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Inhibition of *In Vitro* Lymphoproliferation by Three Novel Iron Chelators of the Pyridoxal and Salicyl Aldehyde Hydrazone Classes

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ABSTRACT. The capacity of three novel iron chelators, namely 1-[*N*-ethoxycarbonylmethylpyridoxylidenium]-2-[2′-pyridyl]hydrazine bromide (EPH), 1-[5′-bromosalicylidene]-2-[2′′-pyridyl]hydrazine (BsPH), and 1-pyridoxylidene-2-[1′-phthalazyl]hydrazine dihydrochloride (PPhH), to inhibit the proliferation of mitogen-stimulated murine lymph node cells was examined *in vitro*. All three are of the aryl hydrazone class, the prototype of which is pyridoxal isonicotinoyl hydrazone. The chelators inhibited lymphoproliferation at low micromolar concentrations. EPH and PPhH had an inhibitory capacity comparable to that of desferrioxamine (IC_{50}: 3 and 2 μM, respectively), whereas BsPH was more potent ($IC_{50} < 1$ μM). The inhibitory effects of the chelator were not due to cell cytotoxicity and could be abrogated by pretreating the chelator with iron. Time–course studies established a site of action for the chelators at the G_1/S phase transition. These agents warrant further investigation for their potential as immunosuppressants. BIOCHEM PHARMACOL **60**;4:581–587, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. iron; iron chelators; lymphocyte; proliferation; aryl hydrazone

T lymphocyte proliferation *in vitro* is iron-dependent, and this requirement can be met by providing transferrin or iron salts in serum-free cell medium [1–3]. Bomford and colleagues [4] have attempted to characterise the distribution of iron taken up by mitogen-activated human lymphocytes. Most of the iron was stored as ferritin or bound to transferrin with only a small percentage present as haem, while some 15% of the iron remained uncharacterised [4], suggesting the possibility of a low molecular weight pool of iron in these cells.

A large number of studies have demonstrated that a variety of iron chelators will inhibit T lymphocyte proliferation *in vitro* [5–25], and several targets for chelator action have been investigated. As potential sites for the inhibitory action of iron chelation, studies assessing the effect of iron chelators on IL-2‖ synthesis and IL-2R expression have produced conflicting results [3, 9–12, 14–16]. Notably, several studies have shown that iron deprivation does not inhibit expression of IL-2R or IL-2 synthesis or of c-*fos* expression in activated T lymphocytes [2, 3, 24], suggesting that these are unlikely to be significant targets for iron chelators.

The iron chelator desferrioxamine inhibited ODC activity in mitogen-activated murine LNC [20]. However, co-treatment of the cells with a relatively high concentration of the polyamine putrescine (the product of the reaction catalysed by ODC) could reverse the effect of desferrioxamine on DNA synthesis only partially, suggesting that in T lymphocytes the antiproliferative effect of iron chelators may include an inhibition of ODC activity but that this cannot be the sole site of action.

Iron deprivation of mitogen-activated T lymphocytes completely blocked synthesis of cyclin A and the cyclin-dependent kinase, cdc2 [3, 22–24]. Inhibition of cyclin A activity by iron chelators may arrest activated T lymphocytes around the G_1/S transition by blocking expression of enzymes involved in DNA synthesis [26–33].

Chelators such as desferrioxamine have been shown to alter the pools of deoxyribonucleotides in mitogen-activated human peripheral blood mononuclear cells [5, 6], suggesting that RR, an iron-containing enzyme, may also be a target for these agents. *Ex vivo* studies have shown direct inhibition of RR, isolated from human lymphocytes, by desferrioxamine [34]. Nyholm and colleagues [35] observed that iron chelators cannot remove iron from the M2 subunit of the enzyme but rather can chelate the iron required for synthesis and regeneration of functional M2 units. Furthermore, transcription of RR may be dependent on cyclin A expression [29], so that its synthesis may also be

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‖ *Abbreviations*: Con A, concanavalin A; DMEM, Dulbecco's Modified Eagle's Medium; IL-2, interleukin-2; IL-2R, interleukin-2 receptor; LNC, lymph node cells; ODC, ornithine decarboxylase; PMA, phorbol myristate acetate; and RR, ribonucleotide reductase.

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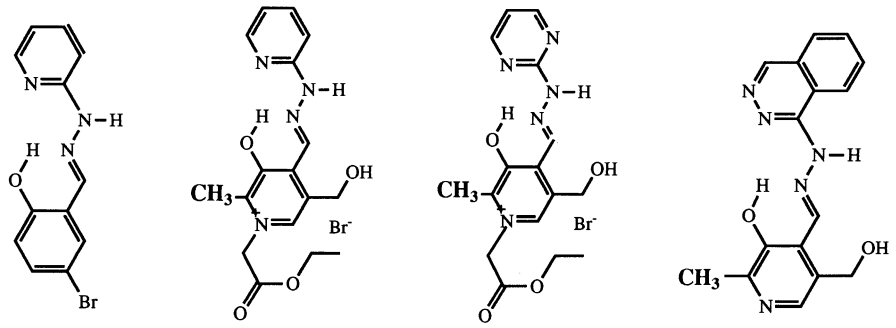

FIG. 1. Some lipophilic chelators of the pyridoxal and salicyl aldehyde classes.

inhibited through a blockade of cyclin A activity induced by iron chelators.

We have investigated the potential immunosuppressive effects of several novel iron chelators of the aryl hydrazone class. These compounds have been synthesised as potential alternatives to desferrioxamine in treating iron overload and as antimalarials [36]. They bind iron preferentially, and the ratio of chelator to metal at neutral pH is 2:1 [36]. They have been shown to promote iron excretion in rats and also to inhibit *Plasmodium falciparum* growth *in vitro* [36], although the antimalarial activity is thought to involve the generation of a carbon-centred radical from the chelator-Fe(II) species rather than a decrease in iron availability [36, 37]. We have shown previously that another related chelator, SAG-15/EPmH (1-[*N*-ethoxycarbonylmethylpyridoxalidenium]-2-[2′-pyrimidinyl]hydrazine bromide, [38]), inhibited *in vitro* proliferation of LNC [21]. The inhibitory effect was blocked by pretreating the chelator with equimolar amounts of either Fe(II) or Fe(III) salts, and any cytotoxic effects were discernible only with prolonged exposure [21]. Now we extend those studies to several other members of this group, namely 1-[*N*-ethoxycarbonylmethylpyridoxylidenium]-2-[2′-pyridyl]hydrazine bromide (EPH), 1-pyridoxylidene-2-[1′-phthalazyl]hydrazine dihydrochloride (PPhH), and 1-[5′-bromosalicylidene]-2-[2″-pyridyl]hydrazine (BsPH) (Fig. 1).

MATERIALS AND METHODS

Reagents

DMEM was purchased from ICN/Flow and was supplemented with folic acid, L-asparagine, L-arginine (all from Sigma), and $NaHCO_3$ (Ajax) as previously described [10, 11]. Complete DMEM was prepared by the addition of 10% (v/v) fetal bovine serum (Commonwealth Serum Laboratories), 10 mM HEPES, 50 μM mercaptoethanol, 2 mM L-glutamine, 1 mM sodium pyruvate (all from Sigma), penicillin (100 U/mL), and streptomycin (100 μg/mL) (both from ICN/Flow) at the time of use. Con A and PMA were purchased from the Sigma Chemical Co., and ionomycin (as the calcium salt) was purchased from Calbiochem. EPH, m.p. 204°, $C_{17}H_{21}N_4O_4Br$ [38]; PPhH, m.p. 283°, $C_{16}H_{18}N_5O_{2.5}Cl_2$*; and BsPH, m.p. 221°, $C_{12}H_{10}N_3OBr$ [39] were synthesised in the laboratory of S. Sarel. Desferrioxamine, as desferrioxamine mesylate (Desferal), was a gift of Ciba Geigy. $FeNH_4(SO_4)_2 \cdot 12H_2O$ was purchased from British Drug Houses.

Protocol for Lymphoproliferation Assays

LNC were harvested from excised lymph nodes of adult male Balb/C mice as previously described [10, 11]. Cells were cultured in round-bottomed 96-well microtitre plates (ICN/Flow) at 5×10^4 cells/well and stimulated with either Con A (4 μg/mL) or the combination of PMA (1 ng/mL) and ionomycin (300 ng/mL). Total volume in each well was 200 μL. Cultures were incubated at 37° and 5% CO_2.

DNA synthesis was assessed by [^{3}H]thymidine incorporation. After 48 hr *in vitro*, 1 μCi [*methyl*-^{3}H]thymidine (ICN/Flow, specific activity 20 Ci/mmol) in 25 μL PBS (Oxoid) was added to each well. After 6 hr (or 4 hr in the thymidine uptake time–course experiments), cells were harvested onto glass fibre discs using a Cambridge PHD Cell Harvester (both from Cambridge Technology). The discs were transferred to plastic scintillation vials (Bacto Labs), 4 mL of Emulsifier Safe liquid scintillant (Canberra Packard) was added to each vial, and the samples were counted on a Beckman LS 60000TA liquid scintillation counter for a maximum of 2 min.

Cell Viability Assays

LNC were harvested and prepared as for the lymphoproliferation studies and were cultured in flat-bottomed 17 mm diameter well tissue culture plates (ICN/Flow) at 6.25×10^5 cells/well in a total volume of 1 mL at 37° and 5% CO_2. After 24 and 48 hr *in vitro*, aliquots of 400–500 μL were transferred to radioimmunoassay tubes (Becton Dickinson).

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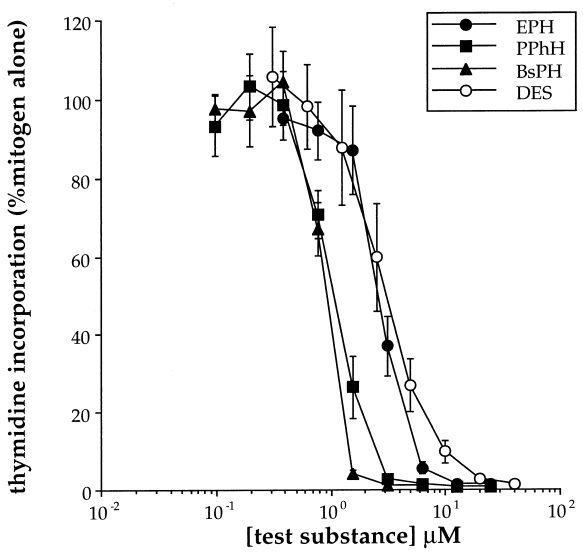

FIG. 2. Inhibition by the four iron chelators of thymidine incorporation in Con A-stimulated LNC. Thymidine incorporation was calculated as (mean counts per minute for mitogen + test substance)/(mean counts per minute for mitogen alone), expressed as %, and results are shown as means ± SEM for 5–9 experiments. The mean counts per minute for mitogen alone were: 70,780 ± 12,680 (mean ± SEM).

The tubes were centrifuged at 180 *g* for 3–5 min, after which the volume of the cell suspension was reduced to approximately 200 μL. Cell viability was assessed by trypan blue exclusion using a Neubauer counting chamber. Just prior to addition to the counting chamber, the cell suspension was taken up into a 1 mL plastic syringe and passed through a 23- to 25-gauge needle 3–4 times to break up clumps of cells. In parallel, cells were cultured in 10-mm diameter well plates (ICN/Flow) at 1.25×10^5 cells/well in 200 μL of complete DMEM and assayed for thymidine incorporation after 48 hr *in vitro*.

Statistical Analyses

The IC_{50} values from individual thymidine incorporation experiments were estimated, and comparisons between those for each of the novel chelators and those for desferrioxamine were made with the Mann–Whitney test.

RESULTS

Inhibition of Lymphoproliferation by the Iron Chelators EPH, PPhH, and BsPH

All three of the new iron chelators were effective inhibitors of thymidine incorporation in LNC stimulated with either Con A or PMA/ionomycin (Fig. 2, Table 1). In terms of their potency, for Con A-stimulated cells EPH and PPhH had an inhibitory capacity comparable to that of desferrioxamine, whereas BsPH was more potent ($P < 0.02$ by the Mann–Whitney test); for PMA/ionomycin-stimulated cells the IC_{50} for all three chelators was significantly lower than that for desferrioxamine (EPH: $P < 0.05$; PPhH and BsPH: $P < 0.01$).

TABLE 1. IC_{50} and IC_{90} values for thymidine incorporation in mitogen-stimulated LNC

	Con A		PMA/ionomycin	
	IC_{50} (μM)	IC_{90} (μM)	IC_{50} (μM)	IC_{90} (μM)
EPH	2.7 ± 0.3	5.7 ± 0.6	1.2 ± 0.2	3.3 ± 0.5
PPhH	1.7 ± 0.4	3.0 ± 0.6	0.3 ± 0.1	1.2 ± 0.1
BsPH	0.9 ± 0.1	1.5 ± 0.0	0.5 ± 0.03	1.1 ± 0.1
DES	3.2 ± 0.6	9.6 ± 2.1	2.9 ± 0.8	14.2 ± 4.8

The concentrations shown are those that led to a 50% (IC_{50}) and 90% (IC_{90}) inhibition of thymidine incorporation as compared with cells treated with mitogen alone and are expressed as means ± SEM (N = 5–9 for Con A and N = 3–6 for PMA/iono). DES = desferrioxamine.

Effect on Cell Viability

The inhibitory effects of these iron chelators were due to cytostatic rather than cytotoxic effects, as there was no significant difference in cell viability between cells treated with Con A plus the chelator and cells treated with Con A alone (Fig. 3). The concentrations tested were those that had been found to lead to >95% inhibition of thymidine incorporation after 48 hr *in vitro* (Fig. 3).

Abrogation of the Inhibitory Effects of EPH, PPhH, and BsPH by Pretreatment of the Chelators with Fe(III)

To confirm that the inhibitory action of the three test compounds was indeed due to the chelation of iron, the effect of pretreating the chelator with an equimolar concentration of Fe(III) ions was assessed. For all three chelators, pretreatment with Fe(III) abrogated the inhibitory response of these compounds (Fig. 4). As also indicated, treating the cells with iron alone did not appear to increase the proliferative response.

Time–Course Studies of Thymidine Incorporation in Mitogen-Stimulated Murine LNC

Further investigation into the mechanisms of action of these chelators was limited to studies using PPhH. To establish the stage of the cell cycle at which these chelators were having their inhibitory effects, a series of preliminary experiments was carried out to estimate the time at which stimulated LNC entered S phase. An increase in thymidine incorporation in LNC was first discernible 16–20 hr after stimulation with either Con-A or PMA/ionomycin (data not shown), continuing to increase up to 40 hr *in vitro*. These results suggest that, under these culture conditions, DNA synthesis and thus entry into S phase in mitogen-activated LNC starts at around 16 hr *in vitro*.

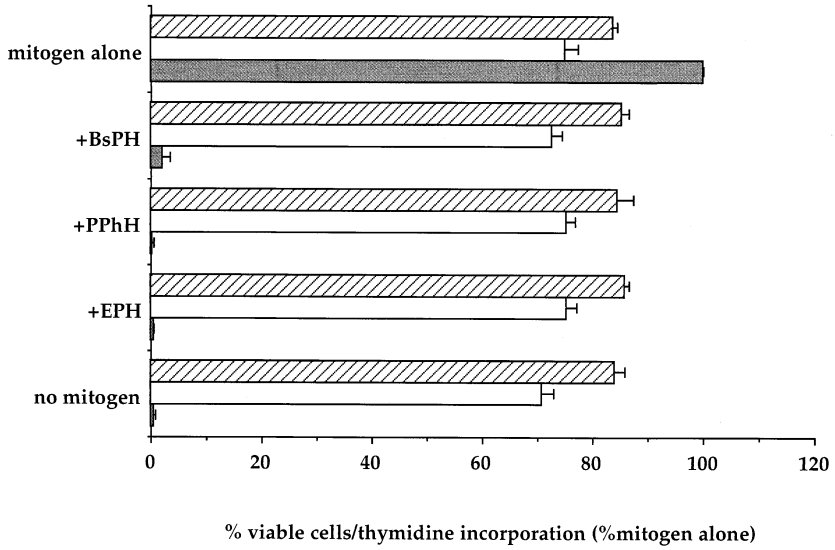

FIG. 3. Cell viability of Con A-stimulated LNC in the presence or absence of the chelators and of unstimulated cells after 24 hr (hatched bars) and 48 hr (open bars) *in vitro* and for thymidine incorporation (filled bars) after 48 hr *in vitro*. The concentrations of iron chelator used were: 1.6 μM (BsPH), 3.1 μM (PPhH), and 12.5 μM (EPH). Results are expressed as means ± SEM for 4–6 experiments. Initial cell numbers were 6.25×10^5 and 1.25×10^5 cells/well for viability and thymidine incorporation assays, respectively. For thymidine incorporation assays in cells stimulated with Con A alone, the mean value (±SEM) was 151,410 ± 34,560 cpm.

Time-Dependent Abrogation of the Inhibitory Effects of PPhH by Fe(III)

The addition of an excess of Fe(III) ions (4-fold for PPhH) up to 8 hr after chelator treatment could block the inhibitory effects of PPhH on thymidine incorporation in Con A-stimulated LNC. After 16 hr of chelator treatment, the capacity of exogenous iron to abrogate the inhibitory effects was attenuated, and this attenuation was greater after 24 hr, whereas at 36 hr the addition of iron had no effect on the activity of the chelators (Fig. 5). Similar results were found with desferrioxamine, where the addition of a 2- to 4-fold excess of Fe(III) up to 8 hr was effective (data not shown).

DISCUSSION

EPH, PPhH, and BsPH are three novel iron chelators, which, in terms of their capacity to inhibit lymphoproliferation *in vitro*, are of similar or greater efficacy compared with desferrioxamine. This may be because they are more lipophilic than desferrioxamine, which could lead to a more efficient uptake by cells [40, 41], a result previously demonstrated for other chelators of this class [42, 43]. Other possible explanations include structural features that facilitate the capacity of these compounds to remove iron from ferritin, or relatively higher binding affinities for iron. The inhibitory effect of all the compounds was not due to cytotoxicity and appeared to involve their capacity to chelate iron.

As members of this group of chelators have been shown previously to be effective when given orally [40, 41] with little evidence of toxicity [40], they have a potential advantage over desferrioxamine in terms of clinical use, as the latter must be administered intravenously [36]. While the major clinical use of chelators has been in treating iron overload, the current as well as previous results suggest their potential as immunosuppressive compounds. Treatment of rats with the iron chelator desferriothiocin resulted in improved survival of cardiac allografts [44]. Additionally, the use of iron chelators as chemotherapeutic agents is suggested by a number of *in vitro* studies demonstrating antiproliferative effects upon leukaemic cell lines of several different classes of iron chelators (reviewed by Richardson [45, 46]), although clinical studies have been restricted to desferrioxamine [47, 48].

The capacity of exogenous iron to abrogate the inhibitory effects of the chelators provided a means of identifying the stage of the cell cycle at which the agents were effective. The mechanism by which the addition of iron facilitated proliferation in chelator-treated cells probably was due to provision of a sufficient excess of metal over what could be complexed by the chelator. Once the sequence of mitogenic activation events had passed the iron chelator-sensitive events, the expectation was that the abrogating effect of exogenous iron would become attenuated. This occurred after the cells had been exposed to PPhH or desferrioxamine for 16 hr. In the LNC, DNA synthesis, as measured by an increase in thymidine incorporation, started between 16 and 20 hr post-stimulation. Thus, the primary inhibitory effects of desferrioxamine and PPhH on LNC proliferation *in vitro* were related to an event(s) late in G_1 and were likely to be linked to inhibition of DNA synthesis. This is supported further by studies showing that addition of an iron chelator can be delayed several hours after mitogenic activation without loss of the inhibitory effects [5, 7, 10, 15, 16].

The current set of studies does not provide further insight into the site of action of the iron chelators. As noted in the introduction, several targets have been identified, namely ODC activity, cyclin A synthesis, and RR synthesis, all of which are necessary for DNA synthesis.

The possibility that the inhibitory effects of the pyridoxal-containing EPH and PPhH are due to their acting as pyridoxal analogs cannot be excluded. However, BsPH is a

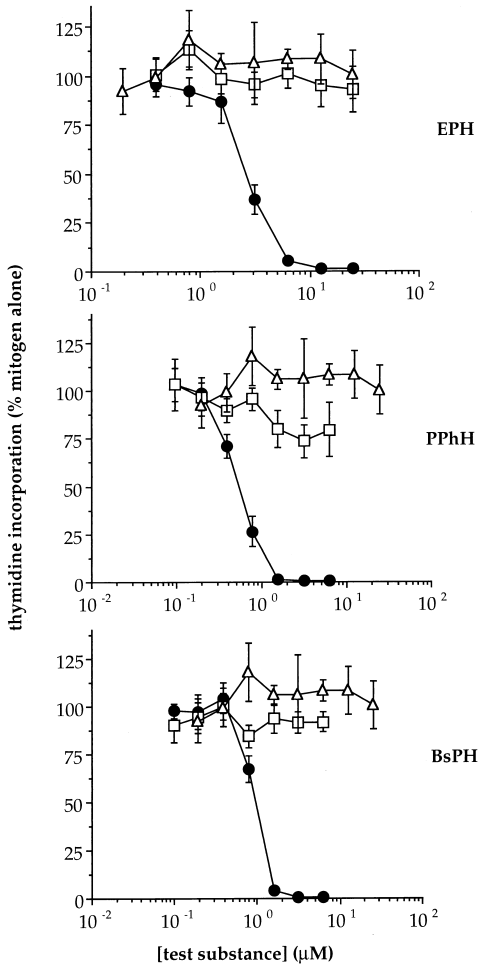

FIG. 4. Comparison of the effects of iron chelator (closed circles), iron chelator pretreated with Fe(III) in a 1:1 ratio (open squares), or Fe(III) alone (open triangles) on thymidine incorporation in Con A-stimulated LNC. Results for all figures were calculated as for Fig. 2 and are expressed as means ± SEM for 4–5 experiments. For cells stimulated with mitogen alone, the mean value (±SEM) was 82,780 ± 12,680 cpm.

salicyl- rather than pyridoxal-based compound, and the IC_{50}/IC_{90} values for all three compounds were similar. Furthermore, they had comparable affinities for iron,* and their inhibitory effects on proliferation were abolished by iron loading (Fig. 4). These observations strongly suggest that it is the capacity to chelate iron that is the basis of the inhibitory effects of all three chelators.

There remains some contrast between the results presented and those supporting pre-commitment events, such as IL-2R expression, as being primary targets for iron chelation [9–11, 16, 17]. However, it should be noted that inhibition of IL-2R expression by iron chelators has required concentrations of chelator in excess of that needed to block DNA synthesis.

In the iron addition studies (Fig. 5, "0–36 hr" conditions), a substantial excess of iron was required to block the inhibitory effect of the chelators, in contrast to the situation where pre-complexing the chelators with an equimolar amount of iron was sufficient to abrogate the effects of the chelators (Fig. 4 and the "pre-Fe" condition in Fig. 5). Previous studies have noted: (i) the rate at which desferrioxamine binds iron, added as $FeCl_3$, is significantly slower in phosphate and HEPES buffers than in Tris [49]; and (ii) in aqueous buffers the formation of the Fe(III):desferrioxamine complex competes with the formation of iron precipitates [35] (also van Reyk D and Hunt N, unpublished observations). Thus, in a complex medium such as complete DMEM (which would contain a number of both protein and low molecular weight chelating species), the capacity of any particular chelator to bind added iron will be determined by the relative strength of competing reactions. Thus, in the studies presented in Fig. 5, a substantial excess of iron was required to saturate the PPhH present and prevent it from interfering with cellular iron stores. The main difference between the studies reported in Figs. 4 and 5 was that in the former set of experiments, premixed iron:chelator stocks were prepared that were diluted upon addition to the cells. In contrast to this, in the latter studies iron stocks were added to chelator that had already been diluted. Thus, the differences in the amount of iron

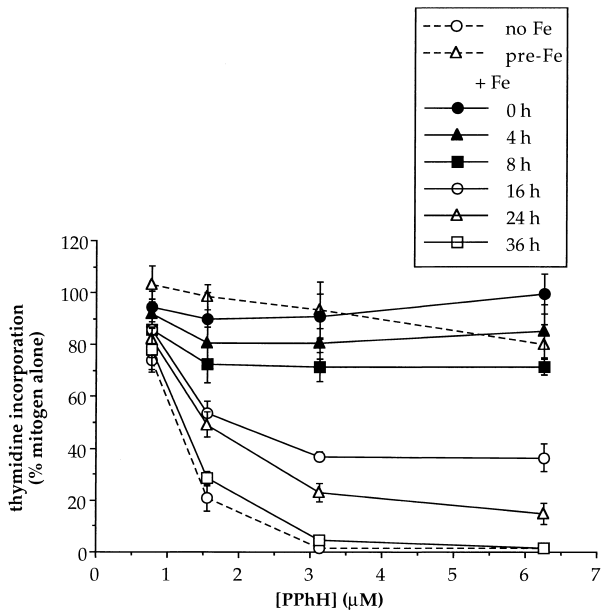

FIG. 5. Effect of the addition of Fe(III) ions to Con A-stimulated LNC treated with PPhH. Cells were treated (i) with the chelator alone (no Fe, open circles and dashed line); (ii) with the chelator precomplexed with an equimolar amount of Fe(III) (pre-Fe, open triangles and dashed line); and (iii) initially with chelator alone and then with Fe(III), in a 4-fold excess (+Fe, solid lines), added to the cells immediately after the chelator (0 hr, closed circles) or after the times specified (4–36 hr after chelator addition). Forty-eight hours after chelator treatment all the cells were assayed for thymidine incorporation. Results were calculated as described in the legend of Fig. 2; values are the means ± SEM for 4–6 experiments. For cells stimulated with mitogen alone, the mean value (±SEM) was 39,740 ± 1,150 cpm.

* Sarel and colleagues, Manuscript submitted for publication.

required may reflect the relative strengths of the different chelating species when the concentration of one species (i.e. PPhH) was varied.

In conclusion, three novel iron chelators have been assayed in terms of their potential antiproliferative activity. In comparison with desferrioxamine they showed a similar, or in some cases greater, capacity to inhibit mitogen-induced thymidine incorporation in LNC without any discernible cytotoxic effect. Further, it was established that the primary effect of these iron chelators upon activated T lymphocytes was related to DNA synthesis rather than to effects on some earlier pre-commitment event.

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