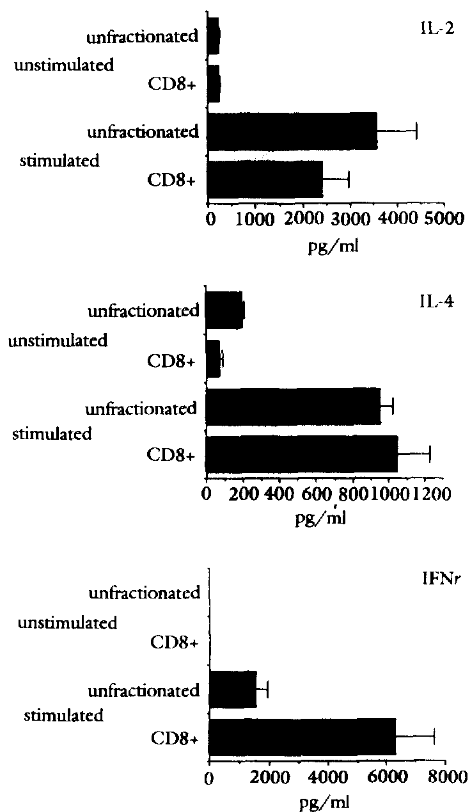

Fig 1. Spontaneous or anti CD3-induced production of lymphokine by unfractionated or $CD8^+$ T cells under serum-free condition

Discussion

Lymphokines secreted by T cells play major roles in the regulation of the responses of both T and B cells and the development of effector cells. Although the synthesis and secretion of different lymphokines or groups of lymphokines are regulated independently[12-14],

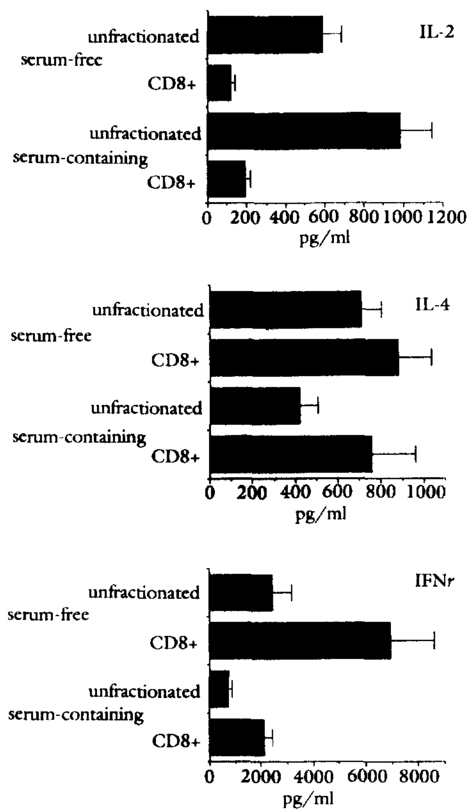

Fig 2. Lymphokine production by stimulated unfractionated or CD8$^+$ T cells under serum-containing or serum-free conditions

IL-2, IL-4 and IFNr are among the best defines. IL-2 is secreted mainly by helper T cells following stimulation by binding of antigen to the T cell receptor. The biological effects of IL-2 have expanded to the generation of cytoxic effector cells[12,15-19]. IL-4 is first described as B cell growth and differentiation factor and has been shown to be active in the development of cytotoxic effectors[17]. IFNr acts as a very potent macrophage-activating factor for tumor cytotoxicity and for enhancing the killing of intracellular parasites. IFNr also acts synergistically with LT and TNFα to produce increased tumor cell lysis *in vitro*[16,17,20,21].

There are a lot of reports regarding the differential production of cytokines by activated T cells in serum-containing conditions. According to these reports[22-26], IL-2 is also produced by murine CD8$^+$ splenocytes although IL-2 synthesis is not always observed. The chief source of IFNr is CD8$^+$ T cell subpopulation based on the contribution of IFNr production by the CD8$^+$ T-cell to the total levels produced by unfractionated splenocytes can very from 50% to greater than 95%[17,24,26-28].

There are no differences between the CD8$^+$ T cells and unfractionated T cells in the IL-4 production. But the absolute amounts of the secreted IL-4 per CD8$^+$ T cell is very low, because the cells, in the present work, heve been sorted from normal unfractionated splenocytes(Fig 1 and 2). The results which are shown here support that the IL-4 is predominantly produced by CD4$^+$ T cells[5,17,25,29].

The informations to be gained from the serum-containing conditions are inconsistant with this study in which CD8$^+$ T cells in serum-free conditions produce very small amounts of IL-2, while significant amounts of IFNr following activation. These discrepancies on the types and quantities of lymphokines produced by activated CD8$^+$ T cells between serum-free and serum-containing conditions may be depend upon the serum-derived factors. Serum contains a number of substances. Among these potent growth factors such as PDGF and the transforming growth factors(α and β) normally sequestered within blood platelets that are released as a consequence of clot formation[6,30,31]. According to the recent report[6], the patterns of lymphokine production are quite different between activated T cells stimulated under serum-free and those stimulated under serum-containing conditions. The presence of serum augmented the amount of IL-2 produced by T cells from lymph node and spleen, while simultaneously reducing the production of IL-4, IL-5 and IFN. And also T-cell hybridoma produced different amounts of IL-2 and IL-4 following activation under serum-free and serum-containing conditions. Based on these results, one of the primary factor in serum responsible for these restrictions in lymphokine production in vitro in known to be PDGF. TGFβ differentially regulate the expression of PDGF-binding sites in 3T3 cells. TGFβ was found to cause the number of α-subunits decreases and the number of β-subunits increases[30]. The most responding T cells display both α and β subunits of the PGDF receptor, with the β-subunit being necessory for modifying cellular beha-

vior[6].

Because lymphocytes are able to exposure to these serum-derived factors during traveling through many types of cellular microenvironments which were created by the infection or trauma, future studies need to be showed that how these factors affect to capacity to produce a number of cytokines by T-cell subpopulations as well as the whole T cells.

Summary

The responsiveness of CD8⁺ T-cell subpopulation according to serum-containing and serum-free conditions were investigated. Cells are freshly isolated from spleen of mature adult BALB/C mice between 13-20 weeks of age. $CD8^+$ T cells in serum-free conditions produce small amounts of IL-2, while significant amounts of IFNr following activation when compared the results of serum-containing conditions. These data indicate that serum-derived factors may play an important role in the alternations of $CD8^+$ T cell responsiveness.

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