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*Review*

# Hyperthermia and Immunity. A Brief Overview

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**Abstract.** *After many years, hyperthermia (HT) is experiencing a new resurgence as seen by the positive results of many randomized trials all over the world. Tumour immunity similarly is suggested as the fourth modality of therapy for metastatic tumours from renal carcinoma and melanoma. An overwhelming amount of data from animal models and human patients indicate that whole body and locoregional hyperthermia exerts many biological and therapeutic effects on immune competent cells and cytokines. Among these effects, hyperthermia has recently been demonstrated to enhance the antigen presentation and consequently the activity of dendritic cells. This improvement is obtained through several mechanisms: a) increased lymphocyte recruitment and trafficking into the tumour area; b) increased immunogenicity of heat treated tumour cells; and c) increased production of the heat-shock proteins and costimulatory molecules. The effects and mechanisms of HT on immunity, lymphocyte recruitment and dendritic cell stimulation by heat shock proteins are reviewed here. Moreover the use of HT as an innate immunity booster in association with biological response modifiers is suggested.*

Tumour regression *in vivo* is mediated by a complex interplay between the innate immune system and adaptive immune response (1). The innate mechanism may trigger inflammatory events in the tumour microenvironment and in the presence of locally adequate cytokine combinations stimulate dendritic cells (DCs), the most specialized antigen presenting cells, to react against tumour specific surface antigens (TAA) (2). DCs are potent antigen presenting cells that exist in virtually every tissue, they capture antigens and migrate to secondary lymphoid organs where they activate naïve T-cells. DCs in the presence of an adequate co-stimulation have the unique ability to activate the naïve CD4+ and tumour specific cytotoxic lymphocytes CD8+ cells (CTLs) and sustain the primary immune response. DCs have been identified in different organs as essential components of the innate and adaptive immune systems. They have the ability to generate CTLs that recognize and kill virally infected or transformed cells. Tumour destruction by CTLs generally occurs in an antigen-specific, major histocompatibility complex (MHC)-restricted fashion. On the contrary CD3-natural killers (NKs) which are part of the innate immunity against tumours, are cells morphologically and functionally distinct from CD8+ and inhibit tumour growth in an MHC-non restricted manner. The killing activity of NK-cells is tightly regulated by a balance of signals integrated from inhibitory and activating receptors expressed on their surfaces (1, 3). NKG2D is one of these surface receptors and has been recognized as a primary cytotoxicity receptor and of pivotal importance for priming a Th1 antitumour T-cell response. NKG2D has been recognized also to be an ancestral defence system and part of the innate immunity reaction against tumours. Furthermore, NK-cells and dendritic cells interact with each other in the coordination of innate and adaptive immune responses as demonstrated by Walzer *et al.* (4). Epidemiological studies and sporadic observations have described spontaneous regression of cancer associated with the induction of fever, and of cellular immunity (5, 6). The crucial importance of fever in these regressions justifies the attempt to induce artificial thermal elevation of body temperature (whole body hyperthermia) for mimicking natural fever effects on cancer (7).

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## Effects of Fever and Hyperthermia on Immune Cells and on Cytokines

*Fever.* In presence of a tumour or invading microorganisms the host occasionally responds by increasing body temperature. Fever is a complex neuroendocrine adaptive

response able to reset the temperature regulator area found in the hypothalamic area (8). After their entry into an organism, bacteria or viruses induce the host cells (primarily macrophages) to produce a series of proinflammatory cytokines such as: interleukins (ILs) -1, 2, 6, tumour necrosis factor alpha (TNF-α), Interferon-α (IFN-α) and IFN-γ (9). These cytokines, with an additional mediator [prostaglandin $E_2$ (PG $E_2$) a cyclooxygenase 2 prostaglandin derivative (COX-2)], act on the thermoregulatory area and reset it to a higher level of temperature, producing the febrile response (9). Temperature elevation has been suggested to be beneficial for the host. In fact, several reports have demonstrated that a few degrees in temperature increase may improve the efficiency of macrophages in killing invading bacteria and in impairing their replication (10).

Herpes virus or rabies infected mice, have a survival benefit, when kept at an average environmental temperature of 38˚C compared to mice kept at a lower environmental temperature (11). Leukocyte adhesion to the endothelium and migration to the site of inflammation are positively affected by heat as are antigen-specific activation, proliferation, differentiation, cytokine expression and antibody secretion by lymphocytes. T-cell responsiveness to mitogens, IL-1, IL-2 and antigens increases linearly until a temperature of 39˚C, beyond this limit a decline is observed (11, 13). Recently, it has been shown *in vitro* that antibody secretion is temperature dependent, but this effect declines in the absence of T-helper cells (12, 13). This means that the two populations are behaving differently in the presence of fever, in fact further elevating the temperature there is impairment in B-cell response with decreased survival of immunoglobulins. In conclusion, the observation that emerges is that temperature in the order of fever range (39-40˚C) is the most favourable for the immune response. In fact many cell types such as NKs, neutrophils, macrophages increase their number and activity in this range of temperature but show a decline when exposed to temperatures beyond ≥40˚C (14).

## Classic Effects of Hyperthermia on Cell-mediated and Humoral Immunity

Depressive effects of hyperthermia (HT) on cell-mediated and humoral immune functions have been reported since 1970. A summary of the *in vitro* and *in vivo* effects of locoregional (LHT) and whole body hyperthermia (WBHT) in more recent studies are illustrated here (7).

*In vitro effects of HT.* NK and lymphokine-activated killer (LAK)-cell's cytotoxicity and viability have been analyzed by many authors *in vitro* and *in vivo*. In some cases their function has been determined in association with IL-2, TNF-α or interferon, to verify the possible abrogation, by these cytokines, of the immune suppressive effects of temperature and their cell rescue activity (15). Fuggetta *et al.* (16) have evaluated *in vitro* the influence of HT (1 h 42˚C) on the cytotoxicity of IL-2 activated NK-cells. HT profoundly reduced the lytic activity of NK-cells. The inhibition of this lytic activity has been demonstrated to be transient and not due to an apoptosis-induced reduction of the effector cells (16). Furthermore these authors have observed that the heat treatment of target cells alone (K 562 and Daudi cells) did not alter their sensitivity to lysis. This in agreement with other authors (17).

Shen *et al*., have demonstrated that LAK cell's cytotoxicity was temperature dependent. It was enhanced in the presence of febrile temperature(≤40˚C), but decreased to exposure for 1 h at 42˚C (17). Investigations by Singh *et al.*, have also shown that LAK-cell cytotoxicity, as well as NK-cell cytotoxicity decreased at a temperature above 38.5˚C. TNF mediated cytotoxicity however was significantly enhanced at 40˚C (18). In contrast, Fritz *et al.* have obtained a certain variability in LAK-cell mediated lysis in the presence of HT and INF-γ (19).

From the various studies analysed it appears that the immune suppressive effects of HT on NK- and LAK-cells become evident at temperatures above 39˚C. The response of NK- and LAK-cells to HT is temperature and dose dependent and not related to cell viability or the absolute number of cells (15, 16, 20, 21). In fact, several authors have noted little loss of cell viability at 39˚C (22, 23), whereas a decrease was demonstrated at temperatures above 42˚C (23). The lytic function is more heat sensitive than the recognition and binding functions. The extent of recovery of activity after exposure to HT is inversely correlated with the temperature, as well as the recovery time and can be complete (16, 23, 24). Human and animal cells show different behaviour during HT exposure. Human and animal NK-cells show similar inhibitory behaviour after HT and among the immune competent cells seem the most sensitive. On the contrary B cells are less affected by heat whereas murine lymphocytes compared to the human counterpart appear to be more heat sensitive (24, 25).

However, the majority of *in vitro* studies are not pertinent to the clinical situation. In fact different parameters are not comparable such as: a) treatment time that is too long (3-4 h; 18 h) or too short (1/2 h), b) the different lytic effector-to-target (E:T) ratios, and c) the pH of the medium. This last factor can condition the lymphocyte reaction. Within the tumour mass lymphocytes, *in situ*, are subjected to a stressful hypoxic and acidic environment that can modify their response to mitogen and cytokine production. Studies by Skeen *et al.* have reproduced this tumour microenvironment and have demonstrated that the pH of the medium (obtained by adding lactic acid) had no effect at 37˚C but showed a synergistic impairment with heat at 41, 42 and 43˚C (24). Future studies with common and more defined experimental conditions are warranted.

*In vivo studies of HT.* Kappel *et al.* (26) have verified the behaviour of NKs and other immune competent cells of healthy volunteers during whole body hyperthermia at 39.5˚C for 2 h. NK-cell cytotoxicity increased according to temperature. Cells incubated with IL-2 or INF-α showed enhancement of their cytotoxicity as well as of their number compared with control values. At temperatures above 39.5˚C a slow decrease in the number of CD3+ was evident with no variations regarding CD19+ cells (B-cells) (26).

Recently, Atanackovic *et al.* have examined during WBHT the following immunological parameters: a) the behaviour of CTLs with a cluster of differentiation (CD) activation markers; b) the serum cytokines; c) the intracellular cytokine levels; and d) the capacity of these cells to proliferate (27). They have divided the effects of WBHT on patient immunity into different phases arbitrarily defined by post-treatment time. Immediately after WBHT a drastic increase in the peripheral blood of NK-cells and CD56 CTLs was noted. This phenomenon was transient and followed after 3 h post WBHT by a short period of reduced T-cell activity indicated by diminished serum levels of soluble interleukin 2 receptors (sIL-2R). In this first phase (the first 5 h following treatment) a short-lived increase in the serum concentration levels of IL-6 was observed. These levels returned to normal after 24 h. TNF-α increased significantly during the first 24 h, associated with a marked increase in the peripheral percentage of CTLs and Cd56. CTLs, CD56, sIL-2R and lymphocytes expressing CD 69 markers reached their maximal concentration 48 h post WBHT. CD69 identifies an antigen expressed early in the activation of lymphoid cells, and is considered to be restricted to activated lymphocytes and undetectable in resting lymphocytes. CD 69 is normally induced after stimulation with mitogens or cytokines like INF-α, INF-γ or TNF-α. As confirmation, the intracellular concentrations in CD8 cells and in serum of INF-γ and TNF-α were found to be elevated 24 h post WBHT in 80% of the patients.

*Effects on cytokines.* During localized hyperthermia no detectable increase in IL-1, IL-6 or TNF-α was found, whereas after WBHT serum levels of IL-1 and IL-6 were increased (28). Robins *et al*., in an attempt to understand why during WBHT (41.8˚C) there is enhancement of radiation or chemotherapy which is not followed by a concomitant increase in myelosuppression, studied an expanded panel of serum cytokines. They reported in different patients an increase of IL-$1_{\beta}$, IL-6, IL-8, IL-10, G-CSF and TNF-α within hours after WBHT (29, 30). Moreover, they reported that bone marrow cells stimulate production of IL-$1_{\beta}$, IL-6 and TNF-α further increasing their plasma levels. This interaction between tumour cells and cytokines, such as interleukin (IL)-6 resulted in a secondary induction in the bone marrow (BM) by IL-3 and GM-CSF. These factors are produced by BM stroma and by T-lymphocytes in the BM. The plasma levels of the cytokine panel increased 1 h following WBHT and diminished after every WBHT application reaching the minimum concentration after 4 cycles (30). Alonso reported in patients undergoing extracorporeal perfusion, a similar cytokine increase with the addition of IL-2, TGF-β, INF-γ and INF-α (31).

The use of IL-2, TNF-α, INF-α and GMCSF as biological response modifiers (BRMs) has been disappointing. This has induced many researchers to use IL-2 and TNF-α with WBHT and LHT. Generally, an additive effect has been demonstrated without an increase in toxicity. The majority of studies have been conducted on animals using IL-2 and TNF-α (32-34). Human studies have been conducted primarily with WBHT and perfusional hyperthermia associated with TNF-α (35, 36).

IL-2 administered before LHT has been demonstrated to be additive and useful for treating mice with lung metastases from melanoma and sarcoma The response was obtained using IL-2 simultaneously with WBHT application (33, 34). The doses and toxicity were lower than those usually reported. Fritz *et al.* have demonstrated that the potentiation of IL-2 combined with HT is mediated by TNF-α induction. In fact, the effect of IL-2 was abrogated by anti-TNF-α antibodies (34).

WBHT or LHT combined with low-dose IL-2 was more effective on reducing tumour growth than either modality alone, and the response was more evident for macroscopic tumours than for microscopic ones. Geehan *et al.* suggested that this phenomena should be ascribed to a selectively increase in permeability in tumour vessels, and to an augmented expression of intercellular adhesion molecule-1 (ICAM-1), followed by an increased homing into tumour tissue of LAK-cells (33).

As reviewed by Klostergaard and Tomasovic (37) studies *in vitro* and *in vivo* on human and animal tumours indicate a sensitization to TNF-α in the presence of HT. Sensitization was greater when tumour targets were treated with TNF-α prior to heat treatment and the effect *in vivo* could be reached with lower dosage and with less toxicity. It appears that the effect of TNF-α *in vivo* is partially due to an increase on plasma membrane receptor expression or affinity and on tumour vasculature. Klostergaard and Tomasovic (37) reported similar effect using INF-α and INF-β, both *in vitro* and *in vivo*. The maximum effect was observed with intratumoural administration and the time of administration with HT was less important. A combination of TNF and INF is possible with additive effect and dosage reduction. As for TNF-α the antiproliferative effect seems to be ascribed to a direct effect on plasma membrane receptor expression or affinity.

Recent studies are more oriented to the use of TNF-α combined with chemotherapy and WBHT or with limb or

organ isolated perfusional HT. As reported by many authors, a synergism between hyperthermia, melphalan (L-PAM) and TNF-α in the clinical setting of limb perfusion for malignant melanoma and sarcoma has been demonstrated. A TNF-α concentration superior to that achieved by bolus administration (10-20 μg/ml) could be given locally (1-2 μg/ml) and was associated only with mild toxicity (grade 1or 2) in the 25% of patients treated (38). A similar combination regimen for therapeutic treatment of unresectable liver malignancies (confined to the liver) by using isolated hepatic perfusion (IHP) has been studied by Alexander *et al*. According to critical evaluation these authors suggested that IHP, L-PAM and TNF-α is the best combination regimen for obtaining a good response rate as compared to other chemotherapeutic regimens using drugs such as FUDR (39).

## Special Effects of Hyperthermia

*Leukocyte recruitment and adhesion.* To obtain tumour regression circulating leukocytes must reach the tumour area (40). The initial step in this process involves recruitment of the leukocyte to the endothelial surface through the association between adhesion molecules, which are found on the surface of both the circulating leukocytes and the endothelium. Generally immune cells are frequently excluded from the intratumoral region due to a decreased expression of ICAM-1 and $\alpha_4\beta_7$ integrins (41-43). Local and WBHT enhance the expression of L-selectin lymphocyte-endothelial cell adhesion, and consequently the leukocyte recruitment to the tumour area (44, 45). Moreover it appears that hyperthermia differs compared to other therapies regarding the increase of adhesion molecules. In fact the increase has been detected only on tumour microvasculature and in peritumour lymphatics not in normal vasculature (45). The mechanism underlying HT control of L-selectin have revealed that febrile temperature does not increase lymphocyte L-selectin surface density or L-selectin dependent recognition of soluble carbohydrates but the avidity of pre-existing adhesion molecules for physiological ligands (44, 45). This up-regulation of the adhesion process endorses the use of LHT and WBHT in the clinical setting for selectively delivering cytotoxic T-cells or gene armed lymphocytes only into the tumour area.

*Hyperthermia, heat stressed cells (danger signal) and antigens presentation.* Recently, due to the work of Matzinger (46), the archetypal view that the immune system exists primarily to distinguish "self" from "non-self" has been replaced by the paradigm that the immune system functions primarily to distinguish dangerous from non-dangerous antigens. Antigen presentation, *in primis* to dendritic cells, provides one of the key-points in the antitumour immune response. The failure to recognize tumour cells "as non-self" or better as "dangerous" determines tumour tolerance and metastatization. For "dangerous cells" every cell submitted to any kind of stress or physical therapy; such as hyperthermia (47), radiotherapy (48) or photodynamic therapy (PDT) (49) must be included. Furthermore, the relationship between tumour cell death, consequent to cancer therapy, and the efficiency of induction of the immune response have been examined both *in vitro* and *in vivo*. Although some reports show that apoptotic cells are more effective than necrotic tumour cells in inducing the immune response others indicate that modes of cancer therapy (*i.e.,* heat, PDT) that predominantly induce necrosis, are the better way for activating the antitumour immune response. Data by Feng *et al.* have demonstrated that heat stressed apoptotic 12B1-D1 cells compared to non-heat stressed cells were more effective in stimulating dendritic cells to secrete interleukin-12 (IL-12) and in enhancing their immunostimulatory functions in mixed leukocyte reactions (50).

In conclusion DCs are able to distinguish between stressed and non-stressed cells undergoing programmed cell death. A tumor tissue that undergoes a stress response (*i.e.,* heat) and goes into apoptosis increases the synthesis of stress proteins on its surface, or releases products during tissue damage that are recognized by tumour-infiltrating lymphocytes as not-self. This may generate potent antitumour T-cell responses.

In contrast, non-stressed apoptotic tumour cells are recognized by the immune system as a physiological process, critical to normal development and able to elicit only a non-inflammatory / or even bland danger signal.

*Heat shock proteins, toll like receptors (TLRs) anticancer innate immunity and vaccination.* When cells are submitted to a variety of stressful events (*e.g.,* heat, hypoxia, glucose deprivation), there is a rapid and coordinated increase in the expression of a group of proteins, the so-called heat shock proteins (HSPs) (51). HSPs are highly conserved constituents of all types of prokaryotic and eukaryotic cells and are classified into several families according to their molecular weight in kilodaltons (*e.g.,* HSPs 100, 90, 70, 60, 40), their induction by a stressful environment and their compartmentalization inside the cell (cytosol or endoplasmatic reticulum) (52, 53). At a molecular level, heat stress increases linearly the synthesis of HSP 70 until a certain threshold temperature that varies according to cell type. Beyond this threshold temperature their synthesis is inhibited and an exponential cell death follows (54). Initially, the role of HSPs, peculiarly of HSP70, appeared to be implicated in thermotolerance (55), recently, they have been recognized to activate the immune system becoming specialized carriers of antigenic peptides *in vivo,* as well as *in vitro* (55-57). In fact, Srivastava (56) has established that

HSPs are not immunogenic *per se*, but when they are complexed with antigenic peptides become powerful immunogens. Once HSP-complexes (MCH1/MCH2+HSPs) are exposed at the outer surface of cancer cells, they interact with macrophages and dendritic cells through specific surface receptors (57, 58). HSP70 binds to the surface of monocytes through the CD14 receptor, whereas gp96 binds with the α-2 macroglobulin/LDL receptor related protein or CD91. Furthermore HSP60 has been demonstrated to be a ligand for the toll-like receptor (TLR4 and TLR2) complex on macrophages (57). These data show that antigen presenting cells (APCs; macrophages, dendritic cells) have evolved receptors for detecting danger signals (HSPs complexes) released during neoplasia. The exposure of the HSP chaperoned peptide MCH1 and MCH2 molecules to macrophages or dendritic cells triggers a secretion of inflammatory cytokines and costimulatory molecules, such as: IL-6, IL-12; TNF-α, B7 that induce the maturation of DCs towards the T helper-1 ($T_H$ 1) phenotype (58, 59). Their association with a broad array of peptides generated within cells make HSPs a good candidate for cancer vaccines. In fact HSP-peptide complexes isolated from a patient's tumour can be utilized as tailored patient specific antigens, which would avoid the search for specific epitopes. HSPs isolated from cancer cells, but not those derived from normal cells can generate an immune response, as observed by Tamura *et al.* (60) and Srivastava (61). The potentiality of HSPs in tumour eradication has been validated in more than ten types of tumour model of different histologies and in different animal species. From these experiments it also emerges, that only necrotic cells or heat stressed cells are able to elicit a tumour-specific immunity (61). This means that the uptake of stress proteins by DCs might constitute a "danger" signal that induces the expression of monokines in the earliest phases of the innate immune response. DCs in addition to the generation of cytotoxic lymphocytes can activate the innate army of the immune system as well. As argued by Basu and Srivastava (62) HSPs and dendritic cells are to be considered the fountainhead of innate and adaptive immune responses.

## Conclusion

Nowadays, it has been clarified that immunity to tumours is composed of innate immunity and acquired immunity, and that dendritic cells (DC) and macrophages, both of which are the participants in the innate immunity, play a critical role in acquired immune responses, *via* their expression of several costimulatory molecules and the production of cytokines (63). Furthermore we suggest that HT is the simplest way for generating the innate arm of immunity and the positive association with IL2 and interferons may represent the most complete method for stimulating an appropriate and tailored antitumour immunity. However, the conditions and the underlying mechanisms of this immune stimulatory effect still remain poorly defined. New clinical non randomized studies are warranted. As suggested by Srivastava (61) the mode of dying by necrosis must be controlled in order to obtain the immunization *in situ*. Following different therapies, the enhancement of immunogenicity *in situ* may ensue; this stimulation is, however, not sufficient to elicit an adequate immune response able to destroy the tumour mass. The failure can partially be ascribed to the insufficient presence of immune stimulants (such as IL-2 or other BRMs), and to the presence of an immunosuppressive moiety inside the tumour mass. Now it is becoming clear that different subpopulations of lymphocytes (T reg cells CD4+CD25, myeloid cells, indoleamine 2, 3-dioxygenase (IDO) cells) are responsible of this immunosuppression (64-66). It, thus, becomes mandatory to use BRMs, such as IL-2, granulocyte-colony stimulating factor or interferon associated with other treatment for controlling these lymphocyte subpopulations. The BRMs aforementioned have been demonstrated *in vivo* and in animal models to be addictive with HT, but not fully active alone (67-69). A study by Prasad *et al.* (70) is a confirmation of this hypothesis. In fact, in mice depleted of CD4+CD25+ regulatory cells, vaccinated with B16-F10 melanoma cells stressed by heat shock, an effective antitumour immunity has been elicited.

Furthermore, new insights into the details of heat-shock proteins – TLR regulation will provide important clues to how immune responses are regulated by hyperthermia. Some HSPs – TLR interactions are immune enhancing, others are immunosuppressive as reviewed by Wang (71). Another aspect to elucidate is how dead cells become immunogenic, are the fragments of membranes more immunogenic than the cytoplasmatic or nuclear substances released after death? Some of these aspects have been reviewed by Proskuryakov *et al.* (72). It is also important to characterize the HSPs receptors on the various subpopulations of lymphocytes and to verify the immunostimulatory effects of HSPs *in vitro* with preparations with less impurities such as lipopolyssaccharide (LPS) (73).

Concluding, we suggest that a correct in terms of time and intensity HT regimen (control of immunosuppressive moiety + BRMs) can be an appropriate method for eliciting an efficient innate/adaptive anticancer immunity.

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