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Immune stimulatory and anti-tumour properties of haemin

A. TSUJI, J. WANG, K. H. STENZEL & A. NOVOGRODSKY* *The Rogosin Institute, Cornell University Medical College and The New York Hospital, New York, NY, USA, *The Rogoff Institute, Beilinson Medical Centre, Petah-Tikva, and Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel*

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SUMMARY

IL-2 induces tumour regression in some patients with metastatic disease, but the dose of IL-2 is limited by severe toxicity. Agents that increase the expression of IL-2 receptors in the effector cells could be used to improve the effectiveness of IL-2 in mediating its anti-tumour effect. We have reported that haemin increased the expression of IL-2 receptors in human peripheral blood mononuclear cells (PBMC) and synergized with IL-2 in the induction of mitogenicity, cytotoxicity and cytokine production. We now report on haemin-induced immune stimulation and tumour regression in mice. Haemin-induced mitogenicity in mouse splenocytes was potentiated up to two-fold by IL-2. The combination of haemin and IL-2 was also effective in inducing cytotoxicity for natural killer (NK)-resistant target cells. Maximal induction of cytotoxicity was attained at an optimal concentration of haemin of 10 μM. Higher concentrations were less effective. Splenocytes isolated from mice that had been treated *in vivo* with haemin and IL-2 incorporated twice the amount of ^{3}H-thymidine compared with splenocytes from mice treated with either haemin or IL-2 alone. Cytotoxicity of splenocytes for NK-resistant target cells was not increased following *in vivo* administration of haemin and IL-2 when fresh splenocytes were tested. Cytotoxicity was enhanced, however, up to five-fold following 48 h *in vitro* incubation with IL-2. Administration of haemin and IL-2 resulted in a significant decrease (40%) of established hepatic metastases in mice. Either IL-2 or haemin alone at the dose used were ineffective. The anti-tumour effect of haemin and IL-2 was enhanced (63% decrease in metastases) by administration of the thiol compound, N-acetylcysteine. Since haemin can safely be administered to patients, it may represent a new class of biologic response modifiers that could enhance IL-2-mediated anti-tumour effects.

Keywords haemin IL-2 cytotoxicity anti-tumour murine splenocytes

INTRODUCTION

IL-2 induces tumour regression in experimental animal models and in patients with metastatic disease [1–3]. In animals IL-2-induced tumour regression is dose-dependent. This effect is potentiated by the administration of autologous lymphokine-activated killer (LAK) cells [1,2]. In clinical trials the dose of IL-2 is limited by severe toxicity [3,4].

Agents that increase the expression of IL-2 receptors in the effector cells could improve the effectiveness of IL-2 in mediating tumour regression. We have found that the oxidizing mitogen, sodium periodate, induces the expression of IL-2 receptors on peripheral blood mononuclear cells (PBMC) and sensitizes them to the effects of IL-2. Combined treatment of cells with IL-2 and periodate, *in vitro*, results in a marked enhancement of both mitogenicity and cytotoxicity [5]. This enhancement is achieved at low levels of IL-2 that alone result in minimal stimulation. These findings provided the basis for a clinical trial in human renal adenocarcinoma using autologous mononuclear leucocytes activated by periodate and IL-2, along with continuous infusion of IL-2 at low dosages [6]. However, the use of activated autologous cells is costly and labour-intensive. It necessitates leukapheresis, cell purification and culture. The procedure requires expensive equipment and disposable supplies, experienced manpower, and carries with it the risk of infection. An alternative to the use of activated cells in adoptive immunotherapy protocols, and a potentially more effective procedure, might be the administration of an agent that was mitogenic or stimulatory for cells of the immune system *in vivo*. A mitogenic agent can increase the number of receptors for IL-2 and render cells more sensitive to its effect in the generation of cytotoxicity and lymphokine production. Mitogen-treated cells may also express different features from those expressed by cells activated by IL-2 alone. These include expression of oncogenes and lymphokine production [7]. Most of the known mitogens, including the mitogenic lectins and the oxidizing mitogens, are unsuitable for *in vivo* use. We have previously

Correspondence: Abraham Novogrodsky MD, PhD, The Rogoff Institute, Beilinson Medical Center, Petah-Tikva 49100, Israel.

reported [8,9] that haemin (iron-protoporphyrin), is mitogenic for human PBMC. Since it has been used in patients with acute intermittent porphyria demonstrating little toxicity [10,11], it could be used as a mitogen *in vivo*.

We have found that haemin, *in vitro*, effectively synergizes with IL-2 in the induction of mitogenicity, cytotoxicity and cytokine production in human peripheral blood mononuclear cells (PBMC) [9]. In this study we evaluated the immune stimulatory and anti-tumour properties of haemin in mice.

MATERIALS AND METHODS

Materials

Haem arginate (Normosang, 25 mg/ml) was obtained from Huhtamaki OY Pharmaceutical (Medica, Helsinki, Finland). Haem arginate is a solution of haemin containing 26·7 mg/ml arginine. This complex of haemin is more stable than haemin itself. Haem arginate was diluted with PBS to the appropriate concentration. Human recombinant IL-2 (3×10^6 Cetus units/mg) was provided by the Cetus Corporation (Emeryville, CA).

Animals

C57Bl/6 mice, 8–10 weeks old, were used. They were obtained from Charles River Lab Inc. (Wilmington, MA).

Tumours

MCA-102 (a gift from Dr Steven A. Rosenberg, National Cancer Institute, Bethesda, MD) is a non-immunogenic fibrosarcoma, syngeneic to the C57Bl/6 strain. The tumour was maintained *in vivo* by serial subcutaneous passages. For experiments, the tumour was excised under sterile conditions, minced with scissors and stirred in a triple enzyme solution of 0·002% deoxyribonuclease, type I, 0·01% hyaluronidase, type V, and 0·1% collagenase, type IV (Sigma Chemical Co., St Louis, MO), at room temperature in Ca^{2+}- Mg^{2+}-free Hanks' balanced salt solution (HBSS) for 3 h. Cells were teased into a single-cell suspension filtered through nylon mesh, washed three times, and resuspended at the appropriate concentration.

Isolation of splenocytes and culture conditions

Spleens were removed aseptically and crushed with the blunt end of a syringe in complete medium (CM), which consisted of RPMI 1640 supplemented with 0·1 mM non-essential amino acids, 1 mM sodium pyruvate, 5×10^{-5} 2-mercaptoethanol, 50 μg/ml gentamycin, 100 μg/ml streptomycin, 100 U/ml penicillin, 0·5 μg/ml fungizone, 0·03% fresh glutamine, and 5% heat-inactivated fetal calf serum. The splenocyte suspension was passed through a layer of nylon mesh and erythrocytes were lysed by suspending the cells in Tris buffer pH 7·4 containing 0·83% NH_4Cl at 37°C for 3 min. The cells were then centrifuged, washed twice with HBSS, and resuspended in CM. Cells (2×10^6/ml) were distributed (0·2-ml aliquots) in flat-bottomed microwells and incubated at 37°C, 5% CO_2 for 68 h. ^{3}H-thymidine incorporation (2 μCi/well) into DNA during the final 20 h of incubation was determined. Triplicate cultures were performed and the means determined.

In vitro *cytotoxicity assay*

Cytotoxicity assays were done as we have previously described [9]. In brief, target cells were labelled with 400 μCi of sodium $^{51}CrO_4$ for 1 h in CM. They were then washed three times and resuspended in CM at 5×10^6 cells/ml. Effector cells, prepared as above, were washed and mixed with target cells at various effector to target ratios and incubated in 96-well, round-bottomed microplates for 3 h at 37°C in a 5% CO_2 incubator. Cell cultures were harvested and supernatants counted in a liquid scintillation counter. Maximum isotope release (MR) was produced by incubation of the targets with 0·1% Triton X-100. Spontaneous release (SR) was measured by incubation of the targets with medium alone. The percentage of cell lysis was calculated by: $ER - SR/MR - SR \times 100$, where ER was the experimental effector release.

Mouse tumour model

Established hepatic metastases using MCA-102 were produced according to the method of Lafreniere & Rosenberg [12]. In brief, C57Bl/6 mice were anaesthetized with 0·2 ml of a 7% pentobarbital solution. The spleens were surgically exposed and 1 ml of tumour cell suspension (5×10^5/ml) in HBSS was injected into the upper pole of the spleen. The spleen was surgically removed after 1 min. Tumour-bearing mice were treated with PBS, IL-2, haemin, and a combination of IL-2 and haemin by i.p. administration. In some experiments mice were injected with N-acetylcysteine (NAC) 500 mg/kg twice a day on days 3 through 7. On day 14, hepatic metastases were counted after i.v. injection of India ink, removal of the livers, and bleaching in Feckete's solution.

Statistical analysis

The significance of differences in numbers of hepatic metastases among groups was determined by the two-tailed Wilcoxon–Mann–Whitney *U*-test.

RESULTS

In vitro *effects of haemin and IL-2 on mouse splenocytes and thymocytes*

Haemin induced mitogenesis in mouse splenocytes and had an additive stimulatory effect when combined with IL-2 (Fig. 1). Peak thymidine incorporation in cells treated with haemin was attained at haemin concentration of 100 μM, and higher levels were less stimulatory (data not shown). The stimulatory properties of haemin were largely dependent on the presence of 2-mercaptoethanol (2-ME) in the culture medium. Haemin augmented IL-2-induced ^{3}H-thymidine uptake in splenocytes over a wide concentration range of IL-2, from 5 to 100 U/ml (Fig. 2). Maximum stimulation was reached at 50 U/ml IL-2. Haemin alone was not stimulatory for mouse thymocytes, but induced blastogenesis in the presence of IL-2 (Fig. 3). High concentrations of haemin were less stimulatory. That is, the stimulatory effect of haemin was maximal at 50 μM and haemin at 100 μM induced significantly less ^{3}H-thymidine uptake (Fig. 3).

Haemin alone induced no cytotoxicity for the natural killer (NK)-resistant target MR-28 (renal adenocarcinoma) cell line, at all haemin concentrations tested. Haemin and IL-2 (50 U/ml) were cooperative in inducing cytotoxicity (Fig. 4). There was an optimal dose of haemin for induction of cytotoxicity (10 μM) and higher doses were less effective. Experiments using the NK-resistant fibrosarcoma MCA 102 and the NK-sensitive lymphone, YAC, gave similar results.

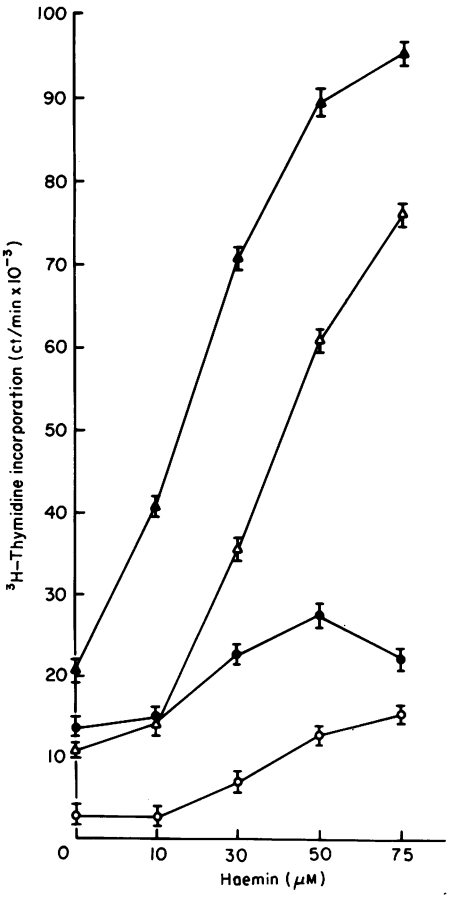

Fig. 1. Stimulation of murine splenocytes by haemin. Cells were incubated with haemin (○); haemin+IL-2 (50 U/ml) (●); haemin+2-mercaptoethanol (2-ME) (5×10^{-5} M) (△); haemin+IL-2+2-ME (▲). Results are expressed as mean ct/min ± s.d. of triplicate cultures from the same experiment. Two additional experiments yielded similar results.

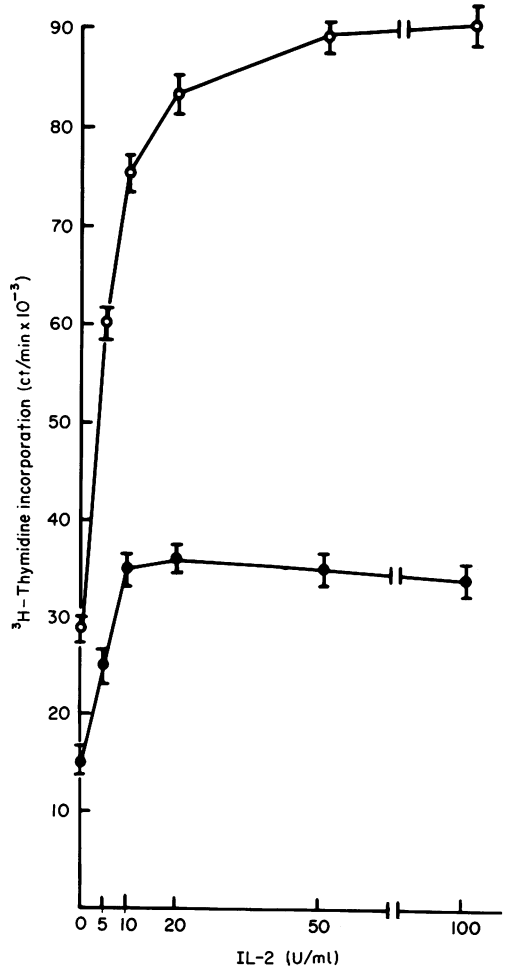

Fig. 2. Effect of IL-2 at different concentrations on haemin-induced stimulation of murine splenocytes. Cells were incubated in the absence (●) and presence (○) of haemin (10 μM). Results are expressed as mean ct/min ± s.d. of triplicate cultures from the same experiment. Two additional experiments yielded similar results.

In vivo *effects of haemin and IL-2 on mouse splenocytes*

Mice were treated with haemin and IL-2, and splenocytes were isolated and tested for proliferative activity. Administration of either haemin or IL-2 alone induced an increased ^{3}H-thymidine uptake in splenocytes, and combined treatment was approximately additive (Table 1). Proliferation was enhanced upon incubation of the splenocytes for 20 h *in vitro* with IL-2 or concanavalin A (Con A) (Table 1).

Splenocytes isolated from mice that had been treated with haemin, IL-2, or the combination of haemin and IL-2, did not exhibit cytotoxicity when fresh splenocytes were tested. However, splenocytes from animals that had been treated with IL-2 and haemin and then incubated *in vitro* in the presence of IL-2 for 48 h, developed cytotoxicity (Table 2). Incubation with IL-2 or haemin alone resulted in only minimal generation of cytotoxicity. Experiments using the NK-resistant MR-28 and MCA 102 cells and the NK-sensitive YAC cells gave similar results.

Anti-tumour effects of haemin and IL-2

Hepatic metastases were induced by intrasplenic injection of a single cell tumour suspension [12] of a methylcholanthrene-induced fibrosarcoma (MCA 102), as described in Materials and Methods. Fourteen days after tumour inoculation, the animals were killed and hepatic metastases counted. Neither haemin nor IL-2 alone, at the doses employed, affected the number of metastases, whereas combined treatment resulted in a significant decrease in the number of lesions (Fig. 5, left panel) ($P=0{\cdot}002$, IL-2 *versus* haemin+IL-2). *In vitro* responses of murine splenocytes are largely dependent on the presence of 2-ME, suggesting that intact thiol moieties are necessary for optimal murine lymphocyte activation. Since haemin could oxidize these groups, we evaluated anti-tumour effects in mice treated concomitantly with the thiol compound N-acetylcysteine. Under these conditions (Fig. 5, right panel), the combined effect of IL-2 and haemin in reducing established hepatic metastases was more pronounced ($P=0{\cdot}05$, haemin+IL-2 *versus* haemin+IL-2+N-acetylcysteine). We observed that the anti-tumour effect of IL-2 alone was modestly enhanced by N-acetylcysteine ($P=0{\cdot}03$), whereas the anti-tumour effect of haemin alone at the dosages employed was not affected.

DISCUSSION

We have previously reported that haemin is mitogenic for human T lymphocytes [8,9] and that it synergized with IL-2 in

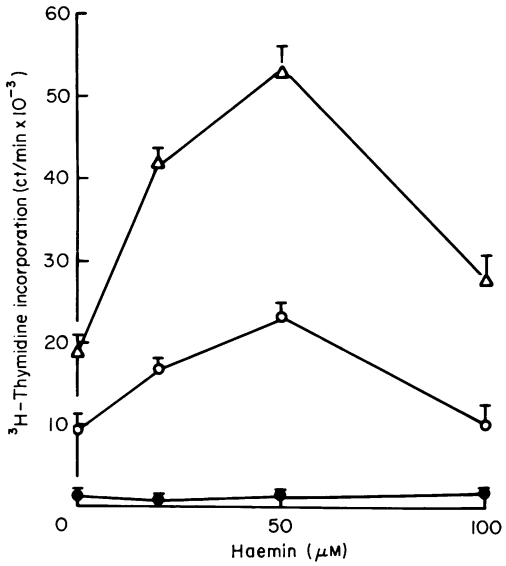

Fig. 3. Haemin and IL-2 induced stimulation of murine thymocytes. Mouse thymocytes were cultured for 48 h in the presence of haemin and IL-2, followed by 20 h pulse with ^{3}H-TdR. ●, No IL-2; ○, IL-2 (20 U/ml); △, IL-2 (100 U/ml). Results are expressed as mean ct/min ± s.d. of triplicate cultures from the same experiment. Two additional experiments yielded similar results.

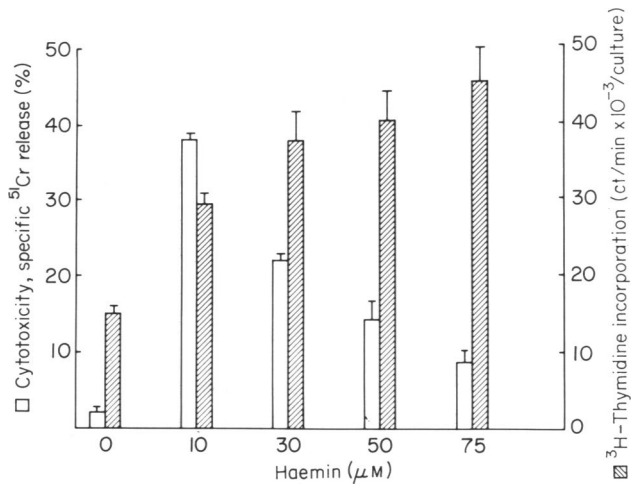

Fig. 4. Haemin-induced cytotoxicity in murine splenocytes. Splenocytes were incubated for 48 h in the presence of haemin at different concentrations and IL-2 (50 U/ml). Cytotoxicity was measured using MR-28 cells as a target at effector to target ratio of 50:1 (□). ^{3}H-thymidine incorporation during the last 20 h of incubation (▨) was measured. Results are expressed as mean ct/min ± s.d. of triplicate cultures from the same experiment. Two additional experiments yielded similar results.

the induction of mitogenicity, cytotoxicity and cytokine production in human PBMC. Results reported here indicated that haemin stimulated murine splenocytes and thymocytes *in vitro* and also elicited lymphocyte stimulation *in vivo*. Stimulation was markedly enhanced on combined treatment with IL-2. Based on our findings in human lymphocytes [9] we assumed

Table 1. Haemin and IL-2 induced mitogenicity *in vivo*

	^{3}H-thymidine incorporation† (ct/min ± s.d.)		
Treatment *in vivo**	None	IL-2 (50 U/ml)	Con A (0·2 μg/ml)
PBS	6560 ± 1071	8069 ± 746	22232 ± 8048
IL-2	14836 ± 1582	19606 ± 319	32725 ± 7590
Haemin	15822 ± 1890	24257 ± 444	25622 ± 6933
Haemin + IL-2	31417 ± 2663	33950 ± 2207	56995 ± 8949

* IL-2 (20000 U intraperitoneally twice daily) was given on days 1-5, and haemin (3 mg/kg intraperitoneally once daily) on days 1, 3, and 5. Mice were killed on day 5 and splenocytes were isolated.

† Splenocytes were cultured for 20 h with no addition, IL-2 (50 U/ml), or concanavalin A (Con A; 0·2 μg/ml) and ^{3}H-TdR incorporation during this period was determined. Results are expressed as mean ct/min ± s.d. of triplicate cultures from the same experiment. Three additional experiments were performed with similar results.

Table 2. Haemin and IL-2 induced immune stimulation *in vivo*

	Specific ^{51}Cr release (%)†				
	Exp. 1		Exp. 2	Exp. 3	
Treatment *in vivo**	100:1	50:1	50:1	50:1	25:1
None	—	—	4·4	0	0
IL-2	12·9	0	4·6	8·4	0·9
Haemin	15·5	1·2	5·8	12·7	7·2
Haemin + IL-2	34·2	14·1	28·4	23·1	17·3

* IL-2 (20000 U intraperitoneally twice daily) was given on days 1, 3, and 5, and haemin (3 mg/kg intraperitoneally once daily) on days 1, 3, and 5. Mice were killed on day 5 and splenocytes were isolated.

† Splenocytes were incubated for 48 h in the presence of IL-2 (50 U/ml) followed by 3 h ^{51}Cr release assay for cytotoxicity, using MR-28 cells as a target. Effector to target ratios were 100:1 to 25:1.

that the cooperative stimulatory effect of haemin and IL-2 on murine lymphocytes resulted from the increase in IL-2 receptors induced by those agents. Large granular lymphocytes are probably mediating the cytotoxicity induced by haemin and IL-2, and T cells are the responding proliferating cells.

Murine splenocyte response to haemin and IL-2, *in vitro*, was markedly enhanced by 2-ME, as was previously shown for other mitogens. The thiol compound N-acteylcysteine was also effective in supporting haemin stimulation, whereas reduced glutathione was less effective.

Fresh splenocytes isolated from haemin- and IL-2-treated animals did not exhibit significant cytotoxicity. However, 48-h incubation with IL-2 generated cytotoxicity, suggesting an increase in LAK cell precursors in treated animals.

Our major goal in these studies was to increase the effectiveness of IL-2 in mediating tumour regression. The data

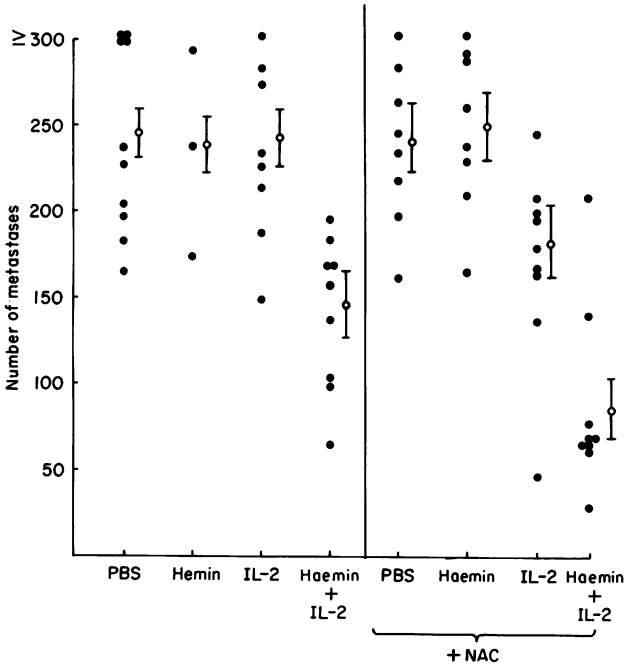

Fig. 5. The anti-tumour effect of haemin and IL-2 against established murine hepatic metastases. Left panel: C57Bl/6 mice were injected intrasplenically with 5×10^5 MCA-102 cells (day 0). Mice were treated on days 3, 5 and 7 with haemin (3 mg/kg per day) interaperitoneally, and on days 3 to 7 with IL-2 (20 000 U/mouse) intraperitoneally twice daily. Mice were killed 14 days after inoculation. Right panel: same as left panel but in addition, N-acetylcysteine (500 mg/kg) was given intraperitoneally twice a day on days 3 to 7. ●, Number of metastases found in each liver; ○, mean number of metastases ± s.e.m. NAC, N-acetylcysteine.

indicate that combined treatment with haemin and a low dose of IL-2 results in a significant decrease in experimental tumour metastases. Preliminary studies also suggested that combined treatment with haemin and IL-2 was more effective than either agent alone in prolonging survival in mice bearing renal cell (MR-28) tumours. The exact mechanism of tumour regression induced by the treatment is unknown. The direct inhibitory effect of the treatment on the growth of the tumour was considered. However, this possibility is less plausible due to the finding that either haemin (50 μM) or IL-2 (500 U/ml) alone or in combination did not inhibit the growth of MCA 102 cells *in vitro*. The anti-tumour effect *in vivo* was attained by combined treatment with haemin and IL-2, suggesting that it is related to the activation of host effector mechanisms. The anti-tumour effect is probably the result of a complex interaction of cellular and humoral events. We found that haemin, in addition to its mitogenic properties and its ability to generate cytotoxicity, effectively stimulated production of tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) in human PBMC [9].

Some of the anti-tumour properties of haemin could result from its ability to activate monocytes. In preliminary experiments we have found that i.p. administration of haemin to mice resulted in activation of monocytes. Activation was assessed by measuring hydrogen peroxide production triggered by tetradecanoyl phorbol acetate (TPA) using a method developed by De La Harpe & Nathan [13]. The biochemical mechanisms responsible for the haemin-mediated lymphocyte activation are unknown. Data from our laboratory suggest that the oxidative properties of haemin are involved in this process. We have found that scavengers of oxygen-derived radicals inhibit haemin-induced mitogenesis [9]. Our recent findings indicated that haemin elicits an insulin-like effect on human PBMC. Namely, haemin enhanced glucose uptake and stimulated protein tyrosine phosphorylation [14]. This process was associated with oxidation-mediated modulation of phosphotyrosine phosphatase. In this respect haemin resembles hydrogen peroxide, which has been found to induce tyrosine phosphorylation in a variety of cell types [15,16].

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