WE investigated the influence of 2-chlorodeoxyadenosine (2-CdA) in combination with tumour necrosis factor- α (TNF α) or its mutein VI, which differs from the native molecule by *N*-terminal amino acid composition, on the survival time of mice inoculated with leukaemia L1210 or P388. Groups of mice with leukaemia L1210 and P388 receiving 2-CdA combined with TNF α had shorter survival times than animals treated with these agents separately. In contrast, the administration of 2-CdA in conjunction with mutein VI, prolonged the survival of mice inoculated with these leukaemias as compared with animals receiving these agents separately. The results of the present study emphasize the importance of the biological activity of the TNF α molecule *N*-terminus.

Key words: 2-Chlorodeoxyadenosine, Experimental leukaemias, Interactions of cytokines and antineoplastic drugs, Muteins of tumour necrosis factor- α , Tumour necrosis factor- α .

The influence of 2-chlorodeoxyadenosine in combination with tumour necrosis factor- α or its mutein on murine leukaemias L1210 and P388

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Introduction

The combination of cytokines and cytotoxic drugs offers a new approach to increasing the therapeutic effect in the treatment of neoplastic diseases. There are many possible levels of interactions between these two groups of anti-neoplastic agents: cellular drug uptake, drug target enzymes, drug metabolizing enzymes.

2-Chlorodeoxyadenosine (2-CdA) is a new antineoplastic drug belonging to the purine analogues, which is especially active in lymphoproliferative disorders. Tumour necrosis factor- α (TNF α), a multimodal cytokine, has a well-known antineoplastic activity. Muteins are TNF α derivatives, in which the first three to seven amino acids of the native molecule have been replaced. *N*-terminal amino acids play an important role in the binding of TNF to its cell surface receptors and in the biological activity of this cytokine.¹

The results of our previous experiments showed that both TNF α and 2-CdA administered separately have therapeutic activity against murine leukaemias L1210 and P388.^{2,3} Our recent studies have demonstrated that mutein VI has the best therapeutic activity against leukaemia P388.⁴

The present study was designed to compare the influence of 2-CdA combined with TNF α or with its mutein VI, on murine leukaemias L1210 and P388.

Materials and Methods

Animals: For the experiments 140 female mice, DBA/2 strain, weighing 18–20 g, 8–10 weeks old, purchased from the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (Wrocław) were used. They were given standard laboratory food and water *ad libitum*.

Leukaemia: L1210 and P388 leukaemia cells were purchased from the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (Wrocław) and were maintained by serial passages in the ascitic fluid of DBA/2 mice. Leukaemic cells from the fluid were resuspended in 0.9% sodium chloride so that 10^6 L1210 or P388 cells were injected i.p. into DBA/2 recipients.

Therapeutics: 2-CdA produced according to the method described by Kazimierzczuk *et al.*⁵ was kindly supplied by the Foundation of Development of Diagnosis and Therapy (Warsaw) as a dry powder. The synthesis and analysis of TNF α and its mutein VI were performed at the Department of Bioorganic Chemistry, Łódź, Poland. TNF had a specific activity of 2×10^7 U/mg. Mutein VI was constructed using synthetic oligonucleotides to induce changes in the cDNA encoding five *N*-terminal amino acids of native TNF- α .⁶ Amino acid sequences were analysed by using automated Edman degradation with an Applied

Table 1. The influence of 2-CdA, TNF α and mutein VI on the survival time of mice with leukaemia L1210

Therapeutics	NaCl (0.9%)			2-CdA (35 mg/kg) ^c		
	$\bar{x} \pm \text{S.D.}^{\text{d}}$	MST^{e}	ILS^{f}	$\bar{x} \pm \text{S.D.}^{\text{d}}$	MST^{e}	ILS^{f}
NaCl (0.9%)	7.22 $\pm$ 0.42	7.0		10.35 $\pm$ 1.05*	10.0*	42*
TNF						
250 $\mu\text{g/kg/24 h}^{\text{a}}$	9.82 $\pm$ 1.22*	10.0*	42*	7.20 $\pm$ 0.95	7.0	0
400 $\mu\text{g/kg/24 h}^{\text{b}}$	8.20 $\pm$ 0.83	8.0	14	6.30 $\pm$ 1.25	6.5	-10
Mutein VI						
250 $\mu\text{g/kg/24 h}^{\text{a}}$	8.50 $\pm$ 0.85	8.0	14	9.50 $\pm$ 1.03*	10.0*	42*
400 $\mu\text{g/kg/24 h}^{\text{b}}$	9.35 $\pm$ 0.90*	9.0	29	12.60 $\pm$ 0.65*	12.5*	78*

Experiments were performed with groups of six animals. Data represent the average of four identical experiments.

^aTreatment, once a day, in eight i.p. injections.

^bTreatment, four i.p. injections on days 2, 4, 6, 8 of experiment.

^cTreatment, once a day, in five i.p. injections.

^dMean values and standard deviation.

^eMedian survival time. Six mice were used per group.

$$^{\text{f}}ILS = \left(\frac{MST \text{ of the treated group}}{MST \text{ of the control group}} - 1 \right) \times 100(\%)$$

*Statistical significance as compared with the control group. $p < 0.05$.

TNF α	Met-Val-Arg-Ser-Ser-Ser-Arg-Tyr-
Mutein VI	Met-Arg-Ile-Arg-Met-

FIG. 1. The *n*-terminal amino acids sequences of TNF α and its mutein VI.

Biosystems ABI 477A protein sequencer. The cDNA was expressed in *E. coli* and the resulting mutein was purified by ion exchange chromatography. Endotoxin contamination amounted to approximately 1.9 ng endotoxin per mg protein, as estimated using a commercially available assay (Sigma Chemical Co., St Louis, MO, USA). Recombinant cytokines were reconstituted using sterile phosphate-buffered saline (PBS).

Drugs were delivered in a volume of 0.01 ml/g mouse weight. Control mice received equivalent volumes of 0.9% solution of PBS.

Antileukaemic assay: Animals received tumour challenges on day 0. All treatments were i.p. and were initiated on the next day. 2-CdA was administered at dose of 35 mg/kg, once a day, for 5 days. Cytokines were administered at dose of 250 $\mu\text{g/kg}$ as daily injections for 8 days or at a dose of 400 $\mu\text{g/kg}$ given 2, 4, 6 and 8 days after the inoculation of leukaemic cells. The control mice received i.p. injections of 0.9% NaCl.

The mice were observed daily for survival for a minimum of 60 days. The median survival time (MST) was assessed according to the equation: $MST = (x + y)/2$, where x denotes the earliest day when the number of dead animals was

$> N/2$, y denotes the earliest day when the number of dead animals was $> (N/2) + 1$, and N denotes the number of animals in the group. The antileukaemic effect of the drugs (measured as the increase in lifespan, ILS) was assessed as the percentage ratio of MST of the treated group (T) to that of the control group (C):

$$ILS = [(MST_T/MST_C) - 1] \times 100$$

As each experiment was repeated four times (six mice per group), data are reported for 24 animals per dose group.

Statistical analysis: Statistical analysis was performed using Student's *t*-test. Results were considered significant when $p < 0.05$.

Results

TNF α given once daily, at a dose 250 $\mu\text{g/kg}$, significantly prolonged the survival time of mice with leukaemia L1210. Nearly the same increase of lifespan in leukemia L1210-bearing mice was seen in the group receiving mutein VI in the sequential treatment schedule. Sequential application of TNF α at a dose of 400 $\mu\text{g/kg}$ and daily application of mutein VI did not change the lifespan of leukaemia L1210-bearing mice as compared with animals of the control group (Table 1).

TNF in both treatment regimes and mutein VI given daily at a dose of 250 $\mu\text{g/kg}$ caused only a slight increase in the lifespan of P388 leukemia-bearing mice. Sequential application of mutein VI

Table 2. The influence of 2-CdA, TNF α and mutein VI on the survival time of mice with leukaemia P388

Therapeutics	NaCl (0.9%)			2-CdA (35 mg/kg) ^c		
	$\bar{x} \pm \text{S.D.}^d$	<i>MST</i> ^e	<i>ILS</i> ^f	$\bar{x} \pm \text{S.D.}^d$	<i>MST</i> ^e	<i>ILS</i> ^f
NaCl (0.9%)	10.25 $\pm$ 0.95	10.0		12.50 $\pm$ 1.55	12.0*	20
TNF						
250 $\mu\text{g/kg/24 h}^a$	10.82 $\pm$ 0.75	11.0	10	8.68 $\pm$ 0.92	9.0	– 10
400 $\mu\text{g/kg/24 h}^b$	10.70 $\pm$ 0.82	10.5	5	9.22 $\pm$ 1.18	9.0	– 10
Mutein VI						
250 $\mu\text{g/kg/24 h}^a$	11.56 $\pm$ 1.02	11.5	15	11.73 $\pm$ 0.78	12.0	20
400 $\mu\text{g/kg/24 h}^b$	13.80 $\pm$ 0.86*	14.0*	40*	15.82 $\pm$ 0.81*	16.0*	60*

Experiments were performed with groups of six animals. Data represent the average of four identical experiments.

^aTreatment, once a day, in eight i.p. injections.

^bTreatment, four i.p. injections on days 2, 4, 6, 8 of experiment.

^cTreatment, once a day, in five i.p. injections.

^dMean values and standard deviation.

^eMedian survival time. Six mice were used per group.

$$^f\text{ILS} = \left(\frac{\text{MST of the treated group}}{\text{MST of the control group}} - 1 \right) \times 100(\%)$$

*Statistical significance as compared with the control group. $p < 0.05$.

at a dose 400 $\mu\text{g/kg}$ significantly prolonged the survival time of mice with leukemia P388 (Table 2).

The combination of 2-CdA and TNF shortened the survival time of animals in all treated groups. In contrast, mice receiving 2-CdA and mutein VI lived for a significantly longer time than did the animals treated with these agents separately (Tables 1 and 2).

Discussion

The interactions between cytokines and anti-neoplastic drugs are very complex. Cytokines can modulate the metabolism of the drug, the expression of adhesion receptors on tumour cells and gene expression.⁷ Cytokines also have a great influence on the host's immune system.

TNF is a polypeptide mediator of inflammation and cellular immune response. It also takes part in the induction of antineoplastic activity of the organism. The mechanism of TNF action is still unclear. Numerous different effects of this cytokine may be explained by both structural and functional heterogeneity of TNF receptors and by a diversification of post-receptor signal transduction pathways.⁸ Although two cell surface receptors for TNF, p75 and p55, have been identified recently, the amino acid residues necessary for the biological activity of TNF are still not known. Klysik *et al.* constructed derivatives of TNF termed 'muteins', in which the first three to seven amino acids of native TNF were replaced

using synthetic cDNA expressed in *Escherichia coli*.⁶

The aim of our study was to compare the anti-neoplastic activity of native TNF + 2-CdA with that of mutein VI + 2-CdA. Our previous results have shown that among muteins III, V and VI, the latter has the greatest activity against murine leukaemias L1210 and P388.⁴ Our results have shown that the combination of 2-CdA and TNF shortened the survival time of mice as compared with that of the control group. In contrast, mice receiving 2-CdA and mutein VI lived for a significantly longer time than did the animals treated with these agents separately. These results are in agreement with our previous observations that mice exhibit much better tolerance to high doses of mutein VI as compared with native TNF.⁴ From other data^{1,9} it is known that *N*-terminal amino acids are critical for both receptor binding and biological activity of TNF. Tchórzewski *et al.* have revealed that mutein VI fails to recognize both TNF receptors. It also fails to increase secretion of cytokines IL-1, IL-6, GM-CSF and IFN- γ , or to activate granulocytes, but it has the highest cytotoxic activity.^{1,9} This may suggest an alternative mechanism of action.

TNF may cause tissue damage by augmenting neutrophil function, both by directly activating neutrophils and by affecting their response to other stimuli.⁹ The lack of these effects after the addition of mutein VI may explain the lower toxicity when combined with 2-CdA as compared with the combination of native TNF and 2-CdA.

It is well known that TNF acts synergistically with topoisomerase target drugs (adriamycin, amsacrine, mitoxantron, etoposide, teniposide). This effect is probably related to the increase in the activity of topoisomerase I and II, resulting in enhanced DNA strand breaks and cleavage complex.⁷ In contrast, TNF does not enhance the antineoplastic effects of bleomycin, hydroxyurea, ara-C, cis-platin, mitomycin, vincristine and vinblastine.⁷ Warzocha *et al.* have shown that TNF potentiates the antineoplastic activity of cyclophosphamide and methotrexate.² The studies of Winkelhake *et al.* have shown that the combination of TNF α and interleukin-2 does not increase the lifespan of L1210 leukaemia-bearing mice, compared with animals treated with these agents separately.¹⁰ The results of our previous experiments revealed that 2-CdA and interferon- α show an additive inhibitory effect on the growth of clonogenic leukaemic blasts *in vitro*.¹¹

The mechanism of action of 2-CdA is still unclear, but it may be related to the activation of apoptosis.¹² Apoptosis (programmed cell death) represents a mechanism of cell removal in embryonic development and in controlling cell populations. Defects of this process may be involved in the production of neoplasms. Apoptosis may be induced by many factors, one of them being TNF.^{13,14} TNF-activated protein kinase C appears to be involved in the induction of the *c-jun*, proto-oncogene which seems to be involved in apoptosis.¹⁴⁻¹⁶ Induction of *c-jun* transcription is also observed in the treatment of myeloid leukaemia cells with new purine analogues, which have also been shown to induce apoptosis.¹⁷ These observations may explain the synergistic action of 2-CdA and the TNF derivative, mutein VI.

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