Inhibitory Effects of Elevated Temperature on Human Cytokine Production and Natural Killer Activity

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ABSTRACT

Febrile reactions often occur in cancer patients given various biological response modifiers such as α- or γ-interferon or interleukin-2. The present studies were undertaken to determine the effects of moderately elevated temperatures (39°C) on various immunological functions related to host defense against malignant cells. The production of the cytokines interleukin-1, interleukin-2, erythrocyte burst-promoting activity, and granulocyte-macrophage colony-stimulating factor from activated human mononuclear cells was assessed in vitro at 34, 37, and 39°C and found to be reduced at 39°C. The natural killer activity of human mononuclear cells preincubated for 18 h at various temperatures was also significantly reduced (P < 0.001) at 39°C. Although the addition of recombinant interleukin-1-β, interleukin-2, and α-interferon during the 18-h incubation augmented natural killer activity at all temperatures, the enhancing effects were least apparent at 39°C. Indomethacin increased cytokine-primed natural killer cell activity at all temperatures but did not reverse the inhibitory effects of elevated temperatures. These results suggest that the fever associated with treatment with pyrogenic cytokines may partially offset the direct stimulatory effects of these substances on cellular immune function.

INTRODUCTION

Anecdotal reports of tumor regression in cancer patients with postoperative infections led Dr. William Coley, a New York City surgeon practicing at the turn of the century, to routinely inject a mixture of bacterial culture filtrates into his patients in an effort to induce the same type of clinical response that he had observed in infected patients (1).

Coley's toxins, a mixture of filtrates from erysipelas strains of Streptococci and endotoxin-producing Serratia marcescens, were widely used before the introduction of radiation therapy for cancer treatment. The immediate clinical response to the injection of these toxins was fever and in many patients hyperpyrexia (fever, 40.5°C). There is little doubt that Coley's toxins were occasionally effective as recent reports have documented cases of tumor regression with toxin therapy (1-3).

Pyrogenic toxins, which are substances (reviewed in Ref. 15), has yet to be used clinically; however, culture supernatants of certain human cell lines which contain large amounts of IL-1 have been injected into humans and have produced dramatic pyrogenic reactions (20). We therefore studied NK activity following exposure to IL-1, IL-2, and IFN at various temperatures in order to mimic the effects of these cytokines on NK activity during a febrile response. The production of the cytokines IL-1, IL-2, BPA, and GM-CSA by human peripheral blood mononuclear cells in vitro was also examined at these temperatures. In contrast to other immune functions that are enhanced at elevated temperatures, we found that cytokine production and both base-line and cytokine-primed NK activity are markedly inhibited at elevated temperatures. Since cytokine production and NK activity are closely associated with host defense against malignancy, these observations suggest that the benefit reported to be derived from toxin therapy is not fever related but, in fact, occurs despite detrimental effects of elevated temperature. These studies support the concept that fever resulting from the injection of an immunostimulant may partially negate any beneficial effects on NK-directed tumor lysis.

MATERIALS AND METHODS

Materials. PHA was purchased from Burroughs Wellcome (Research Triangle, NC) as PHA-P. Endotoxin (National Reference Endotoxin RE-2) used in these studies was supplied by the Bureau of Biologics, Bethesda, MD. Eagle's MEM was purchased from Microbiological

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Associates, Walkersville, MD, and supplemented with 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid buffer (0.01 M), penicillin (100 units/ml), and streptomycin (100 μg/ml; Microbiological Associates). Na_2^{105}CrO_4 (200 to 900 Ci/g Cr) and [H]thymidine (6.7 Ci/mmol) were obtained from New England Nuclear, Boston, MA. Indomethacin was purchased from Sigma (St. Louis, MO), dissolved in 95% ethanol (10 mg/ml), and diluted in MEM prior to use. Recombinant human IFN-α was supplied by Schering Corp. Human IL-1 was purified by immunoabsorption and gel filtration from human monocyte conditioned medium as previously described (21). The activity of IL-1 used in these studies was 100 RPD/ml where 1 RPD produces a fever between 0.6 and 0.9°C in a 2.5-kg rabbit. An IL-1 unit is defined as the concentration (per ml) which doubles the proliferative response of murine thymocytes to PHA. In general, there are 1000 to 2000 IL-1 units per 1 RPD (22, 23). Human IL-2 was purified from PHA-stimulated lymphocyte conditioned medium by sequential anion exchange chromatography and gel filtration (24). Recombinant IL-2 was obtained from Cetus Corp., Emeryville, CA; recombinant IL-1-β was supplied by Cistron Biotechnology, Pine Brook, NJ.

**Incubator Temperatures.** Three separate incubators were used for these studies. Temperature settings were adjusted with the individual control mechanism of each incubator and checked with a single thermometer calibrated at the National Bureau of Standards, Georgetown, MD. The thermometer was placed adjacent to the tissue culture containers. With the exception of one experiment (Fig. 2), temperatures were set at 34, 37, and 39 ± 0.02°C. All incubators contained 5% CO_2-95% humidified air.

**Production of Mononuclear Cell-conditioned Medium.** Human blood MNC were obtained from normal donors by density gradient centrifugation with Ficoll-Hypaque, washed in pyrogen-free saline, and suspended in MEM at 5 x 10^6 cells/ml. For the production of IL-1, cells were suspended in MEM containing 1% (v/v) heat-inactivated human AB serum. One ml of cells was added to flat-bottomed culture wells (2-cm diameter; Costar, Inc., Hamden, CT) with various dilutions of endotoxin, and cultures were incubated at various temperatures. The supernatants were harvested 24 h later and dialyzed against RPMI-1640 medium (Microbiological Associates) in M, 3500 cut-off dialysis tubing.

For the production of IL-2, the MNC were depleted of adherent cells by incubation in Petri dishes at 37°C for 2 h. The nonadherent cells were then suspended at 10^6 cells/ml in RPMI-1640 medium containing 10% fetal calf serum (Hyclone, Logan, UT), indomethacin (1.0 μg/ml), 2 mM hydroxyurea, PHA (1.0 μg/ml), and highly purified human IL-1 (10 units/ml) (see above). The cultures were incubated at the various experimental temperatures for 72 h, and the supernatant media were collected and dialyzed against RPMI-1640 medium. Culture supernatants for BPA and GM-CSA assays were similarly prepared except that the incubation period was extended to 96 h.

**Cytokine Assays.** All assays were carried out at 37°C. IL-1 concentration was assayed by measuring the ability of supernatants to augment murine thymocyte proliferative responses to suboptimal concentrations of PHA (23). IL-2 was assayed in a standard microtiter assay based upon the induction of [H]thymidine incorporation in IL-2-dependent, PHA-activated cultured human T lymphoblasts as previously described (24). BPA was assayed in cultures of human bone marrow cells as previously described (25). Briefly, 0.5 ml of marrow was aspirated from the posterior iliac crests of three hematologically normal donors. The MNC were separated with Ficoll-Hypaque (Pharmacia, Piscataway, NJ), washed 3 times, and cultured on fibrin clots in serum from K562 target cells at an effector:target ratio of 25:1 (16). Some of the experiments were performed with effector cells preincubated with stimulatory cytokines, with indomethacin, or both. The percentage of specific lysis was calculated from the following formula:

\[
\frac{\text{cpm}_{\text{sample}} - \text{cpm}_{\text{spont}}}{\text{cpm}_{\text{max}} - \text{cpm}_{\text{spont}}} \times 100
\]

\(\text{cpm}_{\text{sample}}\) is 51Cr release induced by the effector cells, whereas \(\text{cpm}_{\text{max}}\) and \(\text{cpm}_{\text{spont}}\) are maximal (HCl-induced) and spontaneous release, respectively. In order to pool data from different human donors for each experiment, the specific lysis associated with unstimulated control MNC preincubated at 37°C was arbitrarily assigned a value of 100%. Then each value of effector-induced lysis which was obtained following incubation at other temperatures or with stimulatory lymphokines or indomethacin was calculated as a percentage of this control value. Comparisons were made using the Student unpaired t test. The values for percentage of change are indicated. Asterisks indicate which two values are being compared in the t test.

**Cytotoxic T-Cell Assays.** The Epstein-Barr virus-positive B-cell line JY (HLA-A2,2; B-7,7; DR-4,6), a poor NK target, was utilized as the stimulator cell. Seven x 10^6 MNC from healthy donors and 5 x 10^5 irradiated (4500 rads) JY cells were cocultured in 2.0-ml wells (Nunc, Naperville, IL) at various temperatures in RPMI-1640 medium. Six days later, the nonadherent cells were harvested and assayed for cytolytic activity against 51Cr-labeled JY cells as previously described (27). Standard deviations were less than 5%.

**RESULTS**

**Effects of Temperature on Cytokine Production**

**Interleukin-1.** Human mononuclear cells were incubated at 34, 37, and 39°C in the presence of various concentrations of endotoxin (3, 30, and 300 ng/ml). The supernatants were obtained after 24 h, diluted, and assayed for IL-1 activity (23). Since increased PGE2 has been demonstrated in tissue incubated at febrile temperatures (28) and since cyclooxygenase inhibitors increase IL-1 production in vitro (15), we added indomethacin at concentrations known to suppress PGE2 synthesis in an attempt to eliminate this variable and more directly determine the influence of temperature. As shown in Fig. 1, the addition of indomethacin resulted in increased IL-1 production at all temperatures. However, indomethacin did not offset the effect of elevated temperature on IL-1 production; in fact, the highest level of IL-1 production occurred at 34 rather than 37 or 39°C.

**Interleukin-2.** Human peripheral blood lymphocytes were stimulated with PHA and incubated at 34, 37, and 39°C, and, after 72 h, the supernatant media were dialyzed and assayed for IL-2 activity. Because of decreased IL-1 production at febrile temperatures and the known dependence of IL-2 synthesis on IL-1, the effect of temperature on IL-2 production was studied using adherent cell-depleted mononuclear cell cultures in the

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Fig. 1. IL-1 production at various temperatures. Human MNC were stimulated with endotoxin at concentrations indicated under abscissa. Indomethacin (Indometh) concentration was 1 μg/ml. The results are expressed as the mean cpm ± SD of thymocytes incubated at 37°C with 1 μg of PHA per ml and a 1:20 dilution of the MNC supernatants. ● 34°C; □ 37°C; △ 39°C. *, P < 0.05; **, P < 0.01; ***', P < 0.001.

Fig. 2. IL-2 production at various temperatures. Supernatants were generated as described in “Materials and Methods.” Assay of supernatants was carried out at 37°C with the dilutions indicated under the abscissa. cpm (±SD) of incorporated [3H]thymidine are indicated on the ordinate.

Effect of Temperature on NK Activity

Although natural killer activity is mediated by a subpopulation of LGL, other mononuclear cells are known to influence their cytolytic activity (29). In these experiments, unfractionated MNC rather than isolated LGL were utilized as effectors to permit the detection of such cellular interactions at various temperatures. The MNC were cultured for 18 h at 34, 37, or 39°C, and the cells were subsequently washed, recounted, and assayed in a standard 51Cr release assay with K562 target cells (16). Standard deviations were consistently less than 5% in each assay. In these experiments as well as those with cytokine- or indomethacin-treated cells, the viability of the effector cells was consistently greater than 95% as determined by staining with trypan blue. As shown in Fig. 4, there was a highly significant decrease in NK activity when cells were preincubated at 39°C (control, 37°C versus 39°C; P < 0.001). In parallel experiments involving cells preincubated with indomethacin, a similar temperature dependence was noted (control, 37°C versus 39°C; P < 0.001). At all temperatures tested, indomethacin enhanced the cytolytic activity of MNC against K562 cells but did not dampen the suppressive effects of elevated temperature.

In 2 similar experiments in which cells were placed directly into the standard 4-h cytotoxicity assay at various temperatures without an extended preincubation, little difference in cytolytic activity was evident. Fig. 5 illustrates such an experiment. The effect of IFN, IL-2, or IFN plus IL-2 was not affected by the different temperatures.

Effect of Temperature on IL-1-, IL-2-, and IFN-stimulated NK Activity

IL-1. We previously reported that exposure to IL-1 at 37°C minimally increased the NK activity of human MNC or density gradient-enriched large granular lymphocyte preparations (16). Since several reports have clearly demonstrated augmented T-
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Fig. 5. NK activity of MNC incubated at various temperatures during the cytotoxicity assay. The NK activity against K562 targets incubated during the 4-h assay was carried out at 34, 37, or 39°C. Control NK activity was obtained without added cytokines, whereas addition of IFN (1000 units/ml) or IL-2 (1 unit/ml) is indicated. The data represent an effector:target ratio of 25:1.

Fig. 6. The effects of IL-1 on NK activity at different temperatures. In A, IL-1 activity as assayed on thymocytes is shown (C); NK activity at 37°C is indicated ()]. The IL-1 concentration used during the 18-h preincubation was 5 x 10⁻² RPD/ml. Indometh, indomethacin.

Fig. 7. The effect of IL-2 on NK activity at various temperatures. The data are normalized as in Fig. 4 and represent 3 separate experiments. The IL-2 concentration used during the 18-h preincubation was 1 unit/ml. Indometh, indomethacin.

The inhibition of cyclooxygenase activity with indomethacin also failed to block the inhibitory effects of increased temperature. The NK activity of MNC incubated overnight with IL-1 and indomethacin at 39°C was different from that of control cells incubated at 37°C in the absence of indomethacin.

IL-2. Similar experiments were carried out with IL-2 at various temperatures, with and without indomethacin. As depicted in Fig. 7, IL-2-stimulated MNC lysed the target cells better than unstimulated MNC at all temperatures, and as was the case with unstimulated MNC, elevated temperature had a suppressing effect on cytolytic activity that was not influenced by the addition of indomethacin.

IL-1 and IFN. IL-1 added to either MNC or large granular lymphocytes cultured in the presence of IFN augments the increase in NK activity induced by IFN alone (16). In order to determine the effect of temperature on IL-1 and IFN-primed NK activity, these experiments were repeated at various temperatures. The data presented in Fig. 8 confirm our earlier observation regarding the synergism between these two immunomodulators (16). Furthermore, as shown in similar experiments with unstimulated MNC, incubation at 39°C resulted in decreased NK activity in the presence of two cytokines.

Effect of Temperature on Cytotoxic T-Cell Induction and Cytolytic Activity

In the induction experiments, MNC were incubated with irradiated JY cells at various temperatures and assayed at 37°C for cytolytic activity on Day 6. As shown in Fig. 9, cytolytic activity was maximal with cells incubated with the stimulator cells at 39°C and negligible with cells similarly sensitized at 34°C. In another experiment, cytolytic cells generated at 37°C were assayed on Day 6 for cytolytic activity against JY cells at various temperatures. As shown in Fig. 10, lysis of the allogenic target was optimal at 39°C. Thus, in contrast to NK activity...
The data are depicted as in Fig. 4. The concentration of IFN was 1000 units/ml, and the concentration of IL-1 was $5 \times 10^{-2}$ RPD/ml. Results of 3 experiments incubated with irradiated JY cells at various temperatures and after 6 days assayed for cytolytic activity against JY cells at 37°C.

Fig. 8. The effect of IFN and IL-1 on NK activity at different temperatures. The data are depicted as in Fig. 4. The concentration of IFN was 1000 units/ml, and the concentration of IL-1 was $5 \times 10^{-2}$ RPD/ml. Results of 3 experiments are shown.

Fig. 9. Induction of cytolytic T-cells at various temperatures. MNC were incubated with irradiated JY cells at various temperatures and after 6 days assayed for cytolytic activity against JY cells at 37°C.

Fig. 10. Cytolytic activity of T-cells against JY cells assayed at various temperatures. MNC were incubated with irradiated JY cells at 37°C, and after 6 days assayed for cytolytic activity against JY cells at 37°C.

which is impaired by elevated temperature, both the induction of cytotoxic T-cells and the lysis of the target cells by T-cells are enhanced at elevated temperatures (14).

DISCUSSION

The use of biological response modifiers in clinical trials is frequently associated with fever. In fact, this is the major side effect of both leukocyte-derived and recombinant human IFN-α (18; reviewed in Ref. 31). Recombinant human IFN-γ is also pyrogenic, and the clinical responses to lymphoblastoid and recombinant IL-2 include febrile reactions (19). The studies presented in this paper show that the base-line and cytokine-primed NK activity of human MNC and cytokine production in vitro are both significantly reduced at moderately elevated temperatures (39°C), and they raise questions as to whether the fever induced by BRMs has a negative influence on cytokine production in vivo.

Several investigators have shown that the pyrogen IL-1 stimulates T-cell function and that these effects are enhanced at elevated temperatures (11–13, 30). The $Q_{10}$ for most cellular and biochemical reactions is 2 to 4, but the $Q_{10}$ for IL-1-induced T-cell proliferation was reported to be 50 to 300 (13). In addition, IFN-α inhibition of generation of suppressor T-cells in vitro was optimal at a febrile (39.9°C) temperature (32). Our results with cytolytic T-cells corroborate the view that fever may enhance certain immunological functions. The cytolytic activity of T-cells induced at 37°C was increased when the cytotoxic assay was carried out at 39°C. Furthermore, the 6-day induction of cytotoxic T-cells gave rise to a more cytolytic population when the cocultivation was carried out at 39°C. Thus, both the induction and target cell lysis percentage of cytotoxic T-cells are enhanced by elevated temperature.

In contrast to the several cited examples of enhanced T-cell function at elevated temperature, we found that human T-cell production of IL-2 was decreased at 39°C. IL-1, BPA, and GM-CSF production was also decreased at 39°C. Like IFN production, which is also decreased at elevated temperatures (33), the reduction in IL-1 and IL-2 release at 39°C is not as great as the increase in production which occurs at 34°C. The decrease in IL-1 production at 39°C was only modestly offset when indomethacin was added to the cultures, but relatively large amounts of IL-1 were measured in the supernatant medium when cells were incubated at 34°C in the presence of indomethacin. The injection of neutralizing antibodies to IL-2 or interferon markedly impairs host resistance to neoplasia and virus infection (34–36). These observations suggest that minimal quantities of IL-2 and interferon must be released for optimal cellular immune function but that conditions such as hyperthermia which inhibit the synthesis of immunoregulatory lymphokines may be counterproductive to the host.

A great deal of evidence suggests that the NK cell may be important in the immune surveillance against spontaneously arising malignancy (37). The decrement in NK activity associated with a temperature elevation easily achieved in vivo with the injection of interferon or IL-2 is therefore disconcerting. In another study, a decrease in NK activity was observed following a 1-h exposure to hyperthermic temperatures, and attempts to rescue the cells with an overnight incubation in IFN-α at 37°C were unsuccessful (38). We have been unable to demonstrate a significant variability in cytolytic activity within the narrower range of temperature used in our studies without a more lengthy preincubation period. These studies suggest, however, that a brief exposure to hyperthermia induces a rapid onset of metabolic changes in the NK cell. The response of NK cells to hyperthermia may involve the synthesis of heat shock proteins, which have been detected in chicken and rat blood MNC preparations exposed to elevated temperatures (39, 40). It is unclear why the same level of hyperthermia that initiates heat shock protein synthesis and is associated with enhanced functional activity in T-lymphocytes results in decreased NK activity.

There are few studies examining the in vivo effects of hyperthermia on NK activity. In experiments using an animal model of microwave-induced hyperthermia, hamster spleen cell NK
activity was significantly decreased 4 h following 60 min of hyperthermia (41). However, these animals developed marked lymphopenia and increased plasma glucocorticoid levels. Thus, it remains difficult to assess the effects of elevated temperature on NK activity in vivo independently of other factors. In the present study, we investigated the effects of elevated temperatures on NK activity in vitro without the confounding effects of stress-related substances, such as corticosteroids and hypothalamic hormones, which are elevated during fever or hyperthermia. Our approach was to mimic a brief febrile episode similar to that which occurs following the injection of a BRM. Our results are in agreement with other investigations (38, 42, 43). In addition, we have examined the effects of elevated temperatures on lymphokine-primed NK activity, since these BRMs often cause fever. Thus, it is not surprising that NK activity in human subjects receiving IL-2 is transiently decreased after treatment (19), since patients develop fever lasting several hours following injection.

In conclusion, we have shown that the production of cytokines by stimulated immunocompetent cells and the effects of these immunomodulators on NK activity are dampened by elevated temperature. Indomethacin at concentrations which inhibit cyclooxygenase activity does not reverse the inhibitory effects of elevated temperature on these parameters, and thus we conclude that the suppressive effects are not mediated by effects of elevated temperature on macrophage tumoricidal activity or on lymphokine-activated killers. Furthermore, we did not evaluate the production of tumor necrosis factor nor lymphotxin at different temperatures. It is conceivable that elevated temperature may augment these functions and the production of these cytokines. Nevertheless, NK activity was consistently reduced following 18 h at 39°C. Depending on the relative importance of the various cytolytic populations in the eradication of tumor metastases, there may be a role for antipyretics during treatment with pyrogenic immunomodulators.

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