Effects of Interferons and Cytokines on Melanoma Cells

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This review summarizes recent information on the effects of immunomodulatory cytokines on human melanoma cells. The action of interferon (IFN)-alpha, -beta, and -gamma has been extensively examined in melanoma and melanocyte cultures *in vitro*, and increasing information on the action of other cytokines is now available. All IFNs revealed a dose-dependent antiproliferative effect on melanoma cells with the highest growth inhibition caused by IFN-beta. Proliferation was also inhibited by interleukin (IL) 1-alpha and -beta, and tumor necrosis factor (TNF)-alpha. For IL-4, both growth-stimulatory and -inhibitory properties have been reported. Cellular differentiation in terms of melanin synthesis, formation of dendritelike structures, and antigenic changes was not affected by IFN-alpha or -beta. IFN-gamma, however, induced a more dedifferentiated and biologically more aggressive phenotype of melanoma cells. Histocompatibility antigen (HLA) class I molecules were found upregulated by all IFNs and by TNF-alpha, associated with a marked increase of melanoma cell lysis by tumor infiltrating lymphocytes in vitro. HLA class II molecules were *de novo* expressed or enhanced by IFN-gamma and TNF-alpha. The adhesion molecules ICAM-1, LFA-3, and VLA-2 were upregulated by IFN-gamma, TNF-alpha, and IL-1-beta, whereas melanoma-associated antigens were hardly affected by cytokines. It seems that both antiproliferative and immunomodulatory effects may contribute to the antitumoral activity of cytokines *in vivo*. *In vivo* application of cytokines as well as combinations with cytotoxic drugs, therefore, may be promising for future treatment strategies. *J Invest Dermatol 100:239S–244S, 1993*

Recombinant technology has allowed large scale cytokine production and thereby provided the facilities for new cancer treatment concepts. The finding of direct antiproliferative effects of different interferons (IFNs) on melanoma cells *in vitro* focused the attention on these substances as possible antitumor agents for therapeutic purposes [1,2]. Additionally, the involvement of IFNs in the regulation of the immune system and in cellular differentiation has been described [3–5]. These different mechanisms of action may possibly contribute to the antitumoral activity of IFNs, which are used today for systemic treatment of metastatic melanoma and are combined for this purpose with different cytokines and cytotoxic drugs [6]. Several new cytokines have been detected and characterized in the last years and some of their effects on melanoma cells have been investigated recently (Table I). Interleukin (IL) 1, IL-4, tumor necrosis factor (TNF) alpha, and transforming growth factor (TGF) beta-1 inhibited melanoma cell proliferation *in vitro* [7–13]. IL-6 showed growth-stimulatory properties in murine melanoma cells [14]. The present knowledge on cytokine effects in benign and malignant melanocytes is mainly based on investigations *in vitro*. So far, there are only few systematic investigations on the different effects of single cytokines on melanoma cells. This review summarizes the available data on IFNs and other cytokines on the proliferation, cellular differentiation, the antigenic profile of melanoma cells, and their indirect effects via immune responses of the host.

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Abbreviations:
CD: cluster of differentiation
GD2, GM2: ganglioside D2, M2
HLA: histocompatibility antigen
HMW-MAA: high molecular weight-MAA
ICAM-1: intercellular adhesion molecule-1
IFN(s): interferon(s)
IL(s): interleukin(s)
LFA-3: leukocyte function antigen-3
MAA: melanoma associated antigen
MoAb(s): monoclonal antibody(ies)
NK: natural killer
TGF-beta: transforming growth factor-beta
TIL: tumor infiltrating lymphocytes
TNF-alpha: tumor necrosis factor-alpha
VLA: very late antigen

DIRECT ANTIPROLIFERATIVE EFFECTS

Interferons The role of IFNs as growth inhibitory compounds for human melanoma cells *in vitro* is well established. Czarniecki *et al* described synergistic antiviral and antiproliferative effects of recombinant human IFN-alpha, -beta and -gamma [2]. Both type I and II IFN are capable of inhibiting the proliferation of fresh and long-term cultured melanoma cells either in soft agar or monolayer systems [1,2,15,16]. Contradictory results have been reported on which type of IFN is most effective with respect to antiviral units or molar concentrations. Although some investigators report type II IFN-gamma as the most potent agent [17], we and others found a clearly higher efficiency of IFN-beta compared to both IFN-alpha and IFN-gamma to inhibit the proliferation of established melanoma cell lines (Fig 1) [5,16]. These effects were predominantly cytostatic. Removal of IFN from the culture medium resulted in a rapid onset of proliferation (Fig 2). The combination of type I IFNs, IFN-alpha, and IFN-beta, which bind to the same receptor, resulted in subadditive antiproliferative effects, possibly due to decreased binding of IFN-beta to type I IFN-receptor [16]. On the other hand, combination of type I and II IFN led to a strong synergistic action of both compounds [16]. Comparative studies performed on normal human melanocytes showed a strong antiproliferative activity of IFN-beta and little or no action of IFN-alpha or -gamma [18].

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Table I. Cytokines Investigated for Their Effects on Melanoma Cells

Molecules	Synonyms	Chromosomal Localization	Protein Size Amino Acids	Molecular Weight (kD)	Effects on Melanoma Cells (References)
IFN-α	Lymphoblast IFN	9p13-21	165	15–24	[1,2,6,15,26]
IFN-β	Fibroblast IFN	9p22	166	20	[1,2,6,15,36]
IFN-γ	Immune IFN	12	143	15–45	[2,6,9,36,39]
TNF-α	Cachectin	6p23;q12	157	17	[9,11,13,48]
IL-1 α	Hemopoietin-1	2q14	269;152	31;17	[8,26]
IL-1 β		2q14	269;152	31;17	[8,9]
IL-4	B-cell stimulatory factor	5q	129	15–20	[9,10]
IL-6	IFN-ß2	7q	212	24	[14,20]
TGF-β-1		19q13.1	112	12.5	[7,22,24]

Tumor Necrosis Factor-Alpha No conclusive information exists on the capacity of TNF-alpha to inhibit the proliferation of cultured melanoma cells [9,13]. Apparently, only a subgroup of melanoma cell lines responded to TNF-alpha, and TNF-alpha, having cytotoxic rather than cytostatic effects, may select *in vitro* for melanoma cells with enhanced malignancy [13]. In animal models TNF-alpha revealed a significant antitumoral activity against malignant melanoma [19]. Additional studies performed on melanocyte cultures by Swope *et al* showed that TNF-alpha is able to inhibit both ^{3}H thymidine incorporation into the DNA and cell proliferation [20].

Transforming Growth Factor-Beta Melanoma cells can produce TGF-beta, a potent immunoregulatory mediator [21,22]. Although TGF-beta stimulates proliferation of murine melanoma cells [23], various reports ascribe a growth-inhibitory role for this molecule in the human system [7,22,24]. On cultured melanocytes,

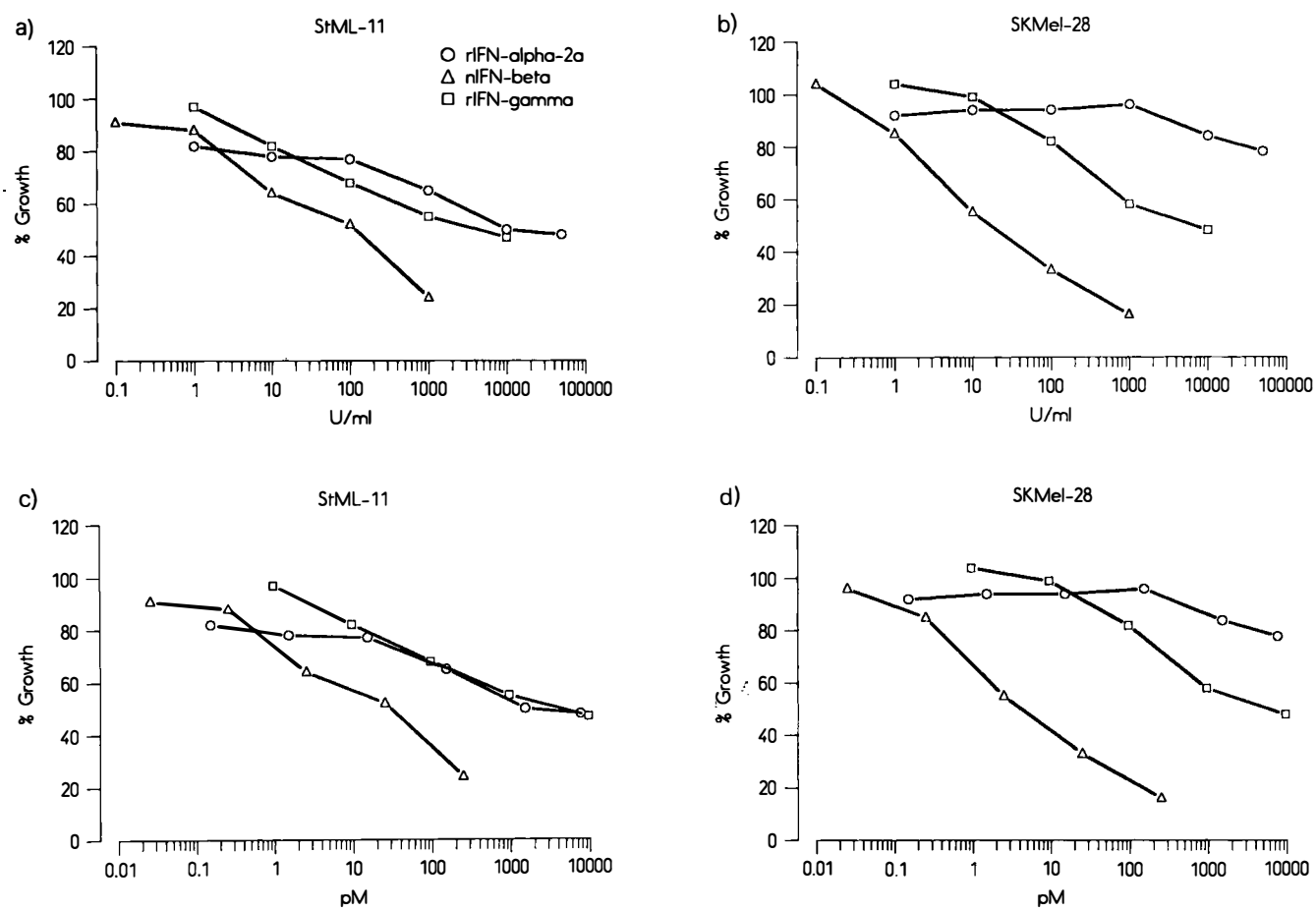

Figure 1. Dose-dependent growth inhibition of melanoma cell lines SKMel-28 and StML-11 by rIFN-alpha-2a, nIFN-beta, and rIFN-gamma. The IFN-doses are expressed in antiviral units (internationally defined units for IFN-alpha and IFN-beta, whereas no international standard exists for IFN-gamma) *(a,b)* and additionally in picomolar concentrations *(c,d)* [16].

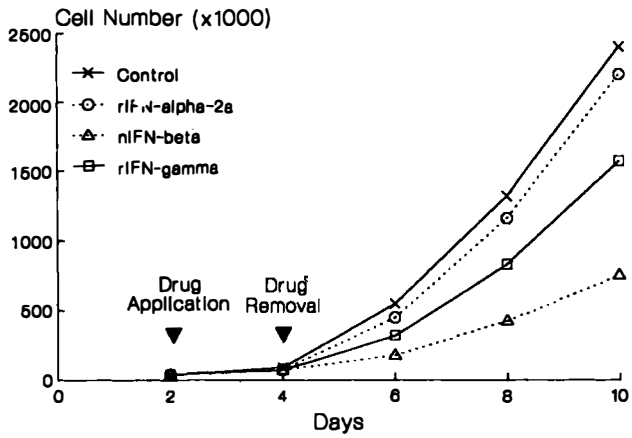

Figure 2. Reversibility of growth inhibition of melanoma cell lines SKMel-28 after a 2-day treatment with rIFN-alpha-2a, nIFN-beta, and rIFN-gamma (100 U/ml) [16].

Table II. Antiproliferative Effects of Cytokines on Melanoma Cells and Melanocytes[a]

	Melanoma Cells	References	Melanocytes	References
IFN-α	++	[1,2,5,6,15,17]	0/+	[18]
IFN-β	+++	[1,2,5,6,15,17]	+++	[18]
IFN-γ	++	[2,6,15,17]	0/+	[18,20]
TNF-α	0/+	[9,10,11,12,13]	++	[20]
IL-1α	0/+	[8,26]	+	[20]
IL-1β	0/+	[9]	ND	
IL-4	*/+	[9,10]	ND	
IL-6	*	[14]	0/+	[20]
TGF-β1	*/+	[22,23,24]	+++	[25]

[a] Crosses indicate inhibition of cellular proliferation as follows: +, little; ++, medium; +++, high, * growth stimulation; and 0, no effect; ND, not done.

TGF-beta prevents cell replication by arresting cells in the G_0/G_1 phase [25].

Interleukins Scant information exists on IL-1 action on pigment cells. Most melanoma cell lines are resistant to IL-1. IL-1alpha sensitive cell lines have been described, such as A375 [8,26]. Mortarini *et al* found three of nine melanoma cell lines to respond to IL-1beta [9]. The same authors found inhibition of melanoma cell proliferation by IL-4 in three of nine cultures tested; however, a slight stimulatory effect was observed in two others. Hoon *et al* also reported that incubation of human melanoma cells *in vitro* with IL-4 resulted in significant inhibition of proliferation [10]. The effects of other cytokines on melanoma cells are unknown so far; there are only two reports on the growth stimulation of murine melanoma cells by IL-6 [14], and of growth inhibition in melanocyte cultures [20].

The mode of antiproliferative action of cytokines such as IFNs seems to be cytostatic without clear cytotoxic effects. The growth control is associated with a decreased probability of the cytokine-treated cells to enter the cell cycle and an increasing portion remaining in the G_0 phase. Possibly, this effect is mediated by increasing levels of (2'-5') oligoadenylate synthetase [27]. Combined cytokine treatment of melanoma cells revealed synergistic antiproliferative effects with IFN-gamma and TNF-alpha or IFN-alpha and IFN-gamma [16,28,29]. These synergistic effects are probably mediated through the induction of cytokine receptors on melanoma cells [29].

CELLULAR DIFFERENTIATION

The term differentiation has been used to describe processes leading to the expression of phenotypic properties characteristic for functionally mature cells *in vivo*. There appears to be an inverse relationship between the expression of differentiation and malignancy associated properties, even to the extent that induction of differentiation has been proposed as a model for cancer treatment [30]. IFNs are known to induce cellular differentiation in different hematologic malignancies [31] and, therefore, the investigation of their differentiating effects in other malignant tissues including melanoma seemed promising.

Mature melanocytes and melanotic melanoma cells possess the ability to synthesize melanin from tyrosine. This process is regulated mainly by a single enzyme, tyrosinase, which catalyses three separate reactions during the conversion process [32]. Therefore, synthesis of melanin as well as the activity of tyrosinase are regarded as highly indicative measures for the expression of differentiated properties in melanocytic cells [5,16,33]. Additionally, melanocytes deliver melanin to keratinocytes via long dendrites *in vivo*. The formation of dendrites can also be observed in cell culture, and dendritelike structure formation may be interpreted as a sign indicating cellular differentiation in melanoma cells [16,33]. Furthermore, the antigenic phenotype is linked to melanocyte maturation and cellular differentiation and certain antibody-defined melanoma-associated molecules were revealed to be useful markers of melanocytic differentiation [4,16,34].

Interferon-Alpha and -Beta Induction of cellular differentiation has been investigated in both murine and human melanoma cells. IFNs are so far the only cytokines that were broadly examined with respect to their differentiating effects on melanoma cells. Fisher *et al* found after the application of IFN-alpha and -beta in both murine and human melanoma cells that there was no increase in melanin production or in dendritelike structure formation [5,35]. Concerning IFN-alpha and -beta, we confirmed these results in different human cell lines [16]; no significant changes of cellular morphology, melanin synthesis, or expression of differentiation markers were detected. Interestingly, a combination of type I IFNs with an inducer of melanoma cell differentiation, such as mezerein, proved to stimulate differentiation and to inhibit cell growth to a significantly higher degree than mezerein as a single agent [5].

Interferon-Gamma On the contrary, application of IFN-gamma significantly reduced dendritelike structure formation and inhibited melanin synthesis [16]. Furthermore, IFN-gamma enhanced progression-associated melanoma markers like the intercellular adhesion molecule-1 (ICAM-1) and very late antigen (VLA)-2 molecules [monoclonal antibody (MoAb) A.1.43] [16]. Therefore, it seemed that a dedifferentiated, biologically more aggressive phenotype of melanoma cells had resulted after treatment with IFN-gamma.

MODULATION OF CELL SURFACE ANTIGEN EXPRESSION

Different classes of antibody-defined melanoma antigens are susceptible to modulation by various cytokines *in vitro*. Among these, three classes of antigens could be relevant in predicting the interaction of individual tumor cells with immune cells, thus being determinants of antitumor response: (1) HLA class I and II antigens, (2) lymphocyte adhesion molecules, and (3) surface antigens preferentially expressed by malignant melanoma cells.

Interferon-Alpha and -Beta Several reports have shown that type I IFN's (IFN-alpha and IFN-beta) enhance the expression of HLA class I on melanoma cells [3,16,36,37]. On the contrary, expression of HLA class II antigens was either not induced by IFN-alpha or -beta [4,16,37] or only moderately enhanced, especially after IFN-beta treatment [3,36,38]. In contrast to IFN-gamma, increased expression of histocompatibility products occurs by a process not requiring *de novo* protein synthesis [39]. In addition to HLA mole-

Table III. Modulation of Melanoma Antigens by Cytokines in Vitro[a]

	HLA-I	HLA-II	ICAM-1	LFA-3	VLA-2	GD3	HMW-MAA
IFN-α	++	0/+	0	0	0	0	0/+
IFN-β	+++	0/+	0	0	0	0	0/+
IFN-γ	++	+++	+++	+	+	0	−
TFN-α	++	+	++	+	+	0	0/+
IL-1	ND	0	++	+	ND	0	0
IL-4	++	++	ND	ND	ND	0	0

[a] Crosses indicate upregulation of antigens as follows: +, little; ++, medium; +++, high; −, downregulation of antigen; 0, no effect; ND, not done.

cules, a number of other antigens preferentially expressed on malignant melanoma cells and designated as melanoma-associated antigens (MAA), can be modulated by IFNs. Giacomini *et al* found an increased shedding of the high molecular weight (HMW)-MAA by type I IFN and an enhanced expression and shedding of three other 100–115 kD MAAs [3].

Interferon-Gamma IFN-gamma is a potent inducer of HLA class I antigens on melanoma cells *in vitro* [4,36–38,40], similar to type I IFN. In addition, IFN-gamma is able to induce strong expression of HLA-DR antigens even in class II negative melanoma cell lines [4,36–38,40]. The capacity of IFN-gamma to induce HLA II antigens is much higher in comparison to type I IFN (up to 3 times for HLA-DR/DP antigens and up to 10 times for HLA-DQ [36]). HLA-DR is more susceptible to upregulation by IFN-gamma than HLA-DQ or -DP antigens [16,38,41,42]. Different mechanisms are seemingly responsible for the modulation of HLA-DR on melanoma cells than for the mediation of antiproliferative effects or for the upregulation of HLA-I by IFN-gamma, as shown in experiments using IFN-gamma resistant melanoma cell lines [43]. Specific modulation by IFN-gamma could be shown for the ICAM-1 by several investigators using monoclonal MoAbs directed against different epitopes of this adhesion molecule, such as MoAbs CL203, CL207, and 84H10 [16,44]. For this process *de novo* RNA and protein synthesis are required [44]. Regarding the modulation of MAA expression by IFN-gamma, Houghton *et al* [4] and Herlyn *et al* [40] found no modulation of MAA by either type I or type II IFN in contrast to HLA. On the other hand, IFN-gamma reduced the synthesis of both HMW-MAA and of 100-kD MAA [45]. Guiffre *et al* reported on a novel MAA inducible by gamma-IFN, a 33 to 38 kD glycoprotein expressed on melanoma cells [34]. Expression of p97 has been found enhanced by both type I IFN-alpha and type II IFN-gamma [46]. We found that IFN-gamma additionally induced the expression of the progression marker A.1.43, which was recently shown to bind to the VLA-2 integrin [47]. On cultured melanocytes, changes of the antigenic profile similar to those described for melanoma cells have been induced by type I and II IFN [18].

Tumor Necrosis Factor-Alpha TNF-alpha has been shown to increase the expression of both HLA-class I and class II DR antigens as well as of ICAM-1 on melanoma cells, although it is less potent than IFN-gamma [9,10,48]. Maio *et al* reported that regulation of HLA-DR expression involved both transcriptional and post-transcriptional mechanisms [48]. TNF-alpha did not influence expression of HMW-MAA [48], but significantly enhanced ganglioside D2 (GD2) expression [10]. Also, it increased the expression of the progression marker VLA-2 antigen and reduced the expression of the early marker K.1.2 [13].

Interleukins The effects of ILs on the immunophenotype of melanoma cells *in vitro* have been far less well investigated. Mortarini *et al* described borderline changes of HLA-DR and of MAA expression on various melanoma cell lines incubated with IL-1 beta, or IL-4 [9]. In those experiments, a hierarchy in cytokine effects could be shown, with IFN-gamma being the most potent immunoregulatory agent, followed by TNF-alpha. Similar results with IL-4 were reported by Hoon *et al*, showing the induction of HLA-I and II antigens and an elevation of the GM3/GD3 ratio and enhancement of GD2 by IL-4 [10].

INDIRECT EFFECTS VIA THE IMMUNE RESPONSE OF THE HOST

Interferon-Alpha and -Beta IFN-alpha is obviously in a position to augment the natural killer (NK) cell activity *in vitro* and *in vivo* [49,50]. Interestingly, an initial and transient decline in blood NK cell activity during the first hours after IFN application has been described that seems to be mediated by suppressor monocytes [51]. An increase of NK activity against melanoma cell lines was only found during the first treatment cycle in melanoma patients, but in subsequent treatment cycles NK activity tended to decrease [52]. Therefore, it remains controversial whether the moderate stimulation of NK activity by type I IFNs may contribute to any relevant antitumoral activity. Recently, the antitumor/antimetastatic effects of immune cells and their modulation by IFNs has been studied in a murine animal model. Depletion of NK cells, alone or in combination with T cells, eliminated the protective effect of IFN treatment; by depletion of CD4-cells alone the protective effect was also eliminated. These results demonstrate the importance of the interaction of different components of the immune systems and their activation by IFNs [53].

Interferon-Gamma The enhancement of HLA class I antigens by IFN-gamma correlated with decreased target susceptibility to lysis by NK cells, whereas the ability of tumor infiltrating lymphocytes (TIL) to lyse the cultured tumor target was markedly increased by pre-incubation of the targets with IFN-gamma [54]. It has been proved in an animal model that the expression of HLA class I antigens is essential for TIL activity [55]. IFN-gamma was shown in a murine model to activate macrophages to kill melanoma tumor targets [56]. IFN-gamma increases the expression of ICAM-1 on melanoma cells and the expression of this antigen was found to be associated with an increase of melanoma cell vulnerability to monocyte-mediated killing [57].

Tumor Necrosis Factor-Alpha TNF-alpha enhances HLA class I antigen expression and increases the expression of ICAM-1 [54,57]. Pre-incubation of melanoma cells with TNF-alpha, therefore, induces resistance against NK-cell lysis, increases lysis by TIL and also the sensitivity of these cells to monocyte-mediated killing [54,57].

Melanoma cells are not only sensitive targets to different cytokines, but they possess the capacity to produce cytokines themselves. IL-1, IL-6, and TGF-beta have been detected to be secreted by cultured melanoma cells [58–61]. Furthermore, additional growth factors are produced by melanoma cells [62]. The influence of exogenous cytokines on the autocrine production of cytokines by melanoma cells has not yet been examined.

FUTURE PERSPECTIVES OF CYTOKINE TREATMENT IN MELANOMA

Mainly the IFNs have been examined in melanoma treatment. High local tissue concentrations after intralesional application into metastases of both IFN-alpha and IFN-beta led to a regression of more than 50% of the metastases [63,64]. Also, after systemic application of IFN-alpha and IFN-beta in over 500 patients, the mean response

rates were between 10% and 15% for the different agents [6,65]. In light of *in vitro* results, it appeared promising to study the effectiveness of IFNs in combination with cytostatic drugs [16]. The results of more than 15 clinical trials have already been published and a number of other clinical studies are under way. Results of *in vitro* trials showed that type I IFNs may produce antagonistic effects combined with some agents (*e.g.*, cisplatin) and synergistic effects when combined with others (*e.g.*, vindesine and BCNU) [6,66]. Clinical trials with combined IFNs and cytostatic drug therapy were started a few years ago and have yielded promising initial results. Various investigators reported a response rate of 25% to 30% with a combination of dacarbazine and IFN-alpha [6]. So far, similar response rates have only been achieved by combining three to four cyostatic agents that involved a much higher toxicity [65]. Based on the published results, this therapeutic concept can be considered safe in practice and seems to be a useful alternative to polychemotherapy.

Recent reports on multidrug regimens including IFN-alpha revealed surprisingly high overall response rates of greater than 50%, which belong to the highest response rates ever described for metastatic melanoma [6]. Interestingly, the addition of IL-2 to combination schedules seemed to augment the response rates further. IL-2 has no direct effects on melanoma cells *in vitro*, but it transforms precursor lymphocytes into lymphokine activated killer cells and stimulates the capacity to lyse tumor cells resistant to NK cells. Improvement of response rates by the addition of IL-2 is in agreement with the results of various IL-2 trials in disseminated melanoma in which the best responses were obtained by combining IL-2 and IFN-alpha [67]. Possibly, future therapeutic schedules for metastatic melanoma will include combinations of cytotoxic drugs with cytokines showing antiproliferative capacity or enhancement of immunologic mechanisms against melanoma cells.

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