Lymphocyte Cyclic Nucleotides and Cellular Immunity in Viral Hepatitis

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Abstract: It has been known that contents of intra-cellular cyclic nucleotides, cyclic 3',5'-adenosine monophosphate (cyclic AMP) and cyclic 3',5'-guanosine monophosphate (cyclic GMP), in T-lymphocytes are closely related with cell-mediated immunity of the host since the first investigations by Lichtenstein & Margolis (1968). However, this problem has never been studied in human viral hepatitis. Therefore, we began the clinical and experimental studies on this problem. It is illustrated in this study that the intracellular cyclic-AMP content of T-lymphocyte in peripheral blood was enhanced by an addition of phytohemagglutinin (PHA) to lymphocyte culture medium. The increase-rate of lymphocytic cyclic-GMP content caused by the addition of similar mitogen was reduced in patients of chronic active hepatitis when compared with normal control. The relationship between the contents of intracellular (lymphocytes) cyclic AMP and serum levels of α_2 -macroglobulin which is thought to be one component of serum inhibitors against the non-specific cell-mediated immunity in our previous data was investigated in this study, and these markers showed the parallel variation with each other in the patients of viral hepatitis. On the other hand, the high cyclic AMP content and low cyclic GMP increase-rate by an addition of mitogen to peripheral T-lymphocyte culture medium (as well as high serum α_2 -macroglobulin levels) were seen in patients with chronic active hepatitis which meant a suppressed cellular immunity. In conclusion, a determination of cyclic nucleotides in T-lymphocytes is very useful to understand the involvement of them in cell-mediated immunity which is closely associated with chronicity of viral hepatitis.

Key words: cyclic nucleotides, cellular immunity, viral hepatitis.

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Introduction

In our previous data (Taoka & Endo, 1978; 1979a, b, c; 1980a, b, c; Taoka, 1980), we have illustrated that the T-cell population and function were mostly decreased in murine and human chronic active hepatitis and progressive liver cirrhosis, and the serum inhibitors against cell-mediated immunity including α_2 -macroglobulin were generally increased. Also, we observed that the suppressor-T cell function was decreased in the cases of chronic active hepatitis and progressive liver cirrhosis, and ⁵¹Cr-labeled T-lymphocytes were transferred to the liver and a high uptake of radio-activities on the liver surface

was observed in chronic active hepatitis.

On the other hand, Hirschhorn *et al.* (1970) suggested that cyclic 3', 5'-adenosine monophosphate (cyclic AMP) mediated the lymphocyte transformation by phytohemagglutinin (PAH) and subsequently, Smith *et al.* (1971a, b) described that aminophylline, isoproterenol and prostaglandin, all of which elevate lymphocyte cyclic AMP levels, inhibited incorporation of ³H-labeled nucleotides and amino acid into the DNA, RNA and protein of PHA-stimulated lymphocytes. Henney *et al.* (1972) illustrated that the treatment of lymphocytes with adenylyl cyclase stimulating agents including histamine, isoproterenol and prostaglandins E₁ and E₂ caused increases in cyclic AMP levels and inhibited cytolytic activity.

Hadden *et al.* (1972) reported that optimal mitogenic concentrations of PHA and concanavalin A (Con-A) produced marked increases in the concentration of lymphocyte cyclic 3', 5'-guanosine monophosphate (cyclic GMP) within the first 20 min of exposure to the mitogens. Also, they asserted that a temporally discrete rise in lymphocyte cyclic GMP concentration is viewed as an active signal to induce proliferation, while the elevation of cyclic AMP concentration in these cells is viewed as a regulatory influence that limits or inhibits mitogenic action.

However, no study has been done on the role of lymphocyte cyclic nucleotides in murine or human viral hepatitis associated with cell-mediated immunity. So, we wanted to confirm these results by basic experiments and clinical studies.

Materials and Methods

Materials

The patients with viral hepatitis who were admitted into our University Hospital and Tokyo Rosai Hospital were tested in this study, and their diagnoses were confirmed by a laparoscopy and liver biopsy.

For the *in vitro* experiment, highly purified materials of α_1 -acid glycoprotein (α_1 -AG) and α_2 -macroglobulin (α_2 -M) were provided from Behringwerke AG, Marburg in W. Germany through the courtesy of Prof. Schwick.

Methods

Lymphocyte materials were separated from peripheral blood taken from the patients with viral hepatitis by the Ficoll-Angioconray method.

An adequate amount of phytohemagglutinin (PHA) for making transformation was added to the samples which contained approximately 1×10^6 lymphocytes, and then the equal volume of 12% Tricarboxylic acid (TCA) was also added for ceasing of reaction.

The samples were provided for the determination at 5, 10, 15 and 20 min before and after the adding of PHA, and were treated with an ultrasonicator for 5 min for the destruction of lymphocyte cell membrane.

Cyclic nucleotides in lymphocyte are contained in supernatant after a centrifugation.

After the centrifugation of samples, an adequate volume of ether was added to the supernatant. After shaking, TCA was transferred into the ether portion whereas the cyclic nucleotides remain in the residual watery portion (precipitate are discarded). This procedure was repeated several times.

Subsequently, the whole of the watery fraction was dried in room temperature for the determination of cyclic nucleotides.

Cyclic AMP was determined by Steiner's method (Steiner *et al.*, 1969) using anti-cyclic AMP serum produced by the Schwarzmann Company.

Cyclic GMP was assayed by Gilman-Murad's method (Gilman, 1970; Murad & Gilman, 1971; Murad *et al.*, 1971) using cyclic GMP binding protein produced by the Diagnostic Products Company.

Results

1. Clinical problem

(1) T cell population and function

Human lymphocytes of normal adults were added to the sera of hepatitis patients who had (or did not have) HBsAg and then were incubated or cultured in a CO₂ incubator and were investigated for the population of rosette forming cells (RFC, T-lymphocytes) and blastogenesis by PHA (Table 1).

As shown in Table 1, the T-cell population is markedly lower in chronic active hepatitis ($26 \pm 11\%$) as compared with normal controls ($47 \pm 10\%$) and chronic persistent hepatitis ($48 \pm 12\%$).

Table 1. Immunological aspect of chronic virus hepatitis
Functions of T-lymphocytes

	Chronic active hepatitis	Chronic persistent hepatitis	
T cell population	26 ± 11%	49 ± 12%	
Blastogenesis by PHA	49 ± 9%	53 ± 13%	
Cyclic nucleotide contents and increase-rate of lymphocytes (p mole/1×10 ⁷ of lymphocytes)			
	cyclic AMP	cyclic GMP	Δc-GMP/15 min
Normal controls	6 ± 3.9 (n=4)	2.2 ± 0.5 (n=2)	40 ± 3% (n=2)
HBsAg asymptomatic carriers	8.0 (n=1)	2.5 (n=1)	16% (n=1)
Chronic active hepatitis with HBsAg	10 ± 6.3 (n=6)	3.5 ± 2.2 (n=4)	25 ± 20% (n=4)
Chronic active hepatitis without HBsAg	8.5 ± 7.5 (n=4)	3.0 ± 1.7 (n=2)	12 ± 6% (n=2)
Chronic persistent hepatitis	7.1 ± 4.2 (n=8)	2.8 ± 1.0 (n=6)	36 ± 8% (n=8)

On the other hand, blastogenesis by PHA is significantly lower in chronic active hepatitis ($49 \pm 9\%$) when compared with normal controls ($61 \pm 8\%$) and chronic persistent hepatitis ($53 \pm 13\%$).

However, no relationship was recognized between HBsAg positive and negative hepatitis patients.

(2) Cyclic AMP contents

Lymphocyte cyclic AMP contents in chronic active hepatitis with HBsAg were comparatively higher (10.0 ± 6.3 p mole/ 1×10^7 of lymphocytes) than normal controls (6.0 ± 3.9), but these values were not significant statistically.

The contents of lymphocyte cyclic AMP in chronic active hepatitis (CAH) with HBsAg, CAH without HBsAg and in chronic persistent hepatitis were 10 ± 6.3 , 8.5 ± 7.5 , 7.1 ± 4.2 respectively, and were a little higher when compared with normal controls (6 ± 3.7) but these values could not be thought to be significant statistically.

(3) Increase-rate of cyclic AMP

Lymphocyte cyclic AMP contents reach maximum at 15 to 20 min after adding PHA to the lymphocyte culture media and the increase-rate by mitogen was higher in normal controls, and progressively lower in HBsAg negative hepatitis patients, asymptomatic carriers of HBsAg and HBsAg positive hepatitis patients in this order (Fig. 1).

Fig. 1. Variation of lymphocyte cyclic AMP levels (control-rates) after PHA stimulation in hepatitis patients.

Fig. 2. Variation of lymphocyte cyclic GMP levels (control-rates) after PHA stimulation in hepatitis patients.

On the contrary, the lymphocyte cyclic AMP contents were lower in normal controls, and progressively higher in asymptomatic carriers of HBsAg, HBsAg negative hepatitis patients, and HBsAg positive hepatitis patients in this order.

(4) Cyclic GMP contents

Lymphocyte cyclic GMP contents were higher in chronic active hepatitis with HBsAg (3.5 ± 2.2 p mole/ 1×10^7 of lymphocytes), and were less higher in chronic active hepatitis without HBsAg (3.0 ± 1.7), chronic persistent hepatitis (2.8 ± 1.0), asymptomatic carrier of HBsAg (2.5) and normal controls (2.2 ± 0.5) in this order (Table 1).

However, these values were not considered to be significant statistically.

(5) Increase-rate of cyclic GMP

Intralymphocytic cyclic GMP contents were increased after adding PHA to the lymphocyte culture media, and reached maximum at 10 to 15 min after the treatment with mitogen, and then the contents were gradually decreased (Fig. 2).

The increase-rate of cyclic GMP ($12 \pm 6\%$) were significantly lower in chronic active hepatitis without HBsAg, and tended to be depressed in asymptomatic carriers of HBsAg (16%), chronic active hepatitis with HBsAg ($25 \pm 20\%$) and chronic persistent hepatitis ($36 \pm 8\%$) in this order.

2. Basic experiment

(1) Correlation between lymphocyte cyclic AMP contents and α_1 -acid glycoprotein (α_1 -AG) and α_2 -macroglobulin (α_2 -M) concentrations in culture media

When authentic samples of α_1 -acid glycoprotein (α_1 -AG) was added to the lymphocyte

Fig. 3. Variation of intralymphocytic cyclic AMP contents by addition of α_1 -acid glycoprotein and PHA to lymphocyte culture media.

culture media, the cyclic AMP contents fluctuated and increased irregularly (Fig. 3).

A similar variation curve to that in α_1 -AG could be seen in α_1 -AG with PHA as shown in Fig. 3.

So, we concluded that cyclic AMP contents showed an increase paralleled with α_1 -AG in the culture media in a lower concentration of α_1 -AG up to 2 mg/dl, however, we could not come to any conclusions concerning the results from adding PHA to the culture media with α_1 -AG.

On the other hand, the cyclic AMP contents in lymphocytes were increased by adding authentic samples of α_2 -macroglobulin (α_2 -M) to the lymphocyte culture media, and were paralleled with α_2 -M concentration in the culture media up to 100 mg/dl as shown in Fig. 4.

(2) Correlation of lymphocyte cyclic AMP contents and suppressor T cell function with α_1 -AG and α_2 -M concentrations in culture media

The suppressor T cell function was estimated by the suppression rate of plaque forming cells (PFC) for sheep erythrocytes after adding lymphocytes which were stimulated by concanavalin-A (Con-A) and were treated with mitomycin-C (MMC) and then were cultured in another system of lymphocyte culture media in which the lymphocytes were stimulated by porkweed mitogen (PWM).

Suppression rates (PFC, %) were increased by adding pure α_1 -AG to the lymphocyte cul-

Fig. 4. Variation of intralymphocytic cyclic AMP contents by addition of α_1 -acid glycoprotein and α_2 -macroglobulin to lymphocyte culture media.

Fig. 5. Variation of intralymphocytic cyclic AMP contents and suppressor T cell functions (suppression rates, PFC %) by addition of α_1 -acid glycoprotein to lymphocyte culture media.

Fig. 6. Variation of intralymphocytic cyclic AMP contents and suppressor T cell functions (suppression rates, PFC %) by addition of α_2 -macroglobulin to lymphocyte culture media.

ture media in a lower concentration of α_1 -AG up to 2 mg/dl paralleled with lymphocyte cyclic AMP contents, and then were gradually decreased up to 4 mg/dl in a concentration of α_1 -AG in the culture media as shown in Fig. 5.

On the other hand, the suppression rates (PFC, %) were increased by adding pure α_2 -M to the lymphocyte culture media and were paralleled with lymphocyte cyclic AMP contents up to α_2 -M concentration of 100 mg/dl in the culture media as shown in Fig. 6.

Discussion

In 1968 Lichtenstein and Margolis presented in "Science" the first evidence implicating cyclic AMP in the control of the leucocyte function. Subsequently, cyclic AMP, acting as a second messenger for vasoactive hormones and as mediators of inflammation, has been shown to modulate a wide variety of immune processes *in vitro* and in experimental models of immediated and delayed hypersensitivity and the humoral antibody response.

In the *in vitro* system, cyclic AMP inhibits an immunologic or inflammatory action of leucocytes, and the production of cyclic AMP is stimulated by β -adrenergic catecholamines, histamin and the E-series prostaglandins.

Hirschhorn *et al.* (1970) and Henney *et al.* (1972) observed the effects of cyclic AMP, dibutyryl cyclic AMP and theophylline on lymphocyte stimulation by PHA and on the specific cytolytic activity of lymphocytes.

Also, Smith *et al.* (1971a, b) and Mendelsohn *et al.* (1973) studied cyclic AMP concentrations in human peripheral blood lymphocytes after stimulation by PHA, isoproterenol,

prostaglandins and aminophylline, and then illustrated these compounds elevated lymphocyte cyclic AMP levels and inhibited incorporation of H-labeled thymidine, uridine and leucine into the DNA, RNA and protein of PHA-stimulated lymphocytes.

In their study, aminophylline inhibition was maximal only when the inhibitor was added within one hour after exposure of cells to PHA, suggesting that a relatively early step in the lymphocyte transformation process may be affected.

In our study, lymphocyte cyclic AMP contents after PHA stimulation were enhanced in chronic active hepatitis (CAH) when compared with chronic persistent hepatitis (CPH) and normal controls, suggesting that cellular immunity in CAH is lower when compared with CPH or a healthy state.

On the other hand, Hadden *et al.* (1972) first reported that cyclic GMP acts as an inhibitory agent of the cyclic AMP analog on induction of the immune response.

They illustrated that optimal mitogenic concentrations of PHA and Con-A produced 10 to 50 fold increases in the concentration of lymphocyte cyclic GMP within the first 20 min of exposure to the mitogens.

Weinstein *et al.* (1974) observed that cyclic AMP inhibited the growth of lymphocytes in culture, as well as the lectin-induced transformation of lymphocytes, and that exogenous cyclic GMP and two of its chemical derivatives could stimulate resting mouse spleen lymphocytes to synthesize RNA and DNA, with a time course similar to that caused by Con-A.

Therefore they supposed that cyclic AMP and cyclic GMP act in opposite directions in biological systems, so that the ratio of the two nucleotides within lymphocytes regulates many cell functions.

Bourne *et al.* (1974) suggest that cyclic GMP and cyclic AMP may act in a 'push-pull' fashion to regulate functions of lymphocytes, neutrophils and even most other cells, that is, wherever cyclic AMP appears to inhibit a reaction, cyclic GMP may enhance it. Similarly, wherever sympathetic neurohormones (catecholamines) appear to act through cyclic AMP, the parasympathetic neurotransmitter, acetylcholine (or its congeners), may

Fig. 7. Hypothetical concept of the role of cyclic AMP on cellular immunity related with α_1 -acid glycoprotein and α_2 -macroglobulin (I).

Fig. 8. Hypothetical concept of the role of cyclic AMP on cellular immunity related with α_1 -acid glycoprotein and α_2 -macroglobulin (II).

act through cyclic GMP.

In our data, lymphocyte cyclic GMP contents after PHA stimulation tended to enhance in CAH when compared with CPH and normal controls, however, these values were not thought to be significant statistically.

On the contrary, the increase-rate of lymphocyte cyclic GMP at 15 min after PHA stimulation were significantly decreased in CAH, especially in a HBsAg negative patient, as compared with CPH or healthy person.

Therefore, we suppose that depressed increase-rates of lymphocyte cyclic GMP after mitogen stimulation express the suppressed cellular immunity in the hepatitis patient, whereas the cyclic GMP contents in T-lymphocytes are not significant markers related to immunity.

In the *in vitro* experiments, we have recognized that the addition of exogenous α_1 -acid glycoprotein and α_2 -macroglobulin to the lymphocyte culture media enhanced the cyclic AMP contents in lymphocyte paralleled with the concentrations of α_1 -AG and α_2 -M in the culture media.

On the other hand, plaque forming cells (PFC, %) were similarly increased to lymphocyte cyclic AMP contents paralleled with the concentrations of α_1 -AG and α_2 -M in the culture media.

These findings suggest that α_1 -AG and α_2 -M may act as serum inhibitors against cellular immunity mediated with enhanced contents of lymphocyte cyclic AMP (and presumably, with the depressed increase-rate of lymphocyte cyclic GMP).

These hypotheses might be true since the suppressor T cell function (PFC, %) was increased and was paralleled with the cyclic AMP contents in lymphocytes and the α_1 -AG and α_2 -M concentrations were increased in lymphocyte culture media (Figs. 7, 8).

Conclusion

Lymphocyte cyclic AMP contents after PAH stimulation were enhanced in chronic active hepatitis (CAH) when compared with chronic persistent hepatitis (CPH) and normal controls (NC).

On the other hand, the increase-rate of lymphocyte cyclic GMP after the mitogen stimulation were depressed in CAH as compared with CPH and NC.

No relationship could be observed between these results and HBsAg.

These findings may be compatible with suppressed cell mediated immunity in chronic viral hepatitis especially in chronic active hepatitis, and suggest that lymphocyte nucleotides may regulate the immune mechanism in the course of viral hepatitis.

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ヒト・ウイルス肝炎におけるリンパ球内サイクリック・ヌクレオチドの動態と宿主免疫機構との関連

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要 旨: 1968年 Lichtenstein らがリンパ球内サイクリック AMP が生体内の免疫機構の調整に関与している可能性を示唆して以来、この問題については今日までに多くの研究があり、サイクリック AMP レベルの増加は T 細胞機能に対して抑制的に、PHA 刺激後のサイクリック GMP レベルの増加は T 細胞機能に対して促進的に働くことが明らかとなり、サイクリック AMP とサイクリック GMP とは生体の免疫機構に対し相互に拮抗的ないし相補的に作用することが明らかとなった。しかし、ヒト・ウイルス肝炎についてこの点を検討した報告は未だなく、この点を中心に追究するとともに、合わせて血清免疫抑制因子との関連についての基礎的検討を行った。著者の成績によれば T-細胞機能の低下がみられる慢性活動性肝炎では慢性非活動性肝炎や健常例に比して、T-リンパ球内サイクリック AMP の増加、PHA刺激後サイクリック GMP 増加率の低下が認められ、細胞性免疫の抑制が示唆された。この場合 HBsAg の有無との間には関連はみられなかった。一方、*in vitro* の実験では T-リンパ球内サイクリック AMP 含量は PFC %よりみた抑制性 T 細胞（サプレッサー T, Ts）機能とよく並行しており、またリンパ球培養液中に添加した細胞性免疫抑制因子と考えられている α_1 -酸性糖タンパク (α_1 -AG) や α_2 -マクログロブリン (α_2 -M) の濃度ともよく並行しており、これら培養液中の血清抑制因子がリンパ球内ヌクレオチドの含量に大きな影響を与えていることが判明した。以上、ヒト・ウイルス肝炎においても T-リンパ球内サイクリック・ヌクレオチドはその増減を介して T 細胞機能を調節し生体の免疫機構に関与していることが強く示唆された。

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