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Increased Intracellular Killing of Bacteria *in vitro* by Monocytes of Patients with Metastatic Melanoma Before and During Treatment with Interferon-γ and Interferon-α

Susanne Osanto, Maria T. van den Barselaar and Jaap T. van Dissel

The effect of recombinant human interferon-γ (rHuIFN-γ) and interferon-α (rHuIFN-α) as *in vivo* stimuli for the activation of human monocytes was investigated on the basis of the bactericidal activity of peripheral blood monocytes in 11 patients with metastatic melanoma before and during treatment with interferons. Patients received increasing doses of rHuIFN-γ and a fixed dose of rHuIFN-α, both administered subcutaneously three times a week. The rates of intracellular killing of *Listeria monocytogenes* and *Salmonella typhimurium* after *in vitro* phagocytosis by monocytes collected from melanoma patients before interferon treatment were increased ($P < 0.01$) by a factor of 1.7 and 1.4, respectively, relative to the rate constants in blood monocytes of healthy donors. During treatment with the interferons, the rates of intracellular killing of the bacteria by patients' monocytes did not further increase. The findings underscore the immunogenicity of malignant melanoma and put into question the macrophage activating activity of IFN-γ with respect to the bactericidal activity of monocytes.
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INTRODUCTION

INTERFERON-GAMMA (IFN-γ) is believed to be the most important cytokine for the activation of mononuclear phagocytes in cell-mediated immune reactions to infectious agents and tumours leading to the expression of increased antimicrobial and tumoricidal activity by these cells [1–5]. Recent findings in peritoneal macrophages of mice have shown, however, that the activation of antimicrobial activity cannot be attributed solely to the action of IFN-γ [6–8].

In earlier studies on macrophage activation [7–11], the rate of intracellular killing of the facultative intracellular microorganisms *Listeria monocytogenes* and *Salmonella typhimurium* was investigated, because non-specifically expressed antibacterial activity probably reflects most closely the state of activation of mononuclear phagocytes [12]. Interferons have been studied in clinical trials against a wide variety of tumours for more than a decade. IFN-γ, more recently prepared by recombinant technology, is the latest IFN species to enter broad clinical evaluation. Combinations of cytokines are under investigation at present because of their additive or synergistic antitumour activity *in vitro* and *in vivo*. The combination of IFN-alpha (IFN-α) and IFN-γ has been shown to have synergistic antiproliferative effects on melanoma and other tumour cells [13–15] and this provides a basis for clinical studies employing the combination of IFN-α and IFN-γ in the treatment of cancer patients. A clinical study on the efficacy of recombinant human interferon alpha (rHuIFN-α) and gamma (rHuIFN-γ) in the treatment of patients with malignant melanoma offered an opportunity to find out whether interferon treatment enhanced the antimicrobial activity of human blood monocytes against *L. monocytogenes* and *S. typhimurium*.

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MATERIALS AND METHODS

Patients and controls

11 patients with metastatic melanoma were studied. No evidence of infection was found in any of the patients. The control group consisted of a total of 59 healthy donors of the blood bank. In each experiment the rate of intracellular killing of bacteria by blood monocytes was determined simultaneously for the patients and controls.

Treatment regimen

Recombinant human (rHu) IFN-γ (2×10^7 IU/mg protein) and rHuIFN-α (3.83×10^8 IU/mg protein), kindly provided by Boehringer Ingelheim BV (Alkmaar and Vienna) were supplied in vials in lyophilised form, and were reconstituted with sterile pyrogen-free water immediately prior to subcutaneous administration. Both interferons were administered simultaneously three times a week by subcutaneous injection at sequentially rotated sites, i.e. a fixed rHuIFN-α dose of 5.2 μg/m² (2×10^6 IU/m²) combined with a starting rHuIFN-γ dose of 0.05 mg/m² followed by escalation of rHuIFN-γ to 0.1 mg/m² on day 15 and to 0.2 mg/m² on day 29.

Microorganisms

Salmonella typhimurium (phage type 510) were grown in nutrient broth No. 2 (Oxoid, London) for 18 h at 37°C, collected by centrifugation (10 min at 1500 *g*), washed twice with phosphate-buffered saline (PBS), and suspended at appropriate bacterial concentrations in Hanks' balanced salt solution (HBSS, Oxoid). *Listeria monocytogenes* (EGD) was maintained in tryptose

phosphate broth (TPB) supplemented with 10% glycerin at −70°C. For each experiment a sample was thawed, inoculated into 10 ml TPB, and incubated for 18 h at 37°C. Suspensions were prepared as described for *S. typhimurium*.

Peripheral blood mononuclear cells

Peripheral blood mononuclear cells were isolated by Ficoll–Hypaque from samples collected by venepuncture before the start of treatment and after 2 and 4 weeks of treatment. The interface layer (monocytes and lymphocytes) was washed four times with PBS, suspended in gelatin-HBSS, and adjusted to appropriate concentrations of 5×10^6 monocytes/ml. The viability of all cells, as checked by trypan blue exclusion, was > 98% before use in phagocytosis and intracellular-killing assays.

Assay of intracellular killing

Intracellular killing of *S. typhimurium* and *L. monocytogenes* by peripheral blood mononuclear cells was measured as described in detail elsewhere [16, 17]. For the assessment of intracellular killing by patient or normal donor control cells, the bacteria were preopsonised with normal AB human serum, after which 5×10^6 bacteria/ml were added to a suspension of 5×10^6 monocytes/ml. Phagocytosis was allowed to proceed for 3 min at 37°C under rotation at 4 rpm, and ingestion was stopped by shaking the tube in crushed ice; the non-ingested bacteria were then removed by centrifugation for 4 min at 110 *g* followed by two washes with ice-cold HBSS. Next, the cells containing ingested bacteria were resuspended in gelatin-HBSS to a concentration of 5×10^6 monocytes/ml and reincubated at 37°C under rotation (4 rpm) in the presence of 10% normal AB serum. At various time-points, a 50 µl sample of the suspension was brought into 450 µl ice-cold distilled water containing 0.01% bovine serum albumin before vigorous mixing to lyse the cells. Finally, the number of viable intracellular bacteria was determined microbiologically.

Calculation of the rate constants of intracellular killing

Rate constants (K_k; per min) were calculated as a measure of the intracellular killing of bacteria according to the equation $K_k = (\ln N_t - \ln N_o)/t$, in which N_o is the number of viable intracellular bacteria at zero time and N_t is the number of viable intracellular bacteria at time t. Values given represent the mean rate constants as calculated after 15, 30, 60 and 90 min incubation during each intracellular killing assay.

Analysis of data

For each experiment the rate constants of intracellular killing by monocytes of patients and healthy control donors were determined simultaneously under identical conditions and were compared by the Student's paired t test. Correlation analysis was performed to detect relationships between clinical parameters and the rates of intracellular killing of monocytes of patients before and during interferon treatment.

RESULTS

Clinical effects of interferon treatment

The clinical characteristics of the patients, including age, sex and clinical condition before entry to the study and survival time since start of IFN treatment are shown in Table 1, as described in detail [18]. No antitumour responses were observed following IFN treatment.

Table 1. Characteristics of the 11 patients entered in the study

No. of patients	11
Men	7
Women	4
Age	
Range (yr)	29–75
Median	45
Performance status (ECOG) 0–2	11
Survival time	
Range (months)	2–16
Median	8.3

Toxicity of treatment with the combination of interferons was mild. Rigours occurred in all patients after the first dose of IFN, but were rare during the further course of treatment. All patients experienced fever between 38 and 40°C at all dose levels. 5 patients complained of fatigue, 2 of anorexia and nausea and 1 patient complained of headache and myalgia during the course of treatment.

Haematological toxicity was slight. The course of granulocyte, lymphocyte and monocyte counts before and during treatment is shown in Table 2. In the majority of patients the granulocyte and lymphocyte counts somewhat decreased, while monocyte counts remained constant during treatment.

Intracellular killing of L. monocytogenes by monocytes of melanoma patients and donors

After *in vitro* phagocytosis of preopsonised *Listeria* for 3 minutes, the number of viable intracellular bacteria amounted to 0.3 to 2.8×10^6 per 5×10^6 monocytes of the patients. This number did not differ ($P > 0.20$) before and during interferon treatment. Incubation of patients' and donor monocytes containing *L. monocytogenes* for 90 min at 37°C led to a curvilinear decrease in the number of viable intracellular bacteria (Fig. 1). The curvilinear decrease allowed calculation of the rate constants of intracellular killing, that for patients' monocytes on average amounted to about 0.034/min (Table 3). After 2 and 4 weeks of treatment with interferons in the majority of cases microbicidal activity of blood monocytes against *L. monocytogenes* was not further enhanced compared to the level of activation before the start of treatment. In 2 patients, however, the rate of intracellular killing of *L. monocytogenes* almost doubled after 4 weeks of treatment.

The rate constant of intracellular killing of *L. monocytogenes* by monocytes of blood donors on average amounted to

Table 2. Course of peripheral blood cells before and during interferon treatment

	Before treatment	During treatment with IFN	
		2 weeks	4 weeks
Granulocytes	4.77 (1.36)	3.33 (1.23)	3.28 (1.28)
Lymphocytes	1.53 (0.48)	1.50 (0.75)	1.33 (0.53)
Monocytes	0.43 (0.20)	0.42 (0.21)	0.40 (0.27)

Values give the absolute number (x 10^9/l) (S.D.) of granulocytes, lymphocytes and monocytes both before and 2 and 4 weeks after the start of treatment for 11 patients.

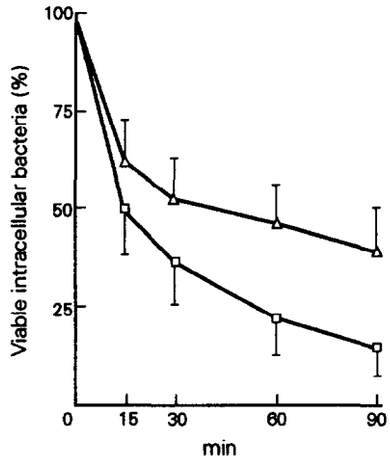

Fig. 1. Course of intracellular killing of *L. monocytogenes* (open triangles) and *S. typhimurium* (open squares) by monocytes of healthy controls. Values represent means (S.D.).

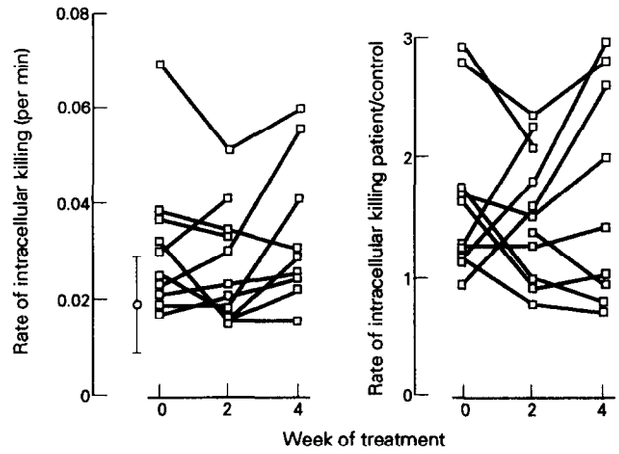

Fig. 2. (Left panel) Rates of intracellular killing and (right panel) ratio of rates of intracellular killing of *Listeria monocytogenes* by blood monocytes of patients and healthy donors, as determined in pairs. Values of individual patients are connected by lines. Open circle and bars indicate mean values (S.D.) of control monocytes.

*Table 3. Rates of intracellular killing of Listeria monocytogenes and Salmonella typhimurium by blood monocytes of melanoma patients and healthy donors**

Microorganisms	Rate of intracellular killing		
	Before treatment	During treatment 2 weeks	4 weeks
L. monocytogenes			
Patients	0.030 (0.014)	0.029 (0.011)	0.044 (0.030)
Controls	0.017 (0.004)	0.020 (0.004)	0.023 (0.007)
Ratio†	1.75	1.45	1.89
S. typhimurium			
Patients	0.049 (0.026)	0.045 (0.019)	0.045 (0.019)
Controls	0.034 (0.007)	0.034 (0.006)	0.033 (0.010)
Ratio†	1.46	1.31	1.36

* Values give the mean (S.D.) constant of intracellular killing by blood monocytes of patients and healthy blood donor controls; each value represents 8–11 patients and an equal number of controls.

† Values give the mean ratio of the rate constants of intracellular killing by blood monocytes of patients vs. healthy controls, as determined in each individual experiment.

0.020/min, both before and during interferon treatment (Table 3; Fig. 2, left panel).

In monocytes of controls the relative coefficient of variation of the assay only amounted to 4–6%, indicating that the interexperimental variability of the killing rates by monocytes of control blood donors is quite low. By contrast, there was a considerable interindividual variability of the results for monocytes of patients' reflecting wide differences in killing efficiency between monocytes of different patients. The ratio of the killing rates by monocytes of patients and donors was on average 1.7, irrespective of the administration of interferon (Table 3; Fig. 2, right panel), indicating that monocytes of patients were far more effective ($P < 0.01$) in the killing of *L. monocytogenes*.

Intracellular killing of S. typhimurium by monocytes of melanoma patients and donors

After *in vitro* phagocytosis of preopsonised *Salmonella* during 3 minutes, the number of viable intracellular microorganisms amounted to 0.5 to 2.7 $\times$ 10^6 per 5 $\times$ 10^6 monocytes of the patients. Incubation of patients' and donor monocytes containing *S. typhimurium* for 90 min at 37°C led to a curvilinear decrease in the number of viable intracellular bacteria (Fig. 1). The curvilinear decrease allowed calculation of the rate constants of intracellular killing, that for patients' monocytes on average amounted to about 0.046/min and similar rate constants were obtained before and during interferon treatment (Table 3; Fig. 3, left panel). The rate constant of intracellular killing of *S. typhimurium* by monocytes of blood donors on average amounted to 0.033/min.

The ratio of the killing rates for monocytes of melanoma patients and healthy donors was on average 1.4, irrespective of the administration of interferon (Table 3; Fig. 3, right panel), indicating that monocytes of patients were more effective ($P < 0.01$) in the killing of *S. typhimurium*.

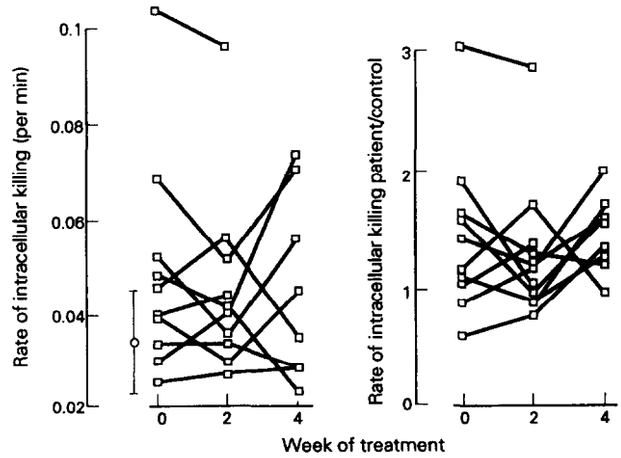

Fig. 3. (Left panel) rates of intracellular killing and (right panel) ratio of rates of intracellular killing of *Salmonella typhimurium* by blood monocytes of patients and healthy donors, as determined in pairs.

In monocytes of controls the relative coefficient of variation of the assay only amounted to 4–7%, but there was a considerable interindividual variability of the results for monocytes of patients.

Correlation analysis

No correlation ($0.16 < P < 0.98$) was found between the capacity of patients' monocytes to kill *L. monocytogenes* and *S. typhimurium* both before and during treatment and any of the parameters already studied, i.e. total numbers of peripheral blood monocytes ($\times 10^9$/l), peak serum levels of rHuIFN-γ or their area under the curve (AUC), mean level of HLA-DR expression on monocytes or changes in that level, and the duration of patient survival after the start of interferon treatment [18].

DISCUSSION

The present findings show that for the functions under study, rHuIFN-γ administered in increasing doses together with a low dose of rHuIFN-α does not activate the monocytes of patients with metastatic malignant melanoma. However, the monocytes of melanoma patients were already more active than those of healthy controls before the start of interferon treatment, whereas granulocytes from patients and controls did not differ with respect to killing activity (data not shown). The peripheral blood monocytes of melanoma patients killed intracellular *Listeria monocytogenes* about 1.7 times more efficiently ($P < 0.01$) and *Salmonella typhimurium* about 1.4 times more efficiently ($P < 0.01$) than normal monocytes did. These killing-rate ratios were not affected by interferon treatment. In parallel with these findings before the start of treatment the mean expression level of HLA-DR of patients' blood monocytes was already higher than that of control monocytes [18].

The inability of rHuIFN-γ to enhance the activity of peripheral human blood monocytes to kill ingested bacteria is in contrast with the observed ability of rHuIFN-γ to enhance the production of reactive oxygen intermediates by monocytes of cancer patients [19, 20]. Moreover, intravenously administered rHuIFN-γ has proven to be a potent inducer of HLA-DR expression in blood monocytes [18–20].

However, rHuIFN-γ-induced changes in biochemical correlates of macrophage activation, such as an enhanced capacity to secrete reactive oxygen intermediates or enhanced HLA-DR expression, are not always accompanied by increased antibacterial activity. In the mouse, divergent responses with respect to the activation of various effector functions of monocytes were seen after activation of macrophages by bacteria or cytokines [7, 8]. For instance, peritoneal macrophages from BCG-infected mice given an intraperitoneal booster with PPD show increased listericidal activity, but similar enhanced activity is not shown by recombinant murine IFN-γ activated macrophages. Although after both methods of activation macrophages display increased oxidative metabolism and toxoplasmostatic activity, they do not kill *S. typhimurium* more efficiently than normal resident macrophages do.

Since monocytes of melanoma patients showed greater antibacterial activity before the start of treatment than monocytes of healthy controls did, the absence of enhancement of killing activity after administration of rHuIFN-γ could be explained if the cells were already maximally activated for this effector function. In this respect, also antagonistic effects of IFN-α and IFN-γ on the activation of certain effector functions of mononuclear phagocytes could be relevant [21, 22]. The literature includes reports of depressed, normal and increased activity of effector functions of mononuclear phagocytes in patients with various malignant diseases [23–29]. In our study the monocytes originated from patients with malignant melanoma, a neoplasm that in particular appears to be capable of eliciting a strong immunological response in the host [30–34]. In this context, it may be significant that the interindividual variability of the killing rates by monocytes was much greater in patients than in control monocytes, even in individual patients at various time points, suggesting that some environmental factors in melanoma patients *in vivo* influenced the subsequent killing rate of bacteria by monocytes *in vitro*.

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Inhibition of Growth of Colon 38 Adenocarcinoma by Vinblastine and Colchicine: Evidence for a Vascular Mechanism

Bruce C. Baguley, Karen M. Holdaway, Lindy L. Thomsen, Li Zhuang and L. Jonathan Zwi

Vinblastine or colchicine, administered intraperitoneally to B6D2F$_1$ mice with advanced subcutaneous colon 38 tumours, induced substantial tumour growth delays with progressive development of haemorrhagic necrosis beginning within 8 hours of treatment. Two multidrug-resistant P388 leukaemia sublines, refractory to vinblastine and vincristine when grown as intraperitoneal ascites, were sensitive to necrosis induction when grown as subcutaneous tumours. Vascular labelling with two fluorescent markers indicated that vincristine substantially reduced tumour blood flow within 4 hours after treatment. The effects of vinblastine, vincristine and colchicine were similar to those of tumour necrosis factor alpha in that: (a) similar tumour necrosis and blood flow changes were induced, (b) coadministration of the serotonin antagonist cyproheptidine prevented tumour necrosis and (c) plasma nitrate levels were elevated, indicative of the stimulation of oxidation of L-arginine to nitric oxide. The results suggest that vinca alkaloids and colchicine act on solid tumours by host cell-mediated vascular effects as well as by direct tubulin-mediated cytotoxicity.
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