Rapid Communication

Mild Hyperthermia Modulates the Relative Frequency of Lymphocyte Cell Subpopulations: an Increase in a Cytolytic NK Cell Subset and a Decrease in a Regulatory T Cell Subset

HIROSHI TERUNUMA[1,2*], AYANO WADA[2], XUEWEN DENG[1], YOSHIHIDE YASUMA[2], TETSURO ONISHI[2], ATSUSHI TOKI[3], HIROYUKI ABE[2]

[1]Biotherapy Institute of Japan, 2-4-8 Edogawa, Koutou-ku, Tokyo 135-0051, Japan
[2]Kudan Clinic, 1-9-5 Kudanshita, Chiyoda-ku, Tokyo 102-0073, Japan
[3]Tama Nanbu Chiiki Hospital, 2-1-2 Nakazawa, Tama, Tokyo 206-0036, Japan

Abstract: Although mild hyperthermia (MHT) cannot directly kill tumor cells, an augmented immunological effect resulting from MHT has been reported to induce injury of malignant tumors. In this study, the impact of regional MHT on lymphocyte subpopulations was investigated. Of particular interest was the effect of MHT on natural killer (NK) cells and T cells, which are important in the innate and adaptive immune systems. Regional MHT treatment was performed using an 8-MHz capacitive heating device, the Thermotron RF8 (Yamamoto Vinita Co., Ltd., Osaka, Japan). An average continuous radio-frequency irradiation of approximately 900 W was applied between two 30-cm electrodes placed on opposite sides of a volunteer's upper abdominal region for 30 min. In healthy volunteers exposed to this thermal treatment, NK cell activity and the percentage of NK cells and cytolytic NK cells ($CD3^-CD56^{dim}$ cells) in lymphocyte populations increased significantly at 1 and 7 days after regional MHT treatment compared with pre-treatment numbers. The number of cytolytic NK cells also increased significantly at 1 day after treatment. The percentage of T cells and $CD4^+$ T cells decreased significantly from 1 to 7 days following the heating procedure. However, no significant changes in the percentage and the number of $CD8^+$ T cells was observed. Interestingly, the percentage and the number of $CD4^+CD25^+$ T lymphocytes which are recognized as regulatory T lymphocytes (T_{reg}) decreased significantly during the 7 day post-treatment period. These results suggest that regional MHT may activate both, the innate and adaptive immune systems, through activation of NK cells and through a decrease in the number of regulatory T cells.

Key Words: hyperthermia, mild hyperthermia, NK cell, NK activity, regulatory T cell

Received 22 November 2006, Accepted 16 February 2007. *Corresponding author; Tel, +81-3-5632-6080; Fax, +81-3-5632-6083; e-mail, terunuma_h@ybb.ne.jp

Introduction

Hyperthermia (HT) has been used as an adjunct to chemotherapy and/or radiotherapy in patients with various malignant diseases. The therapeutic effect of HT may operate through activation of the immune system. Increased body temperature has been shown to stimulate the immune system via the activation of natural killer (NK) cells, T cells and dendritic cells (DC), via modification of the mobility of leukocytes, and augmentation of cytokine production[1-3]. In Japan, the only machine which has been approved for this type of use by the Ministry of Health, Labor and Welfare of Japan is a therapeutic instrument for the non-invasive regional HT treatment of deep-seated malignant tumors. Thus, regional HT is used widely in Japan[4]. Standard regional HT is designed to heat malignant tumors to 42.5°C or higher since this temperature is tumoricidal *in vitro*[5]. Lower temperature HT under 42.5°C does not kill malignant cells directly, but leads to immune system activation[3,6-11], whereas heating at temperatures around 42°C have led to immune system impairments[12-14]. Most studies on the effects of mild hyperthermia (MHT), the so-called fever-range hyperthermia between 39 to 41°C, on the immune response were whole body hyperthermia treatments (WBH)[1,6,8,15]. However, there are few reports describing the effects of regional MHT on the immune system[3,9]. In the study reported here, the effects of regional MHT on human immune responses were observed, especially changes in NK cell and T cell subpopulations.

Materials and Methods

1) Volunteer blood donors

Four healthy volunteers, two males and two females aged between 28 and 47 (average age : 37.5± 9.9) were enrolled in the regional MHT treatment program. None of the volunteers had signs or symptoms of fever or infectious diseases, and were given a thorough explanation of the purpose, procedures, and possible risks of the experimental treatment, and provided their informed consent.

2) Thermal treatment for mild hyperthermia

Thermal treatment was performed using an 8-MHz capacitive heating device, the Thermotron RF-8 (Yamamoto Vinita Co., Ltd., Osaka, Japan). An average continuous radio-frequency irradiation of 900 W was applied between two 30-cm electrodes placed on opposite sides of the upper abdominal region across the liver as described[4]. The time for one thermal treatment session was 30 min. Thermometry was not performed due to medical considerations. The estimated temperature of the liver achieved by this heating procedure was approximately 40°C, based on data collected previously using actual measurements of tissue temperatures during HT treatment of liver tumors[4].

3) Blood cell counts and phenotype analysis of lymphocytes

The absolute numbers of leukocytes, lymphocytes, monocytes and granulocytes were measured with an automated leukocyte counter (LC550, Fukuda Denshi, Tokyo, Japan). To characterize lymphocyte subpopulations, whole blood cells were stained with combinations of the following fluorescent-labeled antibodies : phycoerythrin-cyanin 5.5 (PC5) -labeled anti-human CD3, phycoerythrin (PE) -labeled anti-human CD4, phycoerythrin-cyanin 7 (PC7) -labeled anti-human CD8, PC7-labeled anti-human

CD16, energy coupled dye (ECD) -labeled anti-human CD19, fluorescein isothiocyanate (FITC) -labeled anti-human CD25, PE-labeled anti-human CD56 (Immunotech Coulter, Marseille, France) and isotype-matched monoclonal antibodies for negative controls. After lysing the red blood cells, the stained cells were analyzed with a 5-color digital flow cytometer (Cytomics FC 500, Beckman Coulter) and CXP analysis software. Absolute numbers of each lymphocyte subset were calculated by multiplying the number of lymphocytes by the lymphocyte cell subset percentage.

4) Measurement of cytotoxic activity

To examine the effect of the regional MHT treatment on NK cell functions, analysis was done of the cytolytic activity of NK cells against K562 cells labeled with an immunofluorescent-dye Calcein-AM solution (Dojindo Laboratories, Kumamoto, Japan) at various effector-to-target (E/T) ratios using a Terascan VP (Minerva Tech, Tokyo, Japan) as described[16]. Cytotoxic assay procedures were begun within 2 hours of obtaining blood samples.

5) Statistics

The paired t-test was employed to compare the values at each time-point after regional MHT treatment with pre-treatment values in the same volunteer group by using SPSS (SPSS Japan Inc., Tokyo, Japan). *P* values less than 0.05 were considered as statistically significant.

Results

In healthy volunteers exposed to regional MHT, the absolute numbers of leukocytes, lymphocytes, monocytes and granulocytes did not change significantly (Table I).

The percentage of NK cells in the lymphocyte population significantly increased at 1 and 7 days after treatment with MHT when compared with pre-treatment values (Table I). Although the difference was

Table I. Absolute numbers and the percentages of white blood cells in volunteers exposed to the regional hyperthermia treatment aimed at the upper abdominal region (n=4)

	Cell numbers (/μl blood)			Percentage among lymphocytes (%)		
	Pre-HT	1 day after HT	7 days after	Pre-HT	1 day after HT	7 days after
Leukocytes	5325±634	5425±602	5575±974			
Granulocytes	3225±858	3225±810	3575±1069			
Monocytes	525±222	500±0	575±126			
Lymphocytes	1575±806	1700±469	1425±822			
NK cells ($CD3^-CD56^+$)	138±83	270±143	187±136	8.6±1.1	15.6±4.2*	13.0±2.8*
$CD3^-CD56^{dim}$ cells	134±85	266±143*	180±132	8.3±1.2	15.0±4.4*	12.4±2.6*
$CD3^-CD56^{bright}$ cells	3±2	5±3	5±5	0.3±0.1	0.3±0.1	0.3±0.1
T cells ($CD3^+$)	1140±577	1119±258	962±554	72.2±4.2	66.5±3.6*	67.2±1.8*
$CD4^+$ T cells	702±398	641±217	557±368*	44.0±4.8	37.5±4.6**	38.4±4.9**
$CD4^+CD25^+$ T cells	43±8	36±20	24±9*	3.3±1.7	2.1±0.9	2.1±1.1*
$CD8^+$ T cells	405±173	457±104	389±191	26.3±6.0	27.7±6.2	27.9±5.8
B cells ($CD19^+$)	248±155	236±77	208±137	15.2±3.0	13.8±2.8	13.9±3.2

Significant difference (*$p<0.05$, **$p<0.01$) in comparison with the pre-treatment value

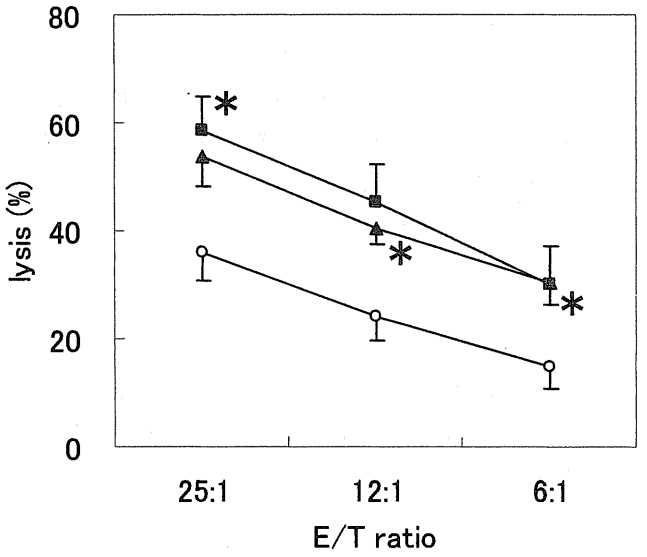

Fig. 1. Natural killer (NK) cell activity versus K562 target cells in mononuclear cells isolated from peripheral blood before (open circles), and 1 day (closed squares) and 7 days (closed triangle) after regional mild hyperthermia. The results are the means and SEM calculated from data obtained from 4 volunteers. *Significantly different from the mean value observed before mild hyperthermia treatment, $p<0.05$.

not significant, the number of NK cells in peripheral blood also increased after MHT treatment (Table I).

Human NK cells can be subdivided into at least two separate subpopulations: cytolytic NK cells and immunomodulatory NK cells[17]. The percentage and the number of $CD3^-CD56^{dim}$ cells which corresponds to cytolytic NK cells which primarily mediate cellular cytotoxicity increased significantly at 1 day after MHT treatment (Table I). The increased percentage of $CD3^-CD56^{dim}$ cells was also present at 7 days post-treatment (Table I). Immunomodulatory NK cells ($CD3^-CD56^{bright}$ cells) which secrete large quantities of inflammatory cytokines displayed no significant change in their percentage and number before and after the MHT treatment (Table I). The change in the percentage of NK cells was thus due primarily to the increase in $CD3^-CD56^{dim}$ cells. At 1 and 7 days post-treatment, NK activity in peripheral blood mononuclear cells also increased significantly (Fig. 1).

The percentage of T cells and $CD4^+$ T cells in the lymphocyte population were found to be significantly decreased on days 1 and 7 following the heating procedure (Table I), and the number of $CD4^+$ T cells showed an additional decrease at 7 days after a MHT treatment (Table I). However, the percentage and the number of $CD8^+$ T cells were found to be slightly increased at 1 day following the treatment, although the changes in comparison with their pre-treatment value were not statistically significant (Table I). Interestingly, significant decreases were observed in the percentage and the number of $CD4^+CD25^+$ T lymphocytes (T_{reg}), which were recognized as regulatory T lymphocytes, at 7 days post-treatment (Table I).

Discussion

It was found that MHT treatment resulted in increased numbers of cytolytic NK cells with NK activity, but that the number of immunomodulatory NK cells in peripheral blood was unchanged. Such an increase of NK cells in blood after HT treatment has been reported repeatedly[11,18,19], and this increase in total NK cell numbers appears to be accounted for primarily by the increase in the number of cytolytic NK cells seen here. There are larger numbers of NK cells, and they may move from the circulation into tissues in response to increased serum levels of cortisol and IL-6 which result from HT[18]. These observations are also supported by a report that increased numbers of NK cells are found in tumor sites

after hyperthermia treatment in mouse models[15]. In the data reported here, the number of NK cells which were thought to be released from the liver, spleen and bone marrow to peripheral blood after MHT treatment was found to be lower on day 7 post-treatment when compared with observations made at 1 day post-treatment. However, these lower levels at day 7 were still higher than the numbers observed prior to MHT treatment. It is possible that MHT also stimulates the generation of NK cells and leads to increases in the number of NK cells present in blood on day 7. Although moderately increased temperatures might enhance NK activation, temperatures as high as 42°C appear to reduce or even abolish NK cytotoxicity *in vitro* and *in vivo*[12,13,14]. NK cells also show delayed apoptosis the day after treatment[19]. Therefore, when relatively immunosuppressed patients, such as those who are being treated with anti-cancer drugs, are exposed to HT at 42.5°C or higher, a combined treatment with adaptive immune therapy with NK cells can be considered.

This study also found a decrease in the number of $CD4^+$ T cells and T_{reg} cells in peripheral blood after regional MHT treatment. The number and the percentage of $CD8^+$ T cell subsets displayed no significant change at 1 and 7 days after hyperthermia treatment. Kida et al has also reported that regional MHT leads to a reduction in the number of $CD4^+$ T cells, while the number of $CD8^+$ T cells was unchanged[3]. Reports have also documented a decrease in the number of $CD8^+$ T cells, as well as of $CD4^+$ T cells in peripheral blood after WBH[7,19], and this change is different from the response seen here to regional MHT. It is unclear if this difference was caused by the differences between healthy volunteers and patients, the difference in the temperatures used, the length of HT treatment, or if the differences are due to the different treatments themselves ; *i.e.* WBH vs MHT. Further investigation is required to resolve the reasons for these different responses.

Regional MHT leads to a decrease in the number of $CD4^+$ T cells in peripheral blood, and a significant rise in apoptosis in $CD4^+$ T cells has been reported to occur after a heating treatment[19]. However, the percentage of T cells undergoing apoptosis after WBH treatment is rather small in peripheral blood[18,19]. The decrease in the number of $CD4^+$ T cells cannot be explained by apoptosis in peripheral blood. WBH causes a decrease in circulating T cells expressing L-selectin (CD62L) or $\alpha 4\beta 7$ integrin adhesion molecules which mediate homing to lymphatic tissues[18]. Fever-range thermal stress increases intercellular adhesion molecule 1 (ICAM-1) and the CCL21 chemokine, exclusively in high endothelial venules which are the main portals for the entry of peripheral lymphocytes into lymphoid tissues[20]. These results suggest that thermal treatment enhances the adhesion molecule-dependent trafficking of lymphocytes to secondary lymphoid tissues[1,6,18]. Although the details of T cell subsets have not been described, WBH has been shown to enhance the homing of antigen-specific T cells to inflamed regions in the mouse model[20]. It is possible that regional heating with regional MHT induced migration of $CD4^+$ T cells from peripheral blood to tissues. This hypothesis is supported by the report that regional HT stimulated the infiltration of lymphocytes into cancerous lesions[21]. If so, then regional MHT treatment may specifically lead to a redistribution of $CD4^+$ T cells.

T_{reg} cells are a subpopulation of $CD4^+$ T cells and inhibit adaptive responses by T cells[22]. It was also found that the number of T_{reg} cells at 7 days after regional MHT treatment decreased significantly in peripheral blood. T_{reg} cells like other $CD4^+$ T cells, might migrate from peripheral blood into lymphoid tissues after MHT treatment. Freshly isolated T_{reg} cells are highly sensitive to

CD95L-mediated apoptosis unlike their resistant $CD4^+$ effector T cell counterparts[23]. To reset the adaptive immune responses, the possible apoptosis of redistributed T_{reg} cells after MHT treatment might occur in lymphoid tissues. This also should be a subject for further investigations.

This study suggests that regional MHT may activate both the innate and adaptive immune systems through activation of NK cells and depression of T_{reg} cells.

Acknowlegements

The authors would like to acknowledge the staff of Biotherapy Institute of Japan for their excellent technical assistance.

References

1) Evans S.S., Wang W.C., Bain M.D., Burd R., Ostberg J.R., Repasky E.A. : Fever-range hyperthermia dynamically regulates lymphocyte delivery to high endothelial venules. Blood, 97 : 2727-2733, 2001.
2) Ostberg J.R., Kabingu E., Repasky E.A. : Thermal regulation of dendritic cell activation and migration from skin explants. Int J Hyperthermia, 19 : 520-533, 2003.
3) Kida Y., Tsuji-Kawahara S., Ostapenko V., Kinoshita S., Kajiwara E., Kawabata H., Yuasa T., Nishide I., Yukawa S., Ichinose M., Miyazawa M. : Increased liver temperature efficiently augments human cellular immune response : T-cell activation and possible monocyte translocation. Cancer Immunol Immunother, 55 : 1459-1469, 2006.
4) Nagata Y., Hiraoka M., Nishimura Y., Masunaga S., Mitumori M., Okuno Y., Fujishiro M., Kanamori S., Horii N., Akuta K., Sasai K., Abe M., Fukuda Y. : Clinical results of radiofrequency hyperthermia for malignant liver tumors. Int J Radiat Oncol Biol Phys, 38 : 359-365, 1997.
5) Dewey W.C, Hopwood L.E., Sapareto S.A., Gerweck L.E. : Cellular response to combination of hyperthermia and radiation. Radiol, 123 : 463-471, 1977.
6) Chen Q., Fisher D.T., Clancy K.A., Gauguet J.-M.M., Wang W.-C., Unger E., Rose-John S., von Andrian U.H., Baumann H., Evans S.S. : Fever-range thermal stress promotes lymphocyte trafficking across high endothelial venules via an interleukin 6 trans-signaling mechanism. Nature Immunol, 7 : 1299-1307, 2006.
7) Atanackovic D., Nierhaus A., Neumeier M., Hossfeld D.K., Hegewisch-Becker S. : 41.8 degrees C whole body hyperthermia as an adjunct to chemotherapy induces prolonged T cell activation in patients with various malignant diseases. Cancer Immunol Immunother, 51 : 603-613, 2002.
8) Appenheimer M.M., Chen Q., Girard R.A., Wang W.-C., Evans S.S. : Impact of fever-range thermal stress on lymphocyte-endothelial adhesion and lymphocyte trafficking. Immunol Invest, 34 : 295-323, 2005.
9) Ostapenko V.V., Tanaka H., Miyano M., Nishide T., Ueda H., Nishide I., Tanaka Y., Mune M., Yukawa S. : Immune-related effects of regional hyperthermia in patients with primary liver cancer. Hepato-Gastorenterol, 52 : 1502-1506, 2005.
10) Miyata K., Hasegawa T., Maeda K., Ando S., Amano M., Fukuyama A., Monzen H., Gu Y., Takahashi T., Yamamoto I. : The activation of immunological activity and anti-tumor effects by mild hyperthermia. Jpn J Hyperthermic Oncol, 21 : 33-45, 2005.
11) Ahlers O., Hildebrandt B., Dieing A., Deja M., Bohnke T., Wust P., Riess H., Gerlach H., Kerner T. : Stress induced changes in lymphocyte subpopulations and associated cytokines during whole body hyperthermia of 41.8-42.2 degrees C. Eur J Appl Physiol, 95 : 298-306, 2005.
12) Kalland T., Dahlquist I. : Effects of *in vitro* hyperthermia on human natural killer cells. Cancer Res, 43 : 1842-1846, 1983.

13) Fuggetta M.P., Alvino E., Tricarico M., D'Atri S., Pepponi R., Prete S.P., Bonmassar E. : *In vitro* effect of hyperthermia on natural cell-mediated cytotoxicity. Anticancer Res, 20 : 1667-1672, 2000.

14) Koga T., Harada H., Shi T.S., Okada S., Suico M.A., Shuto T., Kai H. : Hyperthermia suppresses the cytotoxicity of NK cells via down-regulation of perforin/granzyme B expression. Biochm Biophys Res Commun, 337 : 1319-1323, 2005.

15) Burd R., Dziedzic T.S., Xu Y., Caligiuri M.A., Subjeck J.R., Repasky E.A. : Tumor cell apoptosis, lymphocyte recruitment and tumor vascular changes are induced by low temperature, long duration (fever-like) whole body hyperthermia. J Cell Physiol, 177 : 137-147, 1998.

16) Dewan M.Z., Terunuma H., Toi M., Tanaka Y., Katano H., Deng X., Abe H., Nakasone T., Mori N., Sata T., Yamamoto N. : Potential role of natural killer cells in controlling growth and infiltration of AIDS-associated primary effusion lymphoma cells. Cancer Sci, 97 : 1381-1387, 2006.

17) Cooper M.A., Fehniger T.A., Caligiuri M.A. : The biology of human natural killer-cell subsets. Trends Immunol, 22 : 633-640, 2001.

18) Atanackovic D., Pollok K., Faltz C., Boeters I., Jung R., Nierhaus A., Braumann K.-M., Hossfeld D.K., Hegewisch-Becker S. : Patients with solid tumors treated with high-temperature whole body hyperthermia show a redistribution of naïve/memory T-cell subtypes. Am J Physiol Regul Integr Comp Physiol, 290 : R585-R594, 2006.

19) Dieing A., Ahlers O., Kerner T., Wust P., Felix R., Löffel J., Riess H., Hildebrandt B. : Whole body hyperthermia induces apoptosis in subpopulations of blood lymphocytes. Immunobiol, 207 : 265-273, 2003.

20) Ostberg J.R., Gellin C., Patel R., Repasky E.A. : Regulatory potential of fever-range whole body hyperthermia on Langerhans cells and lymphocytes in an antigen-dependent cellular immune response. J Immunol, 167 : 2666-2670, 2001.

21) Morita M., Kuwano H., Araki K., Egashira A., Kawaguchi H., Saeki H., Kitamura K., Ohno S., Sugimachi K. : Prognostic significance of lymphocyte infiltration following preoperative chemoradiotherapy and hyperthermia for esophageal cancer. Int J Radiation Oncology Biol Phys, 49 : 1259-1266, 2001.

22) Sakaguchi S., Sakaguchi N., Asano M., Itoh M., Toda M. : Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol, 155 : 1151-1164, 1995.

23) Fritzsching B., Oberle N., Eberhardt N., Quick S., Haas J., Wildemann B., Krammer P.H., Suri-Payer E. : Cutting edge : in contrast to effector T cells, $CD4^+CD25^+FoxP3^+$ regulatory T cells are highly susceptible to CD95 ligand- but not to TCR-mediated cell death. J Immunol, 175 : 32-36, 2005.

Abstract in Japanese

局所マイルドハイパーサーミアによるNK細胞の活性化と調節性T細胞数の減少

照沼　裕[1,2]・和田綾乃[2]・邓　学文[1]・安間芳秀[2]
大西哲郎[2]・土岐　敦[3]・阿部博幸[2]

[1]日本バイオセラピー研究所
[2]九段クリニック
[3]多摩南部地域病院・内科

要　　旨：マイルドハイパーサーミアでは悪性腫瘍に対して熱による直接的傷害は期待できないが，免疫系が活性化すれば間接的に悪性腫瘍を傷害できることが報告されている．今回我々は，局所マイルドハイパーサーミアにより，NK細胞やTリンパ球などのリンパ球分画にどのような変化が起こるかを検討した．

局所マイルドハイパーサーミアは，サーモトロンRF8を用いて，30 cmの外部電極で上腹部を挟み，約900 watt，30分間高周波をながす局所加温でおこなった．免疫系の変化として，健常人の血中の白血球数・リンパ球数・その分画の割合と細胞数・NK活性などを加温操作の前後で測定して比較した．

マイルドハイパーサーミアをおこなう直前と比較して，加温1日後と7日後のリンパ球中でのNK細胞百分率・細胞傷害性NK細胞百分率・NK活性，加温1日後の細胞傷害性NK細胞数が有意に増加した．また，加温1日後と7日後のリンパ球中でのTリンパ球百分率・CD4陽性Tリンパ球百分率，加温7日後のCD4陽性Tリンパ球数が有意に減少した．興味深いことに，免疫抑制に関与する調節性Tリンパ球百分率・調節性Tリンパ球数が有意に減少した．

これらの結果は，局所マイルドハイパーサーミアにより，NK細胞を中心とした自然免疫が活性化される一方，調節性T細胞による免疫抑制が解除され，悪性腫瘍に対する免疫活性が増強される可能性が示唆された．