

The Effects of Variations in pH and Temperature on the Activation of Mouse Thymocytes by Both Forms of Rabbit Interleukin-1

PATRICK A. MURPHY, M.D., DANIEL F. HANSON, Ph.D.,*
YAN-NI GUO, M.D.,** AND DEBORAH E. ANGSTER, B.S.

*Department of Medicine, Johns Hopkins University School of Medicine,
Baltimore, Maryland*

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We studied the responses of unpurified mouse thymocytes and of peanut agglutinin-negative mouse thymocytes to the pI 7.3 form of rabbit Interleukin-1. We found that small increases of temperature strongly enhanced the mitogenic effect of this form of IL-1, and that the apparent temperature optimum was 37°C. In both these respects the behavior of the pI 7.3 IL-1 resembled the previously described behavior of pI 5.0 IL-1.

We suspected that the low apparent temperature optimum for IL-1 action was due to inadequate pH control by the bicarbonate-buffered medium. Experiments showed that small decreases in medium pH strongly inhibited the mitogenic action of both forms of IL-1. Furthermore, if thymocytes were stimulated with either form of IL-1 in strongly buffered media, the temperature optimum was at least 39°C.

The pI 7.3 and pI 5.0 forms of IL-1 are known to differ both biochemically and immunologically. Our experimental discovery that their temperature sensitivities are much the same suggests that temperature sensitivity is a property of the T cell rather than of the IL-1 molecules themselves.

INTRODUCTION

Recent studies have delineated many points of similarity between the rabbit monokine Interleukin-1 (IL-1) and endogenous pyrogen (EP) [1]. Rabbit EP exists in two forms; one with an isoelectric point (pI) of 7.3, the other with a pI of about 5 [2]. Studies with highly purified pI 5 EP indicate that both EP and IL-1 activity are properties of the same molecule [3]. This led us to speculate that the central action of EP (fever) might influence its peripheral action (IL-1 mediated mitogenesis).

In a previous paper, we showed that the activation of mouse thymocytes by the pI 5 form of rabbit Interleukin 1 (IL-1) was strongly temperature-dependent [4]. Those experiments were conducted using bicarbonate-buffered medium, and the apparent temperature optimum observed was 38°C. In the discussion, we commented that the apparent temperature optimum at 38°C might be real or might be an artefact of the way in which we performed our experiments.

*Present address: Dept. of Microbiology and Immunology, University of California at Berkeley, Berkeley, CA 94720

**Present address: Inner Mongolian Medical College, Huhehaote, The People's Republic of China

Address reprint requests to: Dr. Patrick A. Murphy, Blalock 1141, Johns Hopkins Hospital, 600 N. Wolfe St., Baltimore, MD 21205

In this paper, we examined the effects of temperature on the activation of the pI 7.3 form of rabbit IL-1. In the first set of experiments we used the same medium as that used for the pI 5.0 pyrogen [4], and we found that the apparent temperature optimum of the pI 7.3 form of IL-1 was only 37°C. Because natural fevers range up to 40 or 41°C, we suspected that the low observed optimum temperatures of IL-1 action were due to artefacts of the *in vitro* tissue cultivation system used. We noticed that the tissue culture media of thymocytes incubated at 39 or 40°C were frankly yellow, suggesting a major drop in pH during the 72 hours of incubation before tritiated thymidine (³HTdR) was added.

We therefore studied the effects of varying medium pH on the activity of both forms of rabbit IL-1. We found that slight lowering of medium pH produced major decreases in the observed activity of both forms of rabbit IL-1. We also showed that strongly buffered tissue culture media changed the apparent optimum temperature for IL-1 action to at least 39°C. The probable significance of these results is discussed.

MATERIALS AND METHODS

IL-1 Preparations

The pI 7.3 and pI 5.0 forms of rabbit IL-1 were obtained from mononuclear blood leukocytes purified by centrifugation on colloidal silica gradients [5] and stimulated with *E. coli* endotoxin (0111:B4, Difco, Westphal process) at a concentration of 0.003 µg/ml. The cells were incubated overnight at 37°C, and the next day were centrifuged down at 400 g for ten minutes. They were resuspended in 0.15 M sodium chloride, and incubated for a further two hours at 37°C. The supernates from both incubations were combined; they contained both the pI 7.3 and pI 5.0 forms of IL-1.

The two forms of IL-1 were precipitated by adding 561 G solid ammonium sulfate per liter of supernate [5], resolubilized in a minimal quantity of ion-free water, and dialyzed against 0.01 M TRIS-HCl, pH 8.3. They were separated by chromatofocusing using two separate gradients, one from pH 8 to pH 6, the other from pH 6 to pH 4. The IL-1 peaks were located by bioassay, and concentrated by dialysis against polyethylene glycol 20,000 (Baker Chemical Co., Phillipsburg, NJ). They were further purified by gel filtration on Sephadex G75 superfine, as described [2] and stored frozen in liquid nitrogen until required.

IL-1 Assay

This has been described previously [4]. Briefly, mouse thymocytes were incubated in supplemented minimal essential medium plus 5 µg/ml phytohemagglutinin in the presence of standard IL-1 preparations or of solutions to be assayed for IL-1. After 72 hours in culture, 1 uCi per well of tritiated thymidine (³HTdR) was added, and incubation was continued for either six hours or overnight, after which the ³HTdR uptakes were determined by harvesting the cells. Our standard preparation of pI 7.3 IL-1 contained about 1,000 u/ml, and our standard preparation of pI 5 IL-1 contained 3,800 u/ml. Neither preparation contained any measurable amount of Interleukin-2 (IL-2), as judged by assay on the IL-2 dependent CTL cell line.

Buffering and pH Variations of Tissue Culture Media

Our standard tissue culture medium was Eagles minimal essential medium (MEM) with Earles salts supplemented with 5 percent fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 u/ml penicillin, 50

$\mu\text{g/ml}$ streptomycin (all of the above from Grand Island Biological Co., Grand Island, NY), and 5×10^{-5} M 2-mercaptoethanol (LKB Producter, Sweden). It was equilibrated in a 5 percent CO_2 incubator at 37°C for about 30 minutes and then adjusted to pH 7.4 with 1.0 N NaOH. Experiments with increased buffering were conducted by adding enough isotonic N-2-hydroxyethylpiperazine-N¹-2 ethane sulfonic acid (HEPES) solution to produce final concentrations of 10 mM or 20 mM before adjusting the pH to 7.4. Thus, the medium remained isotonic, but the concentrations of vitamins and amino acids were somewhat reduced when HEPES was added (3.6 percent reduction at 10 mM and 7.1 percent at 20 mM).

Experiments at pH values other than 7.4 were performed by adding isotonic Good buffers in the amounts suggested by Eagle [6]. The pH values used were 6.5, 6.8, 7.1, 7.2, 7.4, 7.7, 8.0, and 8.3. Each medium was equilibrated with 5 percent CO_2 at 37°C before titration to the desired pH with 1.0 N NaOH or HCl. Experiments showed that temperature variations over the range $29\text{--}39^\circ\text{C}$ produced pH changes in these buffered media of <0.1 pH unit. Therefore, no attempt was made to titrate each buffer to the desired pH at the temperature at which it was to be used.

Conduct of Experiments

Experiments were performed in 96-well Costar plates. The two outer eight-well columns were not used. Each of the 80 interior wells contained 1×10^6 thymocytes in medium buffered to the desired pH. Each concentration of IL-1 was tested in a column of eight wells.

Incubators and Harvesting of Cells

The experiments were conducted using Shell-Lab Model 250 incubators (Sheldon Manufacturing Co., Portland, OR). Each incubator contained an automatic thermostat which was shown to control temperature to within $\pm 0.2^\circ\text{C}$ of the setting. CO_2 was controlled by electronic sensors at 5 percent ± 0.3 percent. After the experiments were concluded, cells were harvested using a PHD harvester (Cambridge Technology, Inc., Cambridge, MA) and $^3\text{HTdR}$ uptakes determined by liquid scintillation counting. Results were expressed as means of eight determinations ± 95 percent confidence limits. All values were expressed as cpm; the average counter efficiency was 40 percent and did not vary appreciably from one vial to the next.

$^3\text{HTdR}$ Uptake Assays on Frozen Plates

When it was inconvenient to determine $^3\text{HTdR}$ uptakes immediately after concluding the experiment, plates were placed in a -20°C freezer. Assays for $^3\text{HTdR}$ uptake were conducted one to four days later. Extensive experiments established that plates which had been frozen in this way gave $^3\text{HTdR}$ uptake values which were identical to those obtained from plates which had been harvested immediately at the end of the experiment: $^3\text{HTdR}$ uptakes were stable for at least a month. Experiments with large numbers of plates were rendered much more practical by freezing, because one could thaw a few plates at a time for harvesting.

RESULTS

Temperature Dependence of PHA-Facilitated Responses to the pI 7.3 Form of IL-1

The dose-response curve for the pI 7.3 form of IL-1 in the presence of $5 \mu\text{g/ml}$ (PHA) was determined at six different temperatures, in bicarbonate-buffered

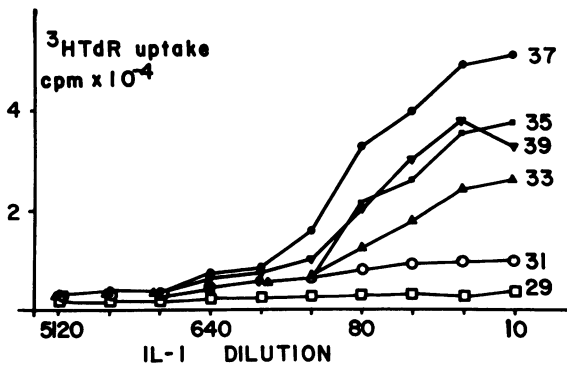


FIG. 1. ³HTdR uptake by mouse thymocytes stimulated with various concentrations of the pI 7.3 form of rabbit EP at six different temperatures. The °C temperature appropriate to each graph is given on the right.

medium. A representative experiment is shown in Fig. 1, which shows that ³HTdR uptake was maximal at 37°C.

In other experiments, thymocytes were incubated in the presence of 5 µg/ml PHA plus variable amounts of the pI 7.3 form of IL-1. Two plates were used for each temperature; both were pulsed with ³HTdR after 72 hours of incubation. One plate of each pair was transferred to a 37°C incubator for the ³HTdR uptake phase; the other was incubated at its original temperature. We found that the ³HTdR uptake of thymocytes was powerfully influenced by the temperature of incubation over the first 72 hours, and that the temperature during the ³HTdR uptake phase made little difference (Figs. 2A,B,C). These experiments established that the response of mouse thymocytes to the pI 7.3 form of IL-1 was strongly temperature-dependent, and there appeared to be no substantial differences from the responses of mouse thymocytes to the pI 5 form of IL-1 [4].

Effect of Improved pH Control

We noticed that thymocytes stimulated with large amounts of IL-1 at high temperatures were somewhat acidic after 72 hours in culture as judged by the yellow color of phenol red in the medium. We wondered whether this acidity would interfere with the ability of the cells to incorporate ³HTdR, and, if so, whether better pH control would allow optimum ³HTdR incorporation to occur at temperatures above 37°C. We therefore tried the effect of adding 20 mM HEPES to the incubation medium of thymocytes being co-cultivated with the pI 7.3 form of IL-1 and found that the optimum temperature for ³HTdR uptake was now 39°C (Fig. 3). This new apparent temperature optimum applied to the pI 5 form of IL-1 as well as the pI 7.3 form (data not shown). In fact, we were able to do an experiment with a single batch of thymocytes, in which the optimum temperature for ³HTdR uptake varied according to the amount of HEPES added to the bicarbonate-buffered medium. With no HEPES, the optimum temperature was 37°C; with 10 mM HEPES, incorporations at 37 and 39°C were equivalent; and, with 20 mM HEPES, incorporation at 39°C was clearly superior (data not shown).

Temperature Optimum for PNA Negative Thymocytes

We also did experiments on thymocytes enriched for IL-1 target cells by the use of peanut agglutinin (PNA) [3]. Those cell suspensions responded to both the pI 7.3 and the pI 5.0 forms of IL-1 in a strongly temperature-dependent fashion, and it was not

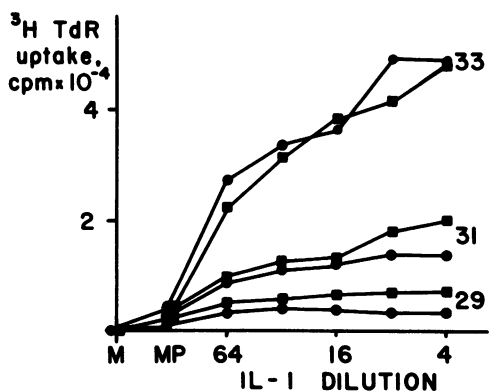
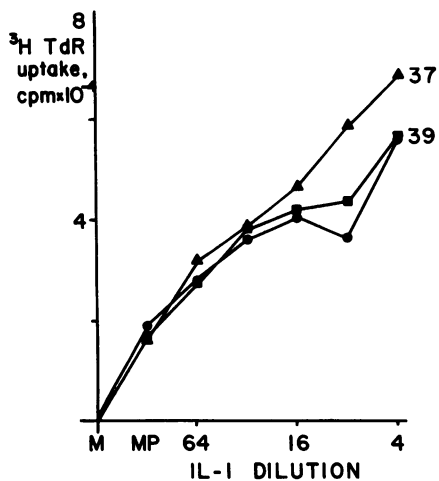
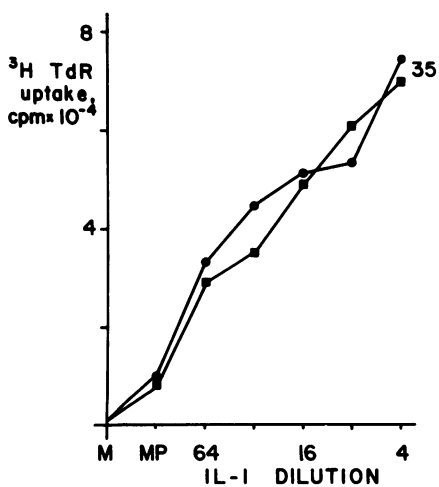


FIG. 2A,B,C. ³HTdR uptake by mouse thymocytes stimulated with various concentrations of the pI 7.3 form of rabbit IL-1 at six different temperatures. ●—●, cells exposed to IL-1 and pulsed with ³HTdR at the indicated temperature. ■—■, cells exposed to IL-1 at the indicated temperature, but pulsed at 37°C. For cells stimulated at 37°C, no temperature switch was possible, and a single curve is given, marked ▲—▲.



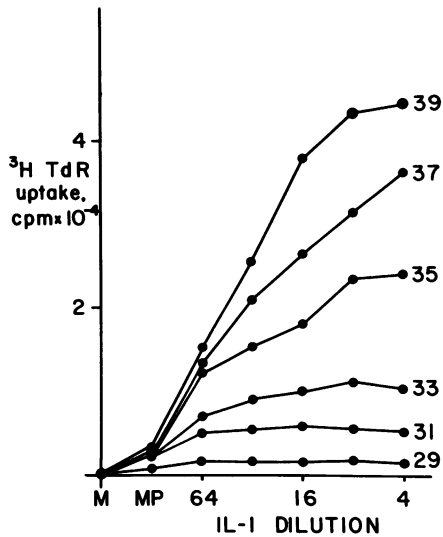


FIG. 3. ^3H TdR uptake by mouse thymocytes stimulated by the pI 7.3 form of IL-1 in medium buffered with 20 mM HEPES, at various temperatures.

necessary to add PHA to detect the responses. For the pI 5 form of IL-1, the optimum temperature for ^3H TdR incorporation was 39°C when the medium was buffered with 20 mM HEPES (Fig. 4). The same optimum was observed with the pI 7.3 form (data not shown).

Effect of Variations in Medium pH on the Mitogenic Action of IL-1

The ^3H TdR uptake of thymocytes exposed to various amounts of IL-1 was determined at eight different values of medium pH. The results were substantially identical for both forms of IL-1; they showed large decreases in mitogenic activity for pH shifts to the acid side, with much smaller decreases in activity with pH shifts to the alkaline side (Figs. 5 and 6).

We also investigated whether changes in incubation temperature altered the optimum pH for IL-1 action. We found that they did not: pH shifts to either side of pH 7.4 were associated with reduced mitogenic activity for both forms of IL-1, and altering the incubation temperature only altered the height of the curve, but not its shape (Figs. 7 and 8). Similarly, variations in medium pH did not alter the optimum temperature for IL-1 action (data not shown).

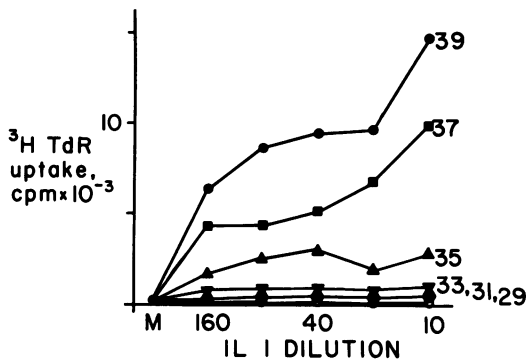


FIG. 4. ^3H TdR uptake by PNA-negative mouse thymocytes exposed to the pI 5.0 form of rabbit IL-1 in HEPES buffered medium, at various temperatures.

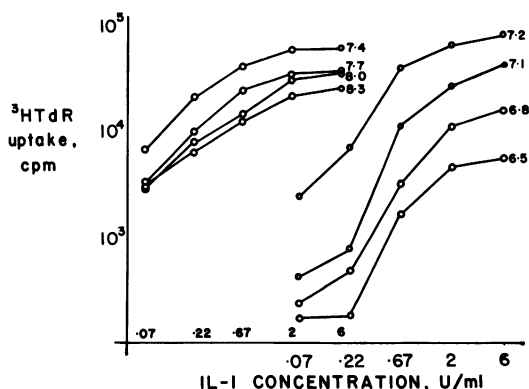


FIG. 5. ³HTdR uptake by mouse thymocytes exposed to various concentrations of the pl 7.3 form of rabbit IL-1 at different values of medium pH. The pH appropriate to each curve is given at the right.

DISCUSSION

Over the last few years it has become obvious that any Interleukin-1 dependent immunological response is strongly accelerated by small increases in the temperature of incubation [4,7,8]. It now seems equally obvious that fever has developed as a response to essentially all infections because it potentiates the immune response. This is, however, not a new idea; experiments on this subject have been done for over 50 years, and there are as many negative experiments as positive ones [9,10]. And in our own work, we initially found that IL-1 action was indeed strongly temperature-sensitive, but that the optimum temperature was only 38°C. Since natural fevers may reach 40 or 41°C, this optimum temperature was disappointingly low.

It is now possible to offer a unitary explanation for these disparate results. All *in vitro* experiments are done on cell populations in which many cells die, and ³HTdR uptake experiments and other studies are done on the survivors. If cells are incubated in high concentrations of mitogens at elevated temperatures, they are so intensely stimulated that, by the time ³HTdR is added after 72 hours of incubation, the medium pH has fallen to values which will not support high rates of DNA synthesis. Thus, the apparent temperature optimum is reduced from 39 or 40°C to 37 or 38°C. In experiments in which the lowest temperature used was 37°C, there may appear to be no enhancement of the response by temperature [10]. Reference to the papers above shows that those in which temperature was reported to have a strong enhancing effect on immunological responses used strongly buffered media.

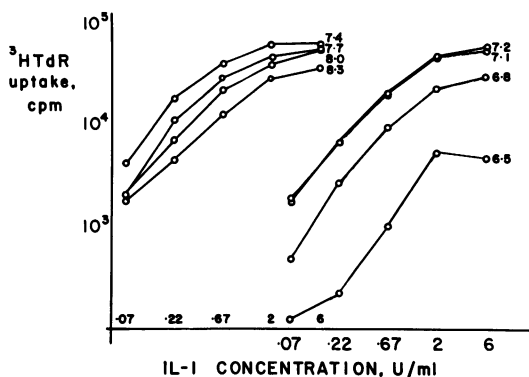


FIG. 6. ³HTdR uptake by mouse thymocytes exposed to various concentrations of the pl 5.0 form of rabbit IL-1 at different values of medium pH. The pH appropriate to each curve is given at the right.

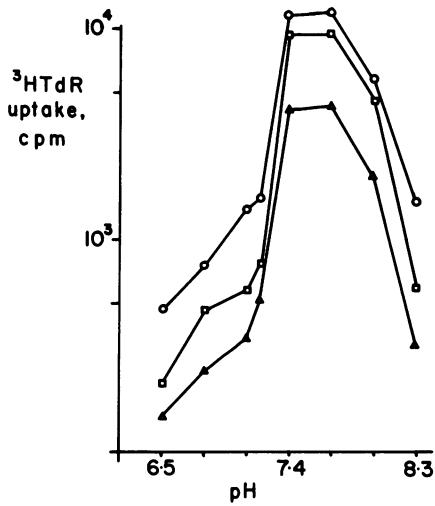


FIG. 7. ³HTdR uptake by mouse thymocytes exposed to 1 U/ml of the pI 7.3 form of rabbit IL-1 at different values of medium pH. Three different incubation temperatures were used: ○—○, 39°C; □—□, 37°C; △—△, 35°C.

It is of course possible that an apparent absence of temperature effect on proliferative immunological responses could reflect exhaustion of some medium component rather than a pH change. We have no way of assessing this in the experiments of others, but the fact that we were able to raise the apparent optimum temperature by pH control alone suggests that fall in medium pH was the main factor in our experiments.

In our original paper [4], we commented that the apparent temperature optimum at 38°C might be an artefact, and it now seems that the hypothesis was correct. Of course, all observations made *in vitro* are artefacts, and there is no intrinsic reason why one artefact should be preferred to another. None the less, fevers observed during infectious illnesses range up to 41°C. Since the mitogenic response of thymocytes to IL-1 correlates with *in vitro* models of important host defense mechanisms, it should be possible to show that it is enhanced by temperatures which are clearly above normal body temperature. By the use of 20 mM HEPES buffer in addition to the usual

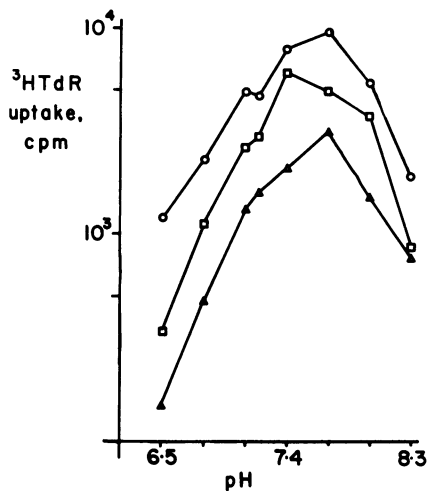


FIG. 8. ³HTdR uptake by mouse thymocytes exposed to 1 U/ml of the pI 5.0 form of rabbit IL-1 at different values of medium pH. Three different incubation temperatures were used: ○—○, 39°C; □—□, 37°C; △—△, 35°C.

bicarbonate, we were able to shift the apparent temperature optimum of the same batch of thymocytes from 37°C to 39°C. We have not yet tried temperatures above 39°C, but the temperature coefficients for the interval 37 to 39°C were rather small, and we think it unlikely that our present medium would show an apparent temperature optimum much above 39°C.

It does seem likely that pH control is better achieved in the body than *in vitro*, because the density of lymphocytes in nodes and other lymphatic tissues greatly exceeds the densities commonly used in tissue culture of lymphocytes. Presumably, the use of HEPES allowed us to approximate physiological conditions more closely. It is possible that even more efficient buffering could shift the optimum temperature above 39°C, and we are currently doing experiments to investigate that possibility.

The other new observation in this paper was that the pH 7.3 form of IL-1 showed a temperature sensitivity which was substantially identical to that of the pI 5.0 form. These two forms of IL-1 are known to differ both biochemically and immunologically [2]. In addition, we have found in unpublished experiments that the pI 5.0 form of IL-1 is very much less sensitive to heat than is the pI 7.3 form. Since temperature affects activity of these two different forms of IL-1 to the same extent, it is suggested that temperature sensitivity is a property of the IL-1 responsive thymocytes rather than of the IL-1 molecules.

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