Modulation of the endocrine and immune systems by well-controlled hyperthermia equipment

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ABSTRACT
Since high levels of hyperthermia induce immunosuppression to a certain extent (i.e., granulocytosis and lymphocytopenia) in patients, we applied mild hyperthermia in volunteers using equipment enabling well-controlled hyperthermia. Restricted control of rectal temperature at 39.4 (±0.2)°C for 30 min was conducted and various parameters of the body were examined. The most prominent change observed during exposure to hyperthermia was elevated levels of pH and PO2 in the blood, even in the venous blood. A transient elevation of ACTH, cortisol and growth hormone in the blood was also seen during this time. In parallel with this phenomenon, the number of total lymphocytes and those of its subsets (especially CD57+ or CD56+ NK cells and NKT cells) increased. More interestingly, the proportion of HLA-DR (MHC class II antigens) increased in NK and NKT cells, and their intensity on the surface of CD20+B cells increased. These results suggest that mild hyperthermia is important for modulation of the functions of the circulatory, endocrine and immune systems.

It is widely known that hyperthermia induces the production of heat shock proteins (HSPs) and that such HSPs are able to induce immunopotentiation via the augmented expression of MHC class I or II antigens on lymphocytes and tissue cells (2, 3, 10, 15). Hyperthermia is therefore expected to have potential as cancer immunotherapy. Another strategy for use of hyperthermia in cancer therapy is based on the notion that cancer cells are sensitive to thermal stress, which results in apoptotic death (5, 20, 23). However, we have encountered severe granulocytosis and lymphocytopenia in patients or animals during the exposure to various types of stress, including hyperthermia (4, 12, 16, 17). This experience is contrary to our expectation for immunopotentiation by hyperthermic therapy.

In light of these findings, we conducted mild hyperthermia in volunteers using the “Thercura System,” a well-controlled hyperthermia device. We found that restricted control of rectum temperature to 39.4 (±0.2)°C for 30 min was able to induce a prominent potentiation of many parameters in the circulation, endocrine system and immune system. Especially, the immunopotentiation induced by the present protocol of mild hyperthermia seems to be of great value for cancer patients and other diseased persons for treatment of their decreased levels of the immune functions.

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MATERIALS AND METHODS

Volunteers and temperature control equipment. Four healthy volunteers, 30 to 60 years of age (52.0 ± 12.1), participated in the present study. The “Thercura System” is a big hot water bath equipped with a temperature control system (Thercure Medical Co., Osaka, Japan). This device is able to maintain the rectal temperature up to the range of 0.1°C (Fig. 1). In this study, the rectum temperature of subjects was controlled at 39.4 (±0.2°C for 30 min (Fig. 2). To reach this level, approximately 30 min was required in each subject.

Parameters tested. Thermal exposure influenced various parameters in the circulation, endocrine system and immune system. To test a change of the circulation, pH and PO$_2$ were measured in the venous blood. The parameters of the endocrine system included ACTH, cortisol and growth hormone in sera, while those of the immune system included the number of leukocytes and their subsets in the blood.

Cell preparations. Peripheral blood lymphocytes (PBLs) were isolated from heparinized blood (22). PBLs were then obtained by Ficoll-Paque PLUS (Amersham Biosciences AB, Uppsala, Sweden) gradient (1.077) centrifugation.

Immunological parameters. The level of lymphocyte subsets, including T cells, B cells, NK cells and NKT cells, was examined by two-color immunofluorescence tests (11). Since thermal exposure was found to induce HLA-DR (MHC class II antigens) on lymphocyte subsets, three-color staining was also conducted to detect the expression of this antigen on cell surface. This flow cytometric analysis was performed as previously described (11). The mAbs used here included FITC, PE, or PerCP-conjugated anti-CD3 (UCHT1), anti-CD56 (N901), anti-CD4 (RPA-T4), anti-CD8 (RPA-T8) (Beckman Coulter Inc., Fullerton, CA), anti-CD57 (HNK-1), anti-CD20 (L27), and anti-HLA-DR (L243) (Beckton-Dickinson Co., Franklin Lakes, NJ). Cells were analyzed by a FACScan (Beckton-Dickinson Co.). Dead cells were excluded by forward scatter and side scatter.

Statistical analysis. The difference between the values was determined by one-factor ANOVA. P values < 0.05 were considered statistically significant.

RESULTS

Parameters of the circulation and endocrine system
All parameters were tested at prethermal exposure (Pre), at peak time (Max, 15 min after reaching 39.4°C of rectal temperature), at the end of thermal exposure (Post), and at 1 h, 2 h and 96 h after thermal exposure (Fig. 3). At the peak time, both pH and PO$_2$ of venous blood increased in all volunteers. Showing a similar pattern, the levels of ACTH, cortisol and growth hormone also increased. Interestingly, this pattern of increase was prominent in two of four cases, case 1 and 2.

Immunological parameters
The number of whole leukocytes (WBC, white blood cells) was found to be relatively stationary during thermal exposure but increased at 1 and 2 h after thermal exposure (Fig. 4). The percentage of granulocytes (Neutro.) tended to decrease during thermal exposure but to increase after the exposure. The percentage of lymphocytes showed the opposite pattern from that of granulocytes. The absolute number of lymphocytes was estimated to increase at the peak time of thermal exposure.

We examined what lymphocyte subsets were activated during thermal exposure (Fig. 5). Two-color staining for CD3 and CD56 (or CD57) was conduct-
Immune modulation by hyperthermia

Expression of MHC class II antigens on NKT cells and B cells by hyperthermia

It is well established that T cells lack the expression of MHC class II antigens, but B and activated T cells expressed these antigens on the surface. However, it was found that HLA-DR⁺NKT cells were newly generated after the exposure of hyperthermia (Fig. 6). In these experiments, three-color staining for CD3, CD57 (or CD20) and HLA-DR was conducted and the DR expression was estimated by the gated analysis for NKT cells or CD20⁺B cells.

In two subjects, case 1 and 2, HLA-DR⁺CD57⁺NKT cells appeared after hyperthermia (especially at Max) (indicated by arrowheads). In the case of CD20⁺B cells, they all expressed HLA-DR irrespective of hyperthermia. However, it was found that the mean fluorescence intensity of HLA-DR-positive cells increased after hyperthermia in all subjects (indicated by arrows).

These experiments were further conducted and the absolute number of HLA-DR⁺ cells on various lymphocyte subsets was estimated (Table 2). CD56⁺NK cells, CD57⁺NKT cells, CD56⁺T cells, CD8⁺T cells and CD20⁺B cells were found to increase in cell number after various points of time of hyperthermia (n = 3, P < 0.05).

Fig. 3 Variation in the parameters of the circulation and endocrine system. Four subjects (case 1 to 4) were monitored as to pH, PO₂, ACTH, cortisol and growth hormone (GH). Max means 15 min after commencement of hyperthermia.

Fig. 4 Variation in the parameters of the immune system. The number of WBC and lymphocytes and the proportion of neutrophils and lymphocytes were examined in four subjects.
**Fig. 5** Two-color staining for CD3 and CD56 (or CD57), that for CD4 and CD8, and that for CD3 and CD20. Lymphocytes isolated from three subjects (cases 1 to 3) were examined by immunofluorescence tests. Numbers in the figure represent the percentages of fluorescence-positive cells.

**Table 1** Comparison of the absolute number of subpopulations in various phases of hyperthermia

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>Absolute number of subpopulations (/μL)</th>
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<tbody>
<tr>
<td></td>
<td>Pre</td>
</tr>
<tr>
<td>CD56(^{+}) NK</td>
<td>207.9 ± 52.4</td>
</tr>
<tr>
<td>CD57(^{+}) NK</td>
<td>133.8 ± 20.2</td>
</tr>
<tr>
<td>CD56(^{+}) NKT</td>
<td>75.8 ± 35.4</td>
</tr>
<tr>
<td>CD57(^{+}) NKT</td>
<td>350.5 ± 63.5</td>
</tr>
<tr>
<td>CD56(^{-}) T</td>
<td>952.9 ± 12.2</td>
</tr>
<tr>
<td>CD57(^{-}) T</td>
<td>598.9 ± 59.6</td>
</tr>
<tr>
<td>CD4(^{+})</td>
<td>523.5 ± 9.8</td>
</tr>
<tr>
<td>CD8(^{+})</td>
<td>358.3 ± 69.6</td>
</tr>
<tr>
<td>CD20(^{+})</td>
<td>150.7 ± 33.1</td>
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\(*P < 0.05, n = 3\)
DISCUSSION

In the present study, we demonstrated that mild hyperthermia (keeping rectal temperature at 39.4°C for 30 min) induced a change of various parameters in terms of the circulatory, endocrine and immune systems. The most prominent change was seen in the circulation, showing elevated levels of pH and PO\(_2\) in the venous blood. At the time of initiation, pH was around 7.35 ± 0.03 and then reached 7.55 ± 0.04 at Max after hyperthermia. PO\(_2\) in the venous blood was primarily very low (< 10 mmHg) in all subjects, but this increased up to 80 mmHg by hyperthermia. Diseased persons, including cancer patients, have low body temperature (e.g., < 35.5°C) due to poor circulation (our unpublished observation). Therefore, the present protocol might be useful in helping such persons to recover good circulation with resultant good body temperature.

Changes in the parameters of endocrine system were of interest. A transient elevation of ACTH and a resultant elevation of cortisols resembled stress-associated responses. This response was prominently seen in two persons but it was not in other two persons. In other words, the applied protocol of hyperthermia in this study was estimated to be a borderline between stress and non-stress. We previously reported that an injection of steroid hormone induces granulocytosis (mainly neutrophils) as well as lymphocytopenia (including thymic atrophy) (9). Granulocytosis is sometimes dangerous in that it induces tissue damage via production of superoxides (6, 8). However, granulocytosis induced by the mild hyperthermia in the present protocol was not so prominent. Inversely, it was possible to induce lymphocytosis by the present protocol.

In addition to the change in this hypothalamus-adrenal axis, the variation in the level of growth hormone was also of interest. At present, we do not know the meaning of this phenomenon. However, there were several reports in which hyperthermia

![Fig. 6 Three-color staining for CD57, CD3 and HLA-DR and that for CD20, CD3 and HLA-DR. By gated analysis, HLA-DR\(^+\) cells were identified on CD57\(^+\)T cells and CD20\(^+\)B cells. In the case of B cells, the mean fluorescence intensity was also indicated.](image)

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>Absolute number of HLA-DR(^+) cells among subpopulations (/μL)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Pre</td>
</tr>
<tr>
<td>CD56 NK</td>
<td>8.8 ± 2.2</td>
</tr>
<tr>
<td>CD57 NK</td>
<td>2.7 ± 2.2</td>
</tr>
<tr>
<td>CD56 NKT</td>
<td>2.2 ± 1.8</td>
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<tr>
<td>CD57 NKT</td>
<td>22.1 ± 2.3</td>
</tr>
<tr>
<td>CD56 T</td>
<td>155.4 ± 8.7</td>
</tr>
<tr>
<td>CD57 T</td>
<td>26.6 ± 1.4</td>
</tr>
<tr>
<td>CD4(^+)</td>
<td>27.0 ± 9.6</td>
</tr>
<tr>
<td>CD8(^+)</td>
<td>12.8 ± 6.0</td>
</tr>
<tr>
<td>CD20(^+)</td>
<td>149.2 ± 32.5</td>
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</table>

*P < 0.05, n = 3
has a potential to increase the level of growth hormone in sera (1, 18, 21). In this study, we examined endocrine system parameters other than growth hormone, for example, the serum levels of insulin, thyroid stimulating hormone (TSH) and thyroxin. Hyperthermia in the present protocol did not change these values.

In a final portion of the present study, we examined various parameters of the immune function. In addition to the increase in the total number of lymphocytes at the exposure time of hyperthermia, the number and proportion of CD56⁺NK cells, CD57⁺NK cells, and CD56⁻ (or CD57⁻) NKT cells were found to increase. It is known that these NK and NKT cells are associated with immune functions in animals and humans with malignancy (7, 13, 14, 19). It is therefore speculated that the present phenomenon of the immune system might be important for the immunopotentiation of cancer patients.

Since hyperthermia is known to be related to the augmentation in the expression of MHC antigens on the cell surface (2, 3, 10, 15), we investigated whether mild hyperthermia in the present protocol had the potential to induce such augmentation. It is well-known that a population of NK, NKT cells and activated T cells, as well as all B cells, carry MHC class II antigens. In this regard, HLA-DR expression on NK cells, NKT cells and B cells as well as on conventional T cells (NK T cells) was examined by three-color immunofluorescence tests. The gated analysis of each lymphocyte subset demonstrated that the proportion of HLA-DR⁺NK cells and that of NKT cells were increased by hyperthermia. In the case of B cells, it was confirmed that all populations expressed HLA-DR on the surface. The mean intensity of immunofluorescence (i.e., the expression level of HLA-DR) on these B cells was elevated during hyperthermia. These results therefore suggest that not only the blood but also the immune system was augmented by mild hyperthermia in the present protocol.

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REFERENCES


