FEVER AND IMMUNOREGULATION

III. Hyperthermia Augments the Primary In Vitro Humoral Immune Response*

By HENRY D. JAMPEL, GORDON W. DUFF, RICHARD K. GERSHON, ELISHA ATKINS, and SCOTT K. DURUM

From the Departments of Pathology and Internal Medicine, and the Howard Hughes Medical Institute at Yale University School of Medicine, New Haven, Connecticut 06510

Fever has been recognized as a sign of disease since ancient times, but as yet, no clear understanding of the role of fever in host defense, if indeed it has one, has emerged (1). Although the growth of certain bacterial pathogens (e.g., *Streptococcus pneumoniae* and *Neisseria gonorrhea*) is impaired at febrile temperatures, it is by no means certain that this is the major selective advantage of the febrile response since many pathogenic organisms replicate even more rapidly at hyperthermic temperatures. A few studies have suggested possible benefits of hyperthermia to host defense mechanisms, e.g., improved in vitro proliferation of human lymphocytes in response to lectins at elevated temperatures (2–5). Whereas little is known about the survival value of fever, great strides have been made in understanding the mechanism of fever production (6). Fever, in response to infection, is thought to result from the release of a soluble product of macrophages known as endogenous pyrogen (EP) (1).[1] Circulating EP then acts on the thermoregulatory centers of the brain, producing a controlled elevation of body temperature (7–9). During infection, the release of EP from monocytes or macrophages can be stimulated by a direct action of microbial products, such as endotoxin (10, 11) or indirectly, in response to soluble products released from activated lymphocytes that are themselves responding to the invading organisms (12). Thus fever can be generated during the course of an immune response. Furthermore, it has recently been reported that EP is probably identical to interleukin 1 (IL-1), a macrophage-derived mediator that powerfully potentiates immune responses in vitro (13, 14). These findings led us to consider the relationship between fever and the immune response. We first postulated that the hyperthermic action of EP/IL 1 in vivo might affect its immune-potentiating activity and therefore tested the temperature dependence of IL-1-induced T cell proliferation. The action of IL-1 as a co-factor in murine T cell mitogenesis in vitro was indeed greatly increased at febrile temperatures (15).

We now examine whether the augmentation of T cell proliferation that we observed previously has any functional consequences in the primary in vitro humoral immune

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[1] *Abbreviations used in this paper:* EP, endogenous pyrogen; IL-1, interleukin 1; PFC, plaque-forming cells; SRBC, sheep erythrocytes; T_h, helper T cells; T_s, suppressor T cells; T_sF, T suppressor factor.

response and conclude that hyperthermia characteristic of fever can dramatically increase this response as well. This temperature dependence of the humoral immune response could therefore contribute to the selective value of fever, a complex physiological response that is phylogenetically both ancient (appearing in teleosts) and enduring.

Materials and Methods

Mice. C57BL/6J and BALB/cJ males, 8–12 wk of age, were purchased from The Jackson Laboratory, Bar Harbor, ME.

Cell Fractionation. Splenic T cells were isolated by the method of Wysocki and Sato (16). Briefly, goat anti-mouse Ig serum was affinity purified on mouse Ig Sepharose (Pharmacia Fine Chemicals, Uppsala) columns, then used to coat plastic petri dishes (8-757-12; Fisher Scientific Co., Pittsburg, PA). Spleen cells (3×10^7 in 3 ml) were incubated in the dishes for 70 min (agitated at the halfway point) at 5°C, then decanted. The nonadherent fraction contained <3% Ig-positive cells by fluorescence staining.

Ly-1 T cells were produced by treating T cells with monoclonal anti-Lyt-2.2 in ascitic fluid (provided by F. W. Shen, Memorial Sloan-Kettering Cancer Center, New York), diluted 1:1,000, for 30 min at room temperature. Cells were washed and then treated with complement, a selected batch of rabbit serum (Dutchland Laboratories Inc., Denver, PA) that had been diluted 1:5 and absorbed with Special Agar-Noble (Difco Laboratories, Detroit, MI), then used to treat cells for 40 min at 37°C, followed by washing. Whole spleen cells were similarly depleted of Lyt-2.2-positive cells by treatment with antibody and complement. B cells were prepared by treating spleen cells with monoclonal anti-Thy-1.2 in ascitic fluid (provided by F. W. Shen), diluted 1:1000, for 30 min at room temperature, washed, then treated with complement.

Medium. RPMI 1640 (Grand Island Biological Company, Grand Island, NY) was supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mM glutamine, 1 mM Hepes, 50 μM 2-mercaptoethanol (Eastman Laboratory and Specialty Chemicals, Eastman Kodak Co., Rochester, NY), and 10% fetal bovine serum (Dutchland Laboratories, Inc.).

Helper T Cells. Ly-1 T cells were cultured (2.5×10^6) with sheep erythrocytes (SRBC) (Colorado Serum Co., Denver CO) (7×10^6) in 1 ml of medium in tissue culture wells (3524, Costar, Data Packaging, Cambridge, MA) for 2 d. The recovered cell population was washed and assayed for helper activity by titration into cultures of B cells (10^6/well) and SRBC (3×10^6/well) which had been initiated in medium 2 d earlier in microtiter wells (3596, Costar) (200 μl final culture volume). 3 d later, triplicate cultures were assayed for plaque-forming cells (PFC) at 37°C by the Cunningham slide modification of the Jerne plaque technique (17).

Suppressor T Cells. The whole T cell fraction was cultured with SRBC (18); this method for generating suppressor T cells differs from helper cell generation, above, in that no prior removal of Lyt-2^+ cells is performed and the cells are cultured 4, rather than 2 d. The recovered population, termed "educated whole T," was washed and assayed for suppression by addition to unfractionated spleen cells (5×10^6) and SRBC (7×10^6) in 1 ml of medium. Cultures were assayed for PFC 5 d later.

Helper Factor. Two methods were used to produce supernatants containing helper activity. One type was produced by the method of Ryser et al. (19). Briefly, BALB/c spleen cells (1.25×10^6/ml) were stimulated in mixed leukocyte culture with irradiated C57BL/6 spleen cells (1.25×10^6/ml) for 12 d. The recovered cell population was restimulated (10^6 responders plus 4×10^6 irradiated stimulators/ml) for 1 d; the supernatant was collected and cleared of cell debris by centrifugation (500 *g*, 10 min) and filtration (0.22 μm pore size; Nalge Co., Nalgene Labware Div., Rochester, NY). The second method of preparing supernatants with helper activity consisted of recovering the conditioned medium at the end of Ly-1 T culture and clearing of cell debris. Helper activity was assayed by titrating supernates into cultures of B cells and SRBC as used to assay for helper T cells.

Suppressor Factor. Supernatants containing T suppressor factor (T_sF) were kindly provided by K. Kondo (Yale University School of Medicine) and prepared as described previously (20). Briefly, C57BL/6 mice were immunized three times with SRBC at 2-wk intervals. 2 wk after

the last immunization, spleen cells were removed, depleted of Lyt-1-positive cells and cultured 48 h. Suppression was assayed by adding supernatants (1:10 final dilution) to primary cultures of unfractionated spleen cells and SRBC, as used to assay for suppressor cells.

Results

To assess the effect of culture temperature on an in vitro immune response without changing other environmental variables such as atmosphere, we used gas-tight boxes that were gassed daily with premixed CO_2 (10%) and air. Later we used incubators with automatic CO_2 controls that were carefully calibrated to maintain 5% CO_2 and were saturated with humidity. The same pattern of temperature effects was observed using either incubation method. Temperature was closely monitored with several thermometers in each incubator and was stable within ±0.2°C throughout the culture period. Two temperatures were chosen for study, based on their physiological relevance in the mouse, 37°C representing the "normal" core temperature and 39.5°C corresponding to a "fever" (21).[2]

Hyperthermia Augments the PFC Response. Mouse spleen cells were cultured 5 d with SRBC and assayed for PFC; the results of four typical experiments are shown in Table I. Incubation at the higher temperature always resulted in an increased response, which over a total of nine experiments has averaged 9.3-fold above the 37°C control, and with no shift in the day of peak response (data not shown). Temperature-

TABLE I

*PFC Response of Spleen Cells is Higher at 39.5°C than at 37°C**

Culture temperature	1	2	3	4
°C				
39.5	6,029 (2,309)‡	1,764 (822)	5,480 (643)	7,725 (395)
37	284 (62)	354 (54)	873 (123)	4,500 (349)
Ratio:§	21.2	5.0	6.3	1.7

* 10^7 spleen cells were cultured with SRBC for 5 d at 37° or 39.5°C, then assayed for PFC.
‡ PFC/culture. A mean of triplicates with SE in parentheses.
§ Ratio: (PFC/culture at 39.5°C)/(PFC/culture at 37°C).

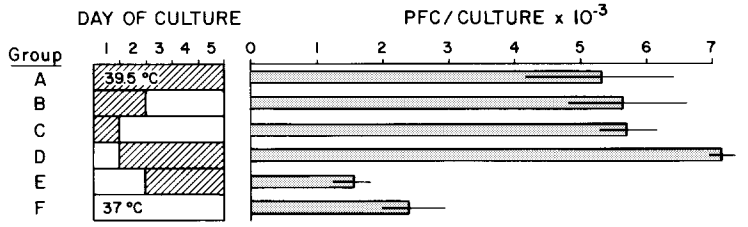

FIG. 1. Temporal requirements of the temperature effect on the PFC response of cultured spleen cells. Cultures of 10^7 spleen cells and SRBC were maintained at 37° or 39.5°C for all or part of the 5-d culture period as indicated in the diagram at left. PFC response, as assayed on day 5, represents the mean (bar equals SE) of triplicate cultures. Period of culture at 39.5°C is shaded, period at 37°C is open.

[2] We have confirmed that in an ambient temperature of 30–32°C (within the thermoneutral zone) a restrained mouse will maintain a rectal temperature of about 37°C and will respond to intravenous injections of lipopolysaccharide or purified EP by mounting a fever of up to 39°C (J. Stitt, S. Durum, and G. Duff, unpublished observations).

sensitive events occur early in the culture period, as shown in Fig. 1. For maximum effect, exposure to the higher temperature must occur during the first or second (or both) days of the 5-d culture period. Thus groups B and C, exposed to higher temperatures early in the culture period, are indistinguishable from group A, exposed for the entire 5 d. There was no benefit in raising temperature beyond the second day since group E, which was at the higher temperature for the last 3 d, was no different from group F, which was maintained throughout at 37°C. In other experiments, higher temperature during the last 3 d was occasionally somewhat deleterious (data not shown).

Effects of Temperature on Helper T Cells (T_h). We next considered three potential sites of action of the temperature effect on immune responses: (*a*) through augmentation of T_h; (*b*) augmentation of B cell responsiveness; or (*c*) impairment of the activity of suppressor T cells (T_s). Our approach to studying temperature effects on T_h takes advantage of the observation that, in the 5-d development of PFC against SRBC in vitro, the events of the first 2 d do not require the presence of Thy-1-positive cells (22, 23); T_h are required during the last 3 d of culture, perhaps to promote the differentiation of activated B cells into Ig-secreting cells. Therefore, since T and B cells need not be co-cultured the first 2 d, and knowing that the temperature-sensitive events occur during this 2-d period (see Fig. 1) we cultured T and B cells separately, each at two different temperatures; precultured Ly-1 T cells (Lyt-2-positive cells—the suppressor phenotype—were removed before preculture) were then titrated into microtiter wells containing 2-d-precultured B cells. Antigen was included in the preculture of both cell types. As shown in Fig. 2, the Ly-1 T fraction precultured at 39.5°C produced a more potent T_h population than their 37°C counterparts. Preculturing B cells at the higher temperature, on the other hand, did not facilitate, but rather impaired their responses to the subsequent delivery of T cell helper signals, as shown. The optimal permutation was achieved therefore, when T_h were raised at 39.5°C and added to B cells raised at 37°C; the poorest responses occurred when T_h were raised at 37°C and added to B cells raised at 39.5°C. Despite the impairment of B cell function by the higher temperature, when both T and B cell populations were

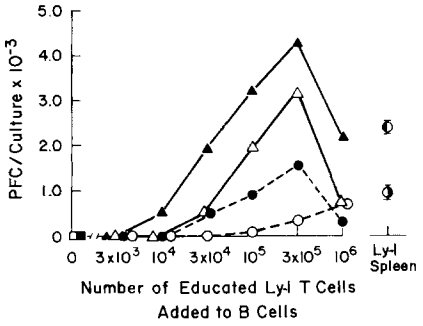

FIG. 2. Influence of culture temperature on the generation of helper T cells. Ly-1 T cells were precultured with SRBC for 2 d; various numbers were then added to assay cultures of 10^6 B cells and SRBC, which had also been precultured for 2 d. PFC responses of triplicate cultures were determined 3 d after addition of T cells. Temperature (°C) of the T cell preculture is designated by the first number, of the B cell preculture by the second number: 37, 37 (●), 37, 39.5 (○), 39.5, 37 (▲), 39.5, 39.5 (△). B cells were precultured with SRBC at 37 (■) or 39.5°C (□) with no added T cells. Ly-1 spleen cells (depleted of Lyt-2-positive cells) were cultured 2 d with SRBC at 37° (◑) or 39.5°C (◐) followed by 3 d at 37°C.

raised at 39.5°C, twice as many PFC resulted than when both T and B cell populations were raised at 37°C. This ratio approximates the benefit of higher temperature on unfractionated spleen cells (depleted of Lyt-2-positive cells) observed in this experiment, also shown in Fig. 2.

Thus the enhancement of PFC responses in unselected spleen cell cultures can be accounted for at least in part through an enhancement of T_h activation, possibly by favoring blast transformation and/or cell division. Once T_h are activated, the higher temperature does not improve the T_h ability to deliver help, since after the separate precultures of T and B cells are complete, the co-culture over the last 3 d is not further benefited by the higher temperature (data not shown).

Effects of Temperature on B Cells. We next examined whether B cell responses to T cell help might also be favored at higher temperature. Supernatants containing T helper factors were added to B cells and SRBC, and cultures were maintained at different temperatures and then assayed for PFC. Fig. 3 shows that the response of B cells was not improved at 39.5° compared with 37°C, but was in fact decreased. It seems unlikely, therefore, that the increase observed in the PFC response of unfractionated spleen cells at elevated temperatures is attributable to increased B cell activation.

Effects of Temperature on T_s. Suppressor T cells represent a third major lymphocyte subset whose function might be temperature sensitive; impairment of the generation or action of T_s, in addition to the favoring of T_h generation noted above, could account for the observed augmentation of the PFC response of unfractionated spleen cells at increased temperature. To test this possibility, we examined the effect of temperature on the generation and delivery of suppression. T cells were educated 4 d (unlike T_h generation, no prior depletion of Lyt-2^+ cells was performed), then assayed for the ability to suppress a primary PFC response of spleen cells. Within the educated whole T population, the active T_s have been previously shown to bear the Lyt-1^- 2^+,IJ^- surface phenotype (24). The effect of temperature on the generation and assay of T_s is shown in Fig. 4 a. No diminution in potency of suppression was seen if T_s were generated at the higher temperature; they may in fact be somewhat stronger. Although

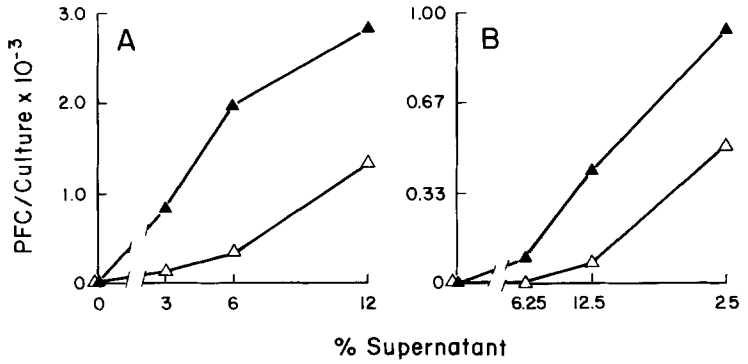

FIG. 3. Influence of culture temperature on the PFC response of B cells to helper factors and SRBC. Supernatants containing helper activity were added in various concentrations to cultures of B cells and SRBC initiated 2 d earlier, then assayed for PFC 3 d after addition of supernatants. Secondary MLC supernate (A) was prepared by stimulating BALB/c spleen cells with irradiated C57BL/6 spleen cells for 12 d, then restimulating 1 d and collecting supernatant. Conditioned medium (B) was collected at the end of 4 d of Ly-1 T cell education with SRBC. B cell cultures were maintained at 37° (▲) or 39.5°C (△).

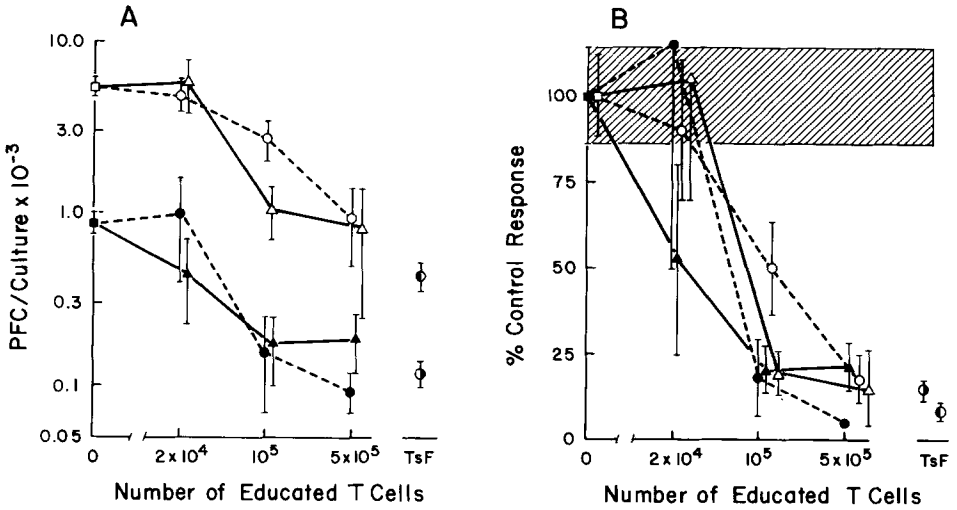

FIG. 4. Influence of culture temperature on the education and assay of suppressor T cells and TsF. T cells were educated with SRBC in vitro for 4 d. TsF was produced by three in vivo immunizations with SRBC; spleen cells were then depleted of Ly-1 T cells and cultured for 2 d, collecting the supernatant. Varied numbers of educated whole T cells or 10% T_sF were added to triplicate assay cultures of 10^7 spleen cells and SRBC; PFC responses were determined at day 5. Data are represented as (A) PFC/culture (bars, SE) and (B) percent of control response for controls at 37° (■) and 39.5°C (□), T_sF assayed at 37° (◑) and 39.5°C (◐), and T_s cells educated (first number) and assayed (second number) at the temperatures (°C) as follows: 37, 37 (●), 37, 39.5 (○), 39.5, 37 (▲), and 39.5, 39.5 (△). Shaded area (B) includes mean and SE for both 37° and 39.5°C controls.

elevating the temperature of the assay culture increases the overall magnitude of responses, either in the presence or absence of T_s, this does not reflect a reduction in delivery of (or a qualitative resistance to) suppression. This point is better made in Fig. 4b in which the same data shown in Fig. 4a are normalized as a percentage of control responses. Compared in this way, there is no apparent difference in the susceptibility to suppression of assay cultures at the two temperatures. Fig. 4b also shows that T_sF was equally effective at either temperature. Thus a given unit of suppression, either in the form of educated whole T cells or T_sF supernatant, inhibits about the same proportion of the response at either temperature. To test further whether reduction of suppression could account for the observed increase in the primary response of unselected spleen cells at the higher temperature, we have found that removal of Lyt-2^+ cells before culture does not diminish the temperature effect (data not shown).

Discussion

Recent findings by others (13, 14) that equate IL-1 and EP prompted us to formulate the hypothesis that the hyperthermia induced by EP/IL-1 might benefit its own action on lymphocytes. To test this hypothesis, we first examined the temperature sensitivity of the in vitro proliferative response of murine lymphocytes to IL-1 and suboptimal concentrations of concanavalin A (15). We found that the response to IL-1 was greatly increased at 39° compared with 37°C, supporting the notion that the pyrogenic action of IL-1 enhances its own action on lymphocytes; we have, therefore, extended the observations of others demonstrating positive effects of hyperthermia on

proliferative responses of human peripheral blood leukocytes to lectins (2–5) and in mixed leukocyte cultures (3).

The results of the present study provide the first demonstration that hyperthermia augments an antigen-specific immune response—the generation of PFC in Mishell-Dutton cultures. By manipulating lymphocyte subsets independently, we conclude that the target of thermal enhancement is likely to be the helper T cell, rather than B cells or T_s, and we suspect that the observed functional augmentation reflects an increase in the number of helper precursors initially triggered. This need not imply that T cells are directly temperature-sensitive, but might represent a property of accessory cells involved in the generation of T_h; this question is under current investigation.

We have, however, recently found an effect of hyperthermia on lymphocytes that is undoubtedly direct, and which may have functional implications—the induction of heat shock proteins (25). As little as 15 min of hyperthermia induces de novo synthesis of four prominent protein species in cloned or normal T cells with molecular weights of ~66, 68, 79, and 88 × 10^3. While the occurrence of heat shock proteins has been well documented in cells from other species (26–29) as well as in other murine cell types (30) their occurrence in lymphocytes, if they account for the observed changes in helper T cell activation, would provide the first evidence for a physiological role for heat shock protein synthesis.

The augmentation of helper T cell generation by hyperthermia is offset to some extent by negative effects on B cells. This is reminiscent of our findings with mitogen stimulation, the response to concanavalin A being greatly improved, the response to lipopolysaccharide being reduced or unchanged, by hyperthermia (15). We assume that the net increase in the response of unselected spleen cells implies an increase in help that more than compensates for deleterious effects on B cells, i.e., T cell help must be sufficiently limiting to account for hyperthermia inducing an average augmentation of 9.3-fold in the PFC response of unselected spleen cells. Since not all subsets of lymphocytes are benefited by hyperthermia, it will be important to extend these observations by examining temperature effects on the generation of other T cell functions, particularly those also attributed to Ly-1 cells, such as the delayed-type hypersensitivity response, providing help to cytotoxic T cells, lymphokine-secretion, etc.

If our observations of the powerful enhancing effect of hypethermia on an in vitro immune response can be extrapolated to in vivo phenomena, then we can now imagine a major selective value for hyperthermia induction during infection. Our results indicate that hyperthermia benefits early events in the course of the immune response. This may suggest that only those fevers arising during the first few days of an infection will be beneficial to the host. Such a situation, in which fever precedes the immune response, could occur during primary infections through the pyrogenic action of any of the various microbial products that can directly induce EP/IL-1 release from macrophages (31). During subsequent infections, EP/IL-1 release from macrophages could be induced both by the direct effect of microbial products and indirectly by signals from activated T cells responding to specific antigenic determinants on the invading organisms (12). The fever response occurring upon re-encounter with foreign antigens may in fact contribute to the characteristic vigor of secondary immune responses through a facilitation of T cell immunity. Similarly, the adjuvan-

ticity of some substances may be attributable to their direct stimulation of EP/IL-1 release, resulting in fever. Hyperthermia has been shown to correlate with increased survival in bacterially infected lizards (32, 33) and fish (34, 35), and in a population of rabbits infected with *Pasteurella multocida*, occurrence of moderate fever was correlated with enhanced survival (36). Interpreted in the light of our findings, these results may be explained as an augmentation in host resistance to pathogens through enhancement of T cell activation. If our findings are relevant to clinical fever in humans, then the suppression of fever with antipyretic drugs during the course of an infection may ultimately prove detrimental to the patient.

In conclusion, hyperthermia, typical of that occurring during natural fever, dramatically increases the primary in vitro immune response. The present study provides further support for our starting hypothesis which can now be restated as follows: The monokine EP/IL-1 may serve a dual role as an endogenous immunopotentiator, first by directly interacting with lymphocytes, and second by inducing hyperthermia.

Summary

We have examined the possibility that hyperthermia, such as that occurring during fever, may benefit the immune response. The effect of temperature on the in vitro immune response of unprimed murine spleen cells against the antigen sheep erythrocytes was tested. Hyperthermia potently augmented the plaque-forming cell response. Temperature-sensitive events occurred early in the culture period. Subsets of lymphocytes were independently assessed for effects of temperature on their activation and function. We showed that the beneficial effect of elevated temperature on the plaque-forming cell response probably occurs during the priming stage of T helper cells, and neither improves the delivery of help or the activation of B cells, nor impairs suppressor T cell generation or function. We propose that this powerful immunopotentiating effect of hyperthermia may account for the selective value of the fever response. This suggests that the monokine interleukin 1, which is the endogenous mediator of fever, may promote immune responses both through a direct action on lymphocytes, and indirectly by an action on the central nervous system resulting in fever.

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