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The Effect of BM 12.531 (Azimexon) on Natural Killer Cell Activity in Myeloma Patients*

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Abstract—*The 2-cyanaziridin derivative, azimexon (E), has previously been shown to have certain immunomodulatory properties. In particular, the induction of leukocytosis, the stimulation of delayed-type hypersensitivity reactions and the synergistic effect of azimexon and antibiotics in the control of lethal bacterial and fungal infections in mice prompted us to test azimexon as an adjuvant to chemotherapy in 14 myeloma patients. In a randomized double-blind cross-over study 3 × 600 mg of azimexon were added to one of two consecutive, identical chemotherapy courses consisting of melphalan/prednisone (MP) or vincristine/cyclophosphamide/melphalan/prednisone (VCMP). Chemotherapy was given during days 1–4 and azimexon or placebo were added on days 6, 10 and 14. Blood counts and natural killer (NK) cell testing were performed on days 0 and 21 of each course. With the exception of a transient taste irritation in two patients, azimexon caused no subjective side-effects. White blood cell counts were not altered by the drug; red blood cells and hemoglobin showed a borderline depression after azimexon. NK activities measured against three target cell lines (K562, IGR3, L1210) tended to increase after azimexon treatment. When added* in vitro *to NK assays azimexon caused a slight increase of NK activity at concentrations of 0.01–0.25 μ/ml, whereas concentrations above 1 μg/ml were inhibitory. The increase of NK activity by azimexon was not due to the induction of interferon in the effector lymphocyte population.*

INTRODUCTION

THE SYNTHETIC compound BM 12.531, inn. azimexon (E) (2-(2-cyanaziridinyl-(1)-2-(2-carbamoylaziridinyl-(1)-propane), has been shown to have certain immunomodulatory properties in the mouse [1–3], rat [2] and man [4, 5]. In particular, a stimulation of lymphoid proliferation to mitogens, an activation of macrophages, a stimulation of delayed-type hypersensitivity (DTH) and a modulation of antibody responses have been reported [1–6]. In addition, azimexon has been shown to have an antitumor effect *in vivo* [1, 7, 8] and to act synergistically with antibiotics in mice lethally infected with *Candida* and Gram-negative bacteria [3]. Currently the most plausible mode of action of azimexon is thought to work via activation of IL-1-producing macrophages [1]. In this study we examined the *in vivo* and *in vitro* effects of azimexon on natural killer (NK) cell activity of peripheral blood mononuclear cells (PBMC) from 14 myeloma patients.

MATERIALS AND METHODS

Patients

After appropriate consent procedures 14 myeloma patients (9 IgG, 3 IgA, 1 IgD myeloma) and one patient with Waldenström's disease were randomized to receive 3 × 600 mg of azimexon or placebo as adjuvant during two consecutive courses of regular, identical chemotherapy. The chemotherapy, for which the patients had not been randomized, consisted of melphalan/prednisone (MP) in 9 patients and of vincristine/cyclophosphamide/melphalan/prednisone (VCMP) in 6 patients (Fig. 1). In the MP regimen melphalan and prednisone were given orally on days 1–4 at daily doses of 8 mg/m^2 and 75 mg/m^2

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respectively, and prednisone was reduced on days 5, 6, 7 and 8 to 75, 50, 25 and 0% of the total dose respectively. The VCMP regimen consisted of 1 mg vincristine i.v. on day 1, 100 mg/m^2 cyclophosphamide p.o. on days 1–4, 5 mg/m^2 melphalan p.o. on days 1–4 and 75 mg/m^2 prednisone p.o. on days 1–4; prednisone was tapered off during days 5–8 in the same fashion as described for the MP regimen. After the first course of chemotherapy a cross-over of the adjuvant therapy was instituted: patients who had been receiving azimexon were given placebo and vice versa. The group of patients comprised 7 males and 8 females; the mean age was 65.1 yr. During the test period one patient progressed and was excluded from the study since his chemotherapy regimen had to be changed so that the second course was different from the first one. Azimexon or placebo were given in a double-blind, randomized fashion as one morning and one evening tablet of 300 mg on days 6, 10 and 14 after the beginning of the chemotherapy course. Subjective side effects were recorded during both courses.

Lymphocyte isolation and NK testing

Blood was collected for routine hematological analysis and for NK testing the day before chemotherapy was instituted (day 0) and 21 days later. PBMC were isolated from 30 ml of defibrinated venous blood by Ficoll-Hypaque gradient centrifugation followed by depletion of mononuclear phagocytes as previously described [9]. Three permanent tumor cell lines were used as target cells for NK tests: the human melanoma line IGR3 [10], the human erythroleukemia line K562 [11] and the murine leukemia line L1210. All three lines were maintained in Eagle's minimal essential medium supplemented with 10% fetal calf serum, antibiotics, vitamins, non-essential amino acids, pyruvate and glutamine (all products for Seromed Lab., Munich, and Flow Lab., Bonn). The melanoma line grew as a monolayer whereas the two leukemia lines were maintained in suspension cultures. Tumor cells (10^6) were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ as previously described [9]. After three washes and appropriate dilution 10^4 tumor cells were incubated in round-bottom microplates with increasing numbers of effector lymphocytes, resulting in effector: target (E/T) ratios of 1:1, 5:1 and 16:1 for K562 and 5:1, 16:1 and 50:1 for L1210 and IGR3. The test volume was 200 μl; all tests were run as triplicates for 12 hr at 37°C and 5% CO_2. Aliquots of cell-free culture supernatants were harvested, counted in an automated gamma-counter and specific ^{51}Cr release was calculated as follows:

$$\frac{\text{counts/min test} - \text{counts/min spontaneous}}{\text{counts/min total} - \text{counts/min spontaneous}} \times 100.$$

In addition, a computerized data analysis system was developed allowing comparison of the cytotoxicity of the patient's lymphocytes with a sex-matched daily control person as well as with a control group of 30 healthy adults [12]. Based on the finding that most individuals at the chosen E/T ratios gave linear cytotoxic dose–response curves on a semilogarithmic scale, the computer calculated three mean cytotoxic indices: (1) patient's NK divided by NK of the daily control (p/dc); (2) patient's NK divided by the mean NK activity of the control group (p/cg); and (3) NK of daily control divided by NK of control group (dc/cg). The control group of 30 healthy individuals was constantly renewed by eliminating the oldest control value each time a new daily control value was entered into the computer.

After termination of the two consecutive, identical chemotherapy courses with either placebo or azimexon as adjuvants, the code of the randomized study was broken and the effects of azimexon and placebo on blood formula and NK activity were compared. Statistical analysis was performed with the one- and two-tailed paired Wilcoxon test ($\alpha \leqslant 0.05$). For the evaluation of NK activities pretreatment values (day 0) in the placebo and azimexon group were substracted from the respective specific ^{51}Cr release values and the cytotoxic index p/cg measured on day 21. The obtained individual differences were compared for a given target cell line and for all three target lines together.

Effect of azimexon on NK activity in vitro

Effector cells from two healthy donors were tested at E/T ratios of 10:1 against K562, IGR3 and L1210 in the presence of decreasing concentrations of azimexon. After preliminary tests, azimexon was assayed in the concentration range of 4.0–0.01 μg/ml. In addition, the capacity of azimexon to induce interferon in the effector lymphocytes was tested under identical conditions. The interferon activities in the culture supernatants were kindly determined by Dr H. Kirchner, Institute of Virology, Deutsches Krebsforschungszentrum, Heidelberg.

RESULTS

Subjective side-effects of azimexon given as adjuvant to chemotherapy in myeloma patients

Table 1 lists side-effects recorded by patients that received azimexon or placebo as an adjuvant to chemotherapy during two consecutive chemotherapeutic courses (see Fig. 1). With the

Table 1. Subjective side-effects under azimexon and placebo

Patient No.	Placebo	Azimexon
1	muscle pain	vertigo
2	nil	nil
3	fatigue	fatigue
4 (excluded)		
5	nil	strange taste
6	nil	nil
7	depressive	fatigue
8	fatigue	nil
9	nil	fatigue, vertigo, strange taste
10	fatigue	fatigue
11	headaches	fatigue
12	bone pain	nil
13	nil	nil
14	nil	nil
15	nil	fatigue

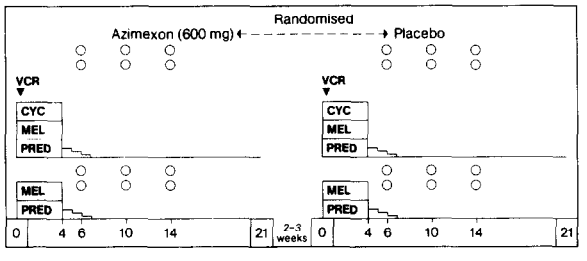

Fig. 1. Nine myeloma patients were treated with a chemotherapy consisting of melphalan (8 mg/m² p.o., days 1–4) and prednisone (75 mg/m² p.o., days 1–4, tapering off during days 5–7) and 6 patients received a combination of vincristine (1 mg i.v., day 1), cyclophosphamide (100 mg/m² p.o., days 1–4), melphalan (5 mg/m² p.o., days 1–4) and prednisone (75 mg/m² p.o., days 1–4, tapering off during days 5–7). The patients were not randomized for their chemotherapy. During two consecutive, identical courses of chemotherapy azimexon or placebo was added in a randomized double-blind fashion on days 6, 10 and 14 of either course. One patient was excluded from evaluation because he had progressive disease and his chemotherapy regimen (MP) had to be changed during the second course. Blood was collected on days 0 and 21 (7 days after the last adjuvant therapy dose) and examined for white and red blood cell counts and for NK activity.

exception of a twice-recorded irritation of the taste, azimexon caused no objective side-effects compared with placebo treatment.

Effect of azimexon on white and red blood cells in chemotherapy-treated myeloma patients

With the exception of a borderline decrease of erythrocytes and hemoglobin on day 21 after azimexon treatment, the comparison of the blood counts revealed no significant differences (Table 2).

In vivo *effect of azimexon on NK activity in* 14 *myeloma patients*

To explain the way of calculating the post- minus pretreatment differences of the specific ^{51}Cr release and the cytotoxic index p/cg, an example is given in Table 3 [PBMC from patient No. 8 (Table 4) reacting against K562]. By adding together all day 21 minus day 0 differences of NK activities measured against the three target cell lines (Table 4) a positive figure was obtained in the azimexon group (specific ^{51}Cr release, +362.6; cytotoxic index p/cg, +17.10), whereas in the placebo group negative values were calculated (specific ^{51}Cr release, -195.8; cytotoxic index p/cg, -6.92). The difference was statistically significant in the paired Wilcoxon test. Similarly, significant differences were seen for the individual NK reactions against L1210 and IGR3 but not against K562 target cells (Table 5). These results suggest an overall positive effect of azimexon on NK activity in a group of 14 myeloma patients treated by chemotherapy.

Effect of azimexon on NK activity in vitro

Admixture of azimexon at concentrations ranging from 4.0 to 0.01 μg/ml to a standard NK assay with normal effector cells produced the type of results shown in Fig. 2. Azimexon alone was not cytotoxic for the three cell lines tested. Addition of a constant effector cell number to the assay showed a slight but consistent increase of NK activity at low concentrations of azimexon. High concentrations of azimexon were rather inhibitory to NK activity. At none of the tested concentrations was azimexon capable of inducing interferon in the effector lymphocyte population (Fig. 2).

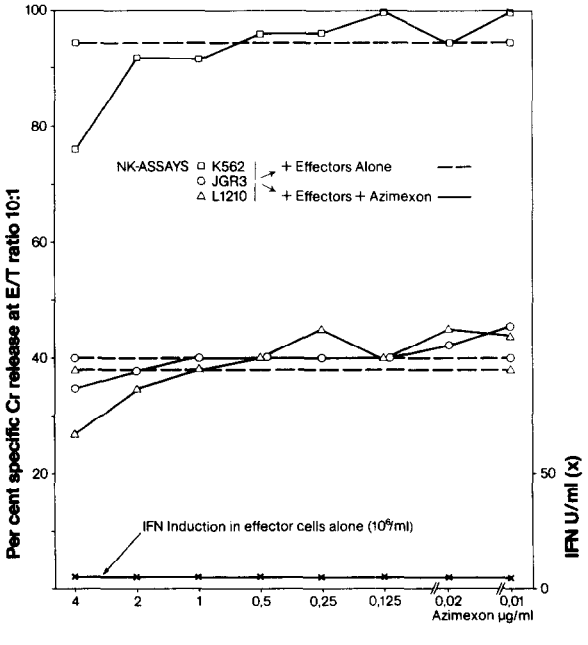

Fig. 2. Effect of azimexon on NK activity and interferon induction in vitro. *Data obtained with PBMC from a healthy adult man.*

Table 2. Effect of azimexon and placebo on white and red blood counts in 14 myeloma patients treated with chemotherapy

Blood component	Placebo		Azimexon	
	Day 0	Day 21	Day 0	Day 21
Leukocytes ($\times 10^5$/mm^3)	3.95* (4.26 ± 0.41)	3.70 (3.79 ± 0.49)	3.75 (3.96 ± 0.43)	3.15 (3.77 ± 0.48)
Erythrocytes ($\times 10^6$/mm^3)	3.70 (3.63 ± 0.15)	3.70† (3.58 ± 0.15)	3.55 (3.59 ± 0.18)	3.40† (3.41 ± 0.12)
Hemoglobin (g Hb/dl)	12.05 (11.89 ± 0.39)	11.95‡ (11.65 ± 0.46)	11.45 (11.79 ± 0.18)	10.95‡ (11.08 ± 0.37)
PMN (%)	64.0 (62.2 ± 3.89)	59.0 (56.3 ± 4.04)	49.0 (49.8 ± 6.17)	53.0 50.6 ± 6.43)
Lymphocytes (%)	23.0 (26.7 ± 3.44)	26.0 (25.8 ± 3.51)	15.0 (20.2 ± 2.59)	28.0 (26.0 ± 3.22)

*Median; in parentheses mean ± S.E.M.
†Borderline significance in the one-tailed Wilcoxon test ($P \leqslant 0.05$) only.
‡Significance in the two-tailed Wilcoxin test ($P \leqslant 0.05$).

Table 3. Example of calculating post- minus pretreatment differences of NK activity in myeloma patients treated with chemotherapy and azimexon

K562	Day 21 1:1	Day 21 5:1	Day 21 16:1	Day 0 1:1	Day 0 5:1	Day 0 16:1	Difference (see Table 4)
			placebo				
Patient No. 8 (p)	4	30	64	25	64	69	
Mean		32.6			52.6		-20
Control group (cg; $n = 30$)	9.9	36.8	61.5	10.3	37.6	61.4	
p:cg	0.39	0.81	1.04	2.35	1.71	1.13	
Mean		0.75			1.74		-0.99
			azimexon				
Patient No. 8 (p)	23	73	93	15	49	51	
Mean		63.0			38.3		+24.7
Control group (cg; $n = 30$)	9.7	35.5	60.9	9.9	36.4	61.8	
p:cg	2.35	1.07	1.53	1.49	1.34	0.83	
Mean		1.98			1.22		+0.76

DISCUSSION

The aim of the present study was to test the effect of azimexon as an adjuvant to chemotherapy in immunocompromised myeloma patients. It is well known that these patients have a depressed bone marrow function due to plasma cell infiltration and repetitive cycles of chemotherapy. They succumb more readily to viral, bacterial and fungal infections and the diminished proliferative capacity of the bone marrow frequently necessitates postponement of due chemotherapy because of prolonged phases of leukopenia. Since azimexon has been shown in animals to increase white blood cell counts [1, 2], to act synergistically with antibiotics in overcoming bacterial infections [3], to enhance NK activity [8, 13] and to exert an antitumor effect [7], we thought it worthwhile to examine azimexon as an adjuvant to regular chemotherapy in myeloma patients. This study aimed at giving an answer to the tolerance of the drug in man, its effect on white and red blood cell counts and its effect on NK activity [14].

Apart from the transient irritation of two patients' taste, no major subjective side-effects could be ascribed to the use of azimexon at the given dosage. An irritation of the taste has not been reported in any other clinical trial with azimexon so far. The reports that azimexon at doses above 600 mg per day might cause transient hemolytic anemia [1] was confirmed in our study, but the changes in the red cell compartment due to azimexon were minute. None of our patients experienced a serious drop in red or white blood cells when tested 7 days after the last of three 600 mg doses of azimexon. When the NK activities were compared before and after treatment with azimexon or placebo a statistically significant increase of cytotoxic activity was noted in the azimexon group for the targets L1210 and IGR3 but not for K562. In addition, there was a trend to

a beneficial effect of azimexon on NK cell activity when the sums of all positive and negative reactions against the three target lines were calculated for the placebo and the verum group (Table 4). It is feasible that changes in the time interval between administration of azimexon and NK testing may result in a more significant NK activity. Since azimexon did not directly induce interferon in normal PBMC, the stimulatory effect of the substance on NK activity remains to be elucidated. The selectivity of the azimexon effect for certain target cell lines, notably for L1210, may be of key importance for the understanding of this phenomenon. It is noteworthy in this context that K562 and IGR3 have previously been successfully freed of mycoplasma infection whereas this goal was not achieved for our L1210 line [15]. The observation that azimexon enhances NK activity *in vitro* at low doses and inhibits at higher ones points to the existence of a therapeutic optimum that is still far from being well established.

The number of observed chemotherapeutic cycles under azimexon was too small to allow any

Table 4. Effect of azimexon and placebo on NK activity in myeloma patients

Patient No.	Target cell lines	Placebo NK on day 21 - day 0		Azimexon NK on day 21 - day 0	
1.	K562	+ 23.6*	+1.07†	- 9,0*	- 0.07†
	L1210	- 23.3	-1.43	- 0.5	- 0.18
	IGR3	- 27.1	-1.26	+ 8.7	- 0.53
2.	K562	+ 5.0	+0.17	+ 28.3	+ 0.98
	L1210	- 27.3	-1.33	+ 10.7	+ 0.25
	IGR3	+ 2.3	-0.31	+ 11.7	+ 0.21
3.	K562	+ 4.3	+0.34	+ 14.6	+0.61
	L1210	+ 7.3	+0.06	+ 5.3	+ 0.08
	IGR3	+ 6.7	+0.23	- 6.4	- 0.78
5.	K562	+ 19.0	+1.23	- 10.6	- 0.52
	L1210	- 9.3	-0.31	- 8.3	- 0.41
	IGR3	+ 8.0	+0.27	- 7.1	- 0.19
6.	K562	+ 8.7	+0.51	- 4.6	- 0.12
	L1210	+ 25.4	+1.55	+ 15.0	+ 0.10
	IGR3	n.t.	n.t.	n.t.	n.t.
7.	K562	- 23.3	-0.77	+ 27.4	+ 0.52
	L1210	8.6	-0.33	+ 19.5	+ 1.86
	IGR3	n.t.	n.t.	n.t.	n.t.
8.	K562	- 20.0	-0.99	+ 24.7	+ 0.76
	L1210	- 7.3	-0.60	+ 46.0	+ 3.26
	IGR3	- 37.3	-1.32	+ 13.4	+ 0.55
9.	K562	- 5.0	-0.49	+ 15.3	+ 0.46
	L1210	+ 43.3	+2.97	+ 57.4	+ 4.49
	IGR3	- 11.0	-0.67	+ 13.4	+ 0.28
10.	K562	- 9.6	-0.39	- 0.4	- 0.09
	L1210	- 12.0	+0.24	+ 28.7	+ 1.69
	IGR3	- 28.3	-1.14	- 10.0	- 0.39
11.	K562	- 42.4	-1.10	+ 20.7	+ 0.83
	L1210	- 16.0	-0.44	+ 26.4	+ 0.72
	IGR3	- 15.6	-0.79	+ 7.0	+ 0.38
12.	K562	- 1.3	+0.23	+ 14.0	+ 0.34
	L1210	- 0.4	+0.10	+ 30.6	+ 0.96
	IGR3	n.t.	n.t.	n.t.	n.t.
13.	K562	+ 17.4	+0.61	- 2.0	+ 0.05
	L1210	+ 15.0	+0.80	+ 6.0	+ 0.48
	IGR3	+ 27.0	+1.30	+ 10.7	+ 0.35
14.	K562	+ 3.3	+0.30	+ 13.3	+ 0.51
	L1210	+ 7.7	+0.58	- 5.4	+ 0.15
	IGR3	+ 11.0	+0.40	+ 35.3	+ 1.32
15.	K562	- 27.6	-0.97	- 23.3	+ 0.69
	L1210	- 55.0	-4.27	- 28.3	- 1.38
	IGR3	- 23.7	-1.17	- 25.6	- 1.03
Sum of all tests		-195.8‡	-6.92§	+362.6‡	+16.57§

*Difference of % specific ^{51}Cr release at day 21 - day 0, mean values of triplicate tests at 3 E/T ratios.

†Difference of cytotoxic index p/cg at day 21 - day 0. The control group (cg) comprised 30 healthy individuals.

‡§Significantly different at $P \leq 0.05$ in the two-tailed Wilcoxon test.

Table 5. Effect of azimexon and placebo on NK activity in myeloma patients

Target cell lines	Results* expressed as	Placebo	Azimexon	P
A. K562†	Difference of specific	1.0§	13.6	N.S.
	^{51}Cr release of day 21 - day 0	(-3.4 ± 5.1)	(7.7 ± 4.0)	
L1210‡	(mean of 14 patients and 3 E/T	-7.9	12.8	≤0.05
	ratios)	(-4.3 ± 6.4)	(14.5 ± 6.0)	
IGR†		-11.0	8.7	≤0.05
		(-8.0 ± 6.1)	(4.6 ± 4.8)	
Sum of all three target lines:		-5.0	10.7	≤0.05
		(-5.0 ± 3.3)	(9.3 ± 3.0)	
B. K562†	Difference of cytotoxic index p/cg	-0.20	0.40	N.S.
	at day 21 - day 0 (p, patient; cg	(-0.02 ± 0.22)	(0.26 ± 0.14)	
L1210‡	control group of 30 healthy	-0.13	0.49	≤0.05
	individuals)	(-0.20 ± 0.43)	(0.89 ± 0.41)	
IGR*		-0.67	0.28	≤0.05
		(-0.41 ± 0.26)	(0.11 ± 0.20)	
Sum of all three target lines:		-0.31	0.38	≤0.05
		(-0.19 ± 0.18)	(0.44 ± 0.17)	

*See Materials and Methods.

†Mycoplasma-free.

‡Mycoplasma-infected.

§Median. In parentheses, mean ± S.E.M. The statistical analysis was performed with the Wilcoxon test; significant differences were seen with the two-tailed test for the target L1210 and the sum of all three targets and with the one-tailed test for the target IGR3.

meaningful judgement as to an effective infection prophylaxis or to an antitumor effect of the substance to be made. However, its good tolerance, the slight side-effects and the observed immunomodulatory properties may justify the use of azimexon as an adjuvant to chemotherapy in randomized, prospective trials.

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