

Differential Effects of Hyperthermia on Human Leukocyte Production of Interferon- α and Interferon- γ ¹ (42383)

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Abstract. Hyperthermia is being used clinically in the treatment of neoplasms. However, there are insufficient data regarding effects of hyperthermia on leukocyte functions potentially important in antitumor immunity. In order to provide such data, human mononuclear leukocytes were exposed to moderate (40.7°C) and marked (42.7°C) hyperthermia for 2 hr. Leukocyte viability, measured by dye exclusion, was not altered by such exposures. Exposure of the cells to moderate hyperthermia did not alter leukocyte production of interferon- α in response to influenza virus or interferon- γ in response to the mitogen phytohemagglutinin. Exposure of the cells to marked hyperthermia significantly depressed production of interferon- α . In contrast, production of interferon- γ was not altered by exposure of the leukocytes to marked hyperthermia. Many studies support a role for interferons (α as well as γ) in antitumor immunity. The current and other data suggest that marked hyperthermia in cancer therapy should be applied locally whenever possible, rather than to the whole body, in order to limit adverse effects on immunity. The data suggest further that interferon- γ may be a heat shock (stress) protein for human leukocytes. © 1986 Society for Experimental Biology and Medicine.

Many studies suggest a survival value of fever or hyperthermia in the settings of infectious or neoplastic challenge (1). Moderate to high temperature elevations within the physiologic range (38.5–41°C) have been shown to enhance several aspects of human leukocyte response to challenge (1–5). However, temperatures above the physiologic range (42.5°C or higher) are commonly required to produce decreased survival of neoplastic tissue *in vitro* or regression of tumors *in vivo* (6–8). We have recently demonstrated that brief exposures (2 hr) to temperatures exceeding 42.5°C result in decreased mitogen-stimulated DNA and RNA synthesis, and decreased unstimulated and mitogen-stimulated total protein synthesis by human mononuclear leukocytes (9). Others have demonstrated that temperature elevations within or exceeding the physiologic range diminish human natural killer cell function (10, 11). Such effects could be abrogated partially by treatment with interferon (IFN). Since mononuclear leukocytes (such as natural killer

cells) may be present within and surrounding a tumor mass that is subjected to hyperthermic therapy (12, 13) and since such cells may represent a major component of host antitumor defense (14, 15) we examined the effects of a moderate (40.7°C) and a marked (42.7°C) hyperthermia on human mononuclear leukocyte production of interferon (IFN)- α and IFN- γ .

Materials and Methods. *Cell source and collection of blood.* Peripheral venous blood was obtained by venipuncture from 30 healthy young adult donors (14 male, 16 female) who were taking no medication at the time of the study. Mononuclear leukocytes were obtained from the heparinized whole blood by Ficoll-Hypaque sedimentation (16). Mononuclear leukocytes obtained by this method consist of 70 to 80% lymphocytes and 20 to 30% monocytes (17); monocytes produce IFN- α in response to influenza virus, and both cell types are required for production of IFN- γ in response to mitogens and antigens (18, 19). Leukocyte cultures were maintained in medium 199 (GIBCO, Grand Island, NY) with modified Earle's salts with glutamine, aqueous penicillin G (100 units/ml), and streptomycin (50 μ g/ml). The medium was further supplemented with 10% autologous serum. For

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studies of total protein synthesis, cells were cultured in leucine-free medium.

Exposure and sham-exposure to microwave-induced hyperthermia. The mononuclear leukocytes were exposed in a waveguide system to 2450 MHz (continuous wave) microwaves at specific absorption rates of 12 or 22.5 mW/ml for 2 hr. The waveguide system used in these studies has been described in detail (20). Exposure and sham-exposure waveguides are located within a water-jacketed, 37°C CO₂ incubator. Temperature inhomogeneity within the cultures is prevented by continuous shaking of the shelf upon which the waveguides rest. In addition to leukocyte cultures enclosed within waveguides for exposure or sham-exposure, we included control cultures located within the same incubator but external to the waveguides and resting upon a stationary shelf. (Such control cultures allow recognition of any effects on cell cultures due to enclosure in the waveguides plus shaking.) Exposures and sham-exposures were monitored continuously using Vitek Electrothermia nonperturbing probes (Vitek, Inc., Boulder, Co.). Earlier studies indicated that exposures of human mononuclear leukocytes to microwaves without moderate or marked increases in culture temperature do not affect cell viability or IFN production (21).

Specific absorption rates (SARs) were determined by analysis of steady-state temperature increments, ΔT_{ss} (20). The mean culture temperature, $\pm SE$, for sham-exposed cultures was 37.35°C \pm 0.05. Cultures were exposed to microwaves at an SAR of 12 mW/ml to achieve a mean change in steady-state temperature, $\pm SE$, of 2.92°C \pm 0.06, with a mean final culture temperature, $\pm SE$, of 40.65°C \pm 0.08. Cultures were exposed to microwaves at an SAR of 22.5 mW/ml to achieve a mean change in steady-state temperature, $\pm SE$, of 5.33°C \pm 0.05, with a mean final culture temperature, $\pm SE$, of 42.73°C \pm 0.07.

Assays of leukocyte viability. Leukocyte viability was determined, from 1 to 6 days after exposure or sham-exposure to microwave-induced hyperthermia, using both total cell counts and assays for percentage of cells able to exclude trypan blue dye or ethidium bromide (21, 22). In the assays for ethidium bromide exclusion, cells were counterstained su-

pravitally with acridine orange (23, 24). There were no significant differences in viability between marked hyperthermia-exposed, moderate hyperthermia-exposed, sham-exposed, and control leukocytes (data not shown).

Assays of interferon production. The interferon activity in supernatant culture fluids of unstimulated and stimulated mononuclear leukocytes was assayed by the inhibition of plaque formation by vesicular stomatitis virus in human foreskin fibroblast cultures, as previously described (18, 25). Cells were cultured immediately after exposure or sham-exposure to hyperthermia, and culture fluids were collected 1, 3, and 5 days later. Production of IFN- α was induced by exposure of the leukocytes to influenza A/AA/Marton/43 H1N1 at a multiplicity of infection of 10. Production of IFN- γ was induced by exposure of the leukocytes to the mitogen phytohemagglutinin (PHA)-M (Difco, Detroit, Mich.) at a concentration of 160 μ g/ml, shown to be optimal in preliminary studies. The characteristics of the induced interferons (IFN- α versus IFN- γ) were confirmed in earlier studies using standard techniques (pH, heat, antisera neutralization susceptibility, etc.) (18, 25). The minimum IFN titer detected in these studies was 5 units/ml. Results are presented in mean log₂ units/ml \pm SE.

Assays of total protein synthesis. Unstimulated and mitogen-stimulated total protein synthesis by the mononuclear leukocytes was assayed by cellular incorporation of the tritiated precursor leucine, using standard methods (17, 21, 26). Mononuclear leukocytes were added to quadruplicate wells of sterile microtiter plates (Costar, Cambridge, Mass.) at a concentration of 5 \times 10⁵ cells/ml (1 \times 10⁵ cells/well). To the cell cultures were added medium alone or medium containing phytohemagglutinin (PHA)-M at an optimal concentration of 160 μ g/ml, shown to yield maximum lymphocyte responses with control mononuclear leukocytes (17, 27). Microtiter plates were then incubated at 37°C in 5% CO₂ and air for 3 days. Cultures were pulsed with the tritiated precursor for the terminal 5 hr of incubation and harvested with a semiautomatic cell harvester (Brandel, Inc., Gaithersburg, Md.). Samples were counted with a liquid scintillation counter. For each individual

experiment, arithmetic mean counts per minute (cpm) of quadruplicate cultures were determined.

Statistical analyses. Student's *t* test was used for analysis of the data. Sham-exposed and control mononuclear leukocytes did not differ in responses. Such observations, for each assay, are not detailed under Results.

Results. Human mononuclear leukocytes were exposed or sham-exposed to moderate (40.7°C) or marked (42.7°C) hyperthermia, and examined for unstimulated and stimulated IFN production. There was no detectable spontaneous production of IFN in any of the cultures from 1 to 5 days after exposure or sham-exposure to hyperthermia.

Virtually all detectable influenza virus-induced IFN- α was present by one day after induction, as expected (18). Equivalent amounts were produced by moderate hyperthermia-exposed and by sham-exposed leukocytes, whether measured 1 day (Fig. 1A), 3 days, or 5 days after induction. Mitogen (PHA)-induced IFN- γ , usually produced by 2–3 days (18, 19), was not detected in any culture supernatant fluid by 1 day after stimulation. By 3 days, IFN- γ was detected in all PHA-induced cultures, with no significant differences between the moderate hyperthermia-exposed and sham-exposed leukocytes (Fig. 1B). Sim-

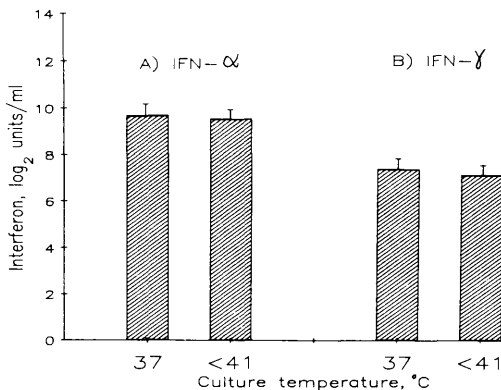


FIG. 1. Influenza virus-induced interferon (IFN)- α (A) and PHA-induced IFN- γ (B) production after exposure or sham-exposure of autologous human mononuclear leukocytes to moderate (40.7°C) hyperthermia for 2 hr. Columns represent mean titers, +SE, from nine experiments measuring IFN- α produced by 1 day, and five experiments measuring IFN- γ produced by 3 days.

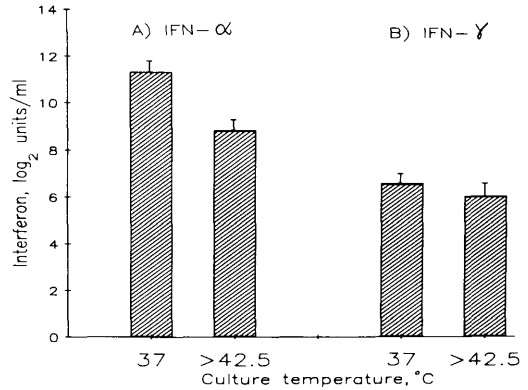


FIG. 2. Influenza virus-induced interferon (IFN)- α (A) and PHA-induced IFN- γ (B) production after exposure or sham-exposure of autologous human mononuclear leukocytes to marked (42.7°C) hyperthermia for 2 hr. Columns represent mean titers, +SE, from nine experiments measuring IFN- α produced by 1 day, and seven experiments measuring IFN- γ produced by 3 days.

ilar titers of IFN were present in culture supernatant fluids 5 days after stimulation.

In contrast, exposure of the leukocytes to marked hyperthermia resulted in a significant reduction in IFN- α production relative to production by sham-exposed leukocytes. IFN- α was again present by 1 day after induction, but leukocytes exposed to marked hyperthermia consistently (9 of 9 experiments) produced less IFN than did sham-exposed leukocytes, whether measured 1 day (Fig. 2A; $P < 0.001$), 3 days ($P < 0.001$), or 5 days ($P < 0.02$) after induction. While the reduction in titers produced by marked hyperthermia-exposed leukocytes was approximately sixfold at 1 day, the difference in titers was reduced to approximately 3.5-fold by 5 days.

IFN- γ again was not produced spontaneously, or by 1 day after mitogen stimulation. In contrast to the depression of IFN- α production caused by exposure to marked hyperthermia, mitogen-induced IFN- γ production (by 3 days) was not reduced by exposure of the leukocytes to the marked hyperthermia (Fig. 2B). Although cells exposed to marked hyperthermia produced lower titers than did sham-exposed cells in five of seven experiments, the differences were minimal, and not statistically significant ($P > 0.2$).

Because IFN- γ synthesis (specifically) was

not reduced by exposure to marked hyperthermia, unstimulated and mitogen-stimulated total protein synthesis was examined 3 days after exposure in order to confirm the expected concomitant depression of total protein synthesis resulting from exposure to marked hyperthermia. Exposure to moderate hyperthermia did not affect unstimulated total protein synthesis (Fig. 3A), and tended to enhance mitogen-stimulated total protein synthesis (Fig. 3B), although differences were not significant. In contrast, exposure to marked hyperthermia resulted in depressed total protein synthesis, both unstimulated (Fig. 4A; $P < 0.02$) and in response to mitogen stimulation (Fig. 4B; $P < 0.001$).

Discussion. Hyperthermia is currently being used in the therapy of neoplasms in view of the heat susceptibility of many neoplastic tissues, as well as the ability of hyperthermia to enhance the efficacy of chemotherapy and radiation therapy (1, 7, 9, 28–30). Since mononuclear leukocytes may be present within and surrounding a heated tumor, we examined the effects of brief exposure (2 hr) of mononuclear leukocytes to moderate (40.7°C) and marked (42.7°C) hyperthermia. The degree of moderate hyperthermia was chosen to represent the upper limits of normal human physiolog-

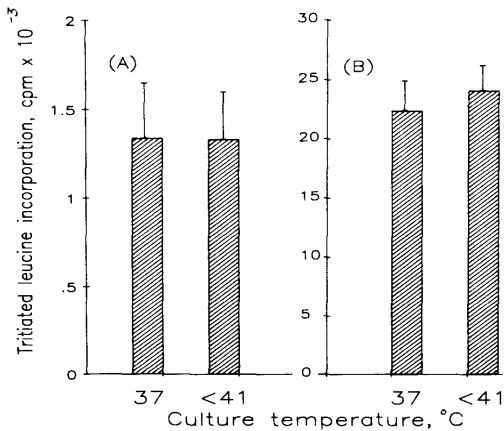


FIG. 3. Unstimulated (A) and mitogen-stimulated (B) total protein synthesis after exposure or sham-exposure of autologous human mononuclear leukocytes to moderate (40.7°C) hyperthermia for 2 hr. Columns represent mean responses after 3 days in culture, +SE, from four experiments.

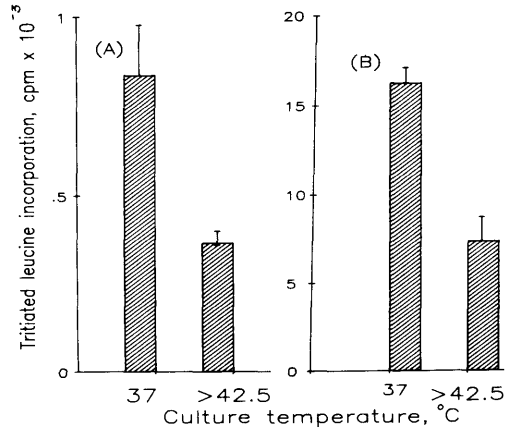


FIG. 4. Unstimulated (A) and mitogen-stimulated (B) total protein synthesis after exposure or sham-exposure of autologous human mononuclear leukocytes to marked (42.7°C) hyperthermia for 2 hr. Columns represent mean responses after 3 days in culture, +SE, from five experiments.

ical (febrile) temperature elevations, while the degree of marked hyperthermia was chosen to represent the lower limits of supraphysiological temperature elevations believed necessary to exhibit direct antitumor effects. Examination of the effects of hyperthermia on interferon production appeared to be warranted in view of two related facts. First, there is extensive, although not definitive, evidence that the interferon system can play a substantial role in antitumor immunity; and second, interferons and interferon inducers have shown sufficient promise to be used in numerous tumor therapy clinical trials (31–35). The current data suggest that brief exposure to moderate hyperthermia does not affect human mononuclear leukocyte production of IFN- α and IFN- γ , but exposure to marked hyperthermia significantly alters such leukocyte responses. Production of IFN- α was significantly reduced following exposure to marked hyperthermia. In contrast, production of IFN- γ was not altered by exposure to the marked hyperthermia.

Human natural killer cells may be quite sensitive to hyperthermia, with activity substantially abolished by exposure to 42°C for 1 hr (11). In fact, human natural killer cell activity may be significantly diminished when measured at 40°C and compared to activity

at 37°C, although the effect is partially abrogated by treatment of the heated cells with human leukocyte interferon (IFN- α) (10). The current studies suggest that IFN- α and IFN- γ production are not altered by exposures to 40.7°C hyperthermia for as long as 2 hr. Thus, diminished natural killer cell activity at 40°C is apparently not due to altered IFN production. The current studies do not exclude the possibility that decreased IFN- α production by leukocytes exposed to 42.7°C hyperthermia (with a reduction in total IFN titers) may contribute to, or prevent abrogation of, effects of marked hyperthermia on natural killer cell activity.

In the current studies, mitogen-induced IFN- γ production was not reduced by exposure of the leukocytes to marked (42.7°) hyperthermia despite depression of both unstimulated and mitogen-stimulated total protein synthesis. Both the degree and duration of exposure to hyperthermia were similar to those which have been used to induce synthesis of so-called heat shock (or stress) proteins in studies using a wide variety of cells, including human cell lines. Synthesis of the proteins is induced by heat shock, or alternately the proteins continue to be synthesized to a degree at least equivalent to that by control cells; in contrast, synthesis of other proteins by the cells is markedly depressed (38–40). IFN- γ has such a high specific activity that its production would not readily be demonstrated by the standard methods (radiolabeled precursor incorporation and gel electrophoresis) for demonstrating synthesis of heat shock proteins. The current studies raise the possibility that IFN- γ should be considered as a heat shock protein for human mononuclear leukocytes insofar as its production was maintained despite the exposure to marked hyperthermia, and despite the marked reduction (by such exposure) of unstimulated and stimulated total protein synthesis [(9) and current data], including synthesis of IFN- α (current data). Supporting such a concept is a recent preliminary report (M. W. Taylor *et al.*, Abstracts of the Annual Meeting, American Society for Microbiology, 1984) that heat shock (42°C) applied to a human lymphoblastoid cell line induced production of IFN- γ . The cell line spontaneously produced large amounts of

IFN- α ; after the heat shock, the cells produced even greater amounts of IFN, which was shown to be IFN- γ by standard methods.

Several investigators have reported that effective heating of a tumor at one site can lead to its regression at other anatomical sites (30). In contrast, in animal studies but not yet proved in man, whole-body heating can cause immunosuppression; enhanced metastatic spread may follow in the tumor-bearing host (30). The current studies, combined with our earlier observations (9) as well as those by others, suggest that marked hyperthermia in cancer therapy should be applied locally whenever possible, rather than to the whole body, in order to limit potential adverse effects on overall host immunocompetence.

The frequent use of hyperthermia in tumor therapy, the evidence of *in situ* immunity in tumors, and the effects of hyperthermia on human leukocyte functions such as are detailed in the current and other studies, all suggest that potential hyperthermia-induced alterations of human leukocyte function warrant further investigation.

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