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Muteins of human interleukin-1 that show enhanced bioactivities

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Using recombinant DNA techniques, we have made a series of amino-terminal muteins of human interleukin-1 (IL-1). Two of the muteins demonstrated 4–7-fold increase in bioactivity as compared to that of the native IL-1. The enhanced biological potency coincides with an increase in both receptor binding affinity and in vivo tumor inhibitory activity. By site specific mutagenesis, we have shown that the arginine at the fourth position of IL-1 is one of the key residues in the function of IL-1. Circular dichroism studies of the amino-terminus analogs showed little structural rearrangement. The change in bioactivity might be due to a change in the stability of the muteins, in the side chain interactions with receptors or in the minor change in folding near the receptor binding site.

Interleukin-1; Site-directed mutagenesis; Antitumor agent

1. INTRODUCTION

Human interleukin-1 (IL-1) is a nonglycosylated cytokine, released by activated monocytes and macrophages, which mediates a diverse array of biological activities [1–3]. These activities include induction of thymocyte proliferation and B-lymphocyte differentiation, prostaglandin and collagenase release, induction of fever and possible involvement in cytotoxicity against certain tumor target cells [4–8].

At present, there are at least two different forms of IL-1, designated α and β, based on isoelectric point heterogeneity [9,10]. Complementary DNA encoding IL-1α and -β have been cloned, sequenced and expressed in *Escherichia coli* [10–16]. The nucleic acid and amino acid sequence analyses revealed that the homology between the two IL-1 species is very limited, approx. 45 and 23%, respectively [10]. The limited homology appears to be clustered within two structurally conserved domains, thought to be the potential active sites of IL-1 [17]. It is striking that two distinct and distantly related molecules compete for the same receptor [18,19] and mediate the same spectrum of immunological and inflammatory responses [20]. It is therefore of interest to define specific residues which are involved in receptor binding and are important for biological activities.

Through the use of an expression system in *E. coli* large amounts of fully active recombinant IL-1 (rIL-1) are now available for structural and functional characterization [13–16]. However there is a sparsity of information on the structure/function relationship of IL-1 [21]. In a previous communication, we showed that the deletion of residues at the amino-terminus led to a total loss of biological activity of IL-1. This suggests that this

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region of the molecules is essential for structure and/or function [16]. These studies have prompted us to construct a series of N-terminal analogs of IL-1 with which to further define the role of this region of the molecule.

2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

E. coli strain JM101, complementary DNA encoding IL-1β and the parental plasmid (pDP506Δ) used in this study are described in [11,16]. Plasmid pDP506Δ was digested with *Eco*RI and *Hin*dIII and replaced with an oligomer (AATTCCATAGAGGGTATTACATATGCTGGAGCATAGATCTCTGAACTGCACGCTCCGGGACTCACAGCAAAAA) to generate the mature form of IL-1β. The oligomer (73-mer), flanked by *Eco*RI and *Hin*dIII restriction sites, includes a Shine Dalgarno (S/D) sequence (dotted line), translational initiation codon, ATG (underlined), and a silent change at Arg_4-Ser_5 coding sequence to generate a new restriction site *Bgl*II. A series of 35-mers (from *Eco*RI to *Bgl*II) was made, with proper coding sequence mutated, and used to generate various amino-terminal mutants.

2.2. *Purification and bioassay of mutein IL-1*

The rIL-1 was purified by a published procedure [16] and the biological activity was evaluated by the standard murine (C3H/HeJ) thymocyte proliferation assay [22]. rIL-1β possessed specific activity comparable to that of native monocyte IL-1 [9,13,16] as measured by fibroblast prostaglandin E_2 production, thymocyte proliferation and EL4/9 interleukin-2 production assays.

2.3. *Circular dichroism spectroscopy*

Muteins were kept in 20 mM phosphate buffer and 0.1 M NaCl at pH 7.0. The protein concentrations, 0.10 $\pm$ 0.005 mg/ml, were determined by quantitative amino acid analysis. The spectra were acquired by averaging three repetitive scans on a Jobin-Yuon Mark V auto-dichrograph.

2.4. *Receptor binding assay*

IL-1 was iodinated using the Bolton Hunter reagent and the binding of ^{125}I-IL-1 to EL 4/9 cells was carried out according to Dower et al. [18].

2.5. *Anti-tumor study*

Inhibition of B16 melanoma in vivo by recombinant IL-1 was followed [23]. Briefly, C57BL/6 mice (7 mice/clone tested) were transplanted i.d. with B16 melanoma cells (5×10^5 cell/mouse) on day 0, rIL-1 was injected once a day on day 8 through to 14 and inhibition rates were calculated on day 15.

3. RESULTS AND DISCUSSION

We took advantage of recombinant DNA technology to generate a series of N-terminal mutants with which to test the functional significance of IL-1's amino-terminal sequence. The mutant plasmids were constructed, isolated, sequenced and expressed in *E. coli* as described earlier [16]. Recombinant protein from individual constructs was purified to homogeneity and its bioactivity evaluated [16].

Table 1 shows the biologic activity of the analogs determined by the thymocyte proliferation assay [22]. Clone 22a, which had the substitution alanine to threonine at the amino-terminus of IL-1, showed a 4-fold increase in bioactivity. Another mutant (clone 18), in which the first two amino acids have been replaced, demonstrated 7-fold enhanced activity. It has been shown that the flexibility and accessibility of the amino-terminus of a protein modulate its stability and function [24]. Bachmair et al. [25] speculated that the stability of a given protein is largely dependent on its amino-terminal residues. It is possible that the improved activity of these analogs is due to structural rearrangements that result in enhanced receptor binding. However, from circular dichroism (CD) measurements, no gross structural modification can be detected (fig.1). It is feasible that the modification of the amino-terminus can contribute to a more stable configuration or a minor change in structure which influences activity.

The three-dimensional structure of a polypeptide chain is determined by the total number of interatomic interactions and hence by the amino acid sequence. Examining the amino-end of the IL-1 sequence revealed that a protonated arginine (position 4 of IL-1β) might be involved in salt-bridge formation or hydrogen bonding which plays a role

Table 1

Bioactivity of recombinant IL-1 purified from amino-terminal mutants

Clone designation	Amino-terminus sequence of IL-1	Spec. act.[a] (units/mg)	Relative specificity[b] (%)
IL-1β	Ala-Pro-Val-Arg-Ser-Leu-	$1.0 \pm 0.0 \times 10^7$	100
C-22a	Thr-Pro-Val-Arg-Ser-Leu-	$3.8 \pm 1.1 \times 10^7$	380
C-18	Thr-Met-Val-Arg-Ser-Leu-	$7.0 \pm 1.5 \times 10^7$	700
Glu-4	Thr-Met-Val-Glu-Ser-Leu-	$1.4 \pm 1.2 \times 10^4$	0.14

[a] One unit equals the amount of material which gives half of the maximum stimulation. Values are means ± SE from 5 independent experiments and are normalized against IL-1β (DP516) [16]
[b] Calculated from spec. act. related to IL-1β

in stabilizing the protein structure. Substitution of arginine by glutamic acid (Glu-4), which is a single amino acid replacement compared to clone 18, resulted in a total loss of bioactivity. This suggested that the positively charged arginine residue is important in the structure and/or biological activity of IL-1.

The receptor binding affinity of these mutants correlated well with the bioactivity (fig.2). Clone 18 which showed enhanced activity, also demonstrates a higher receptor binding affinity to the EL4/9 cells, a murine T-lymphoma cell line which responds to IL-1 with IL-2 production [26]. The analog Glu-4 which exhibited an almost total loss of activity showed a low receptor binding affinity as evidenced by the dramatic rightward shift in the competition assay (fig.2). Individual competition curves were paralleled suggesting that receptors are of a single class. Thus, the altered bioactivity of these analogs appears to result from changes in receptor binding rather than to post-receptor-mediated events.

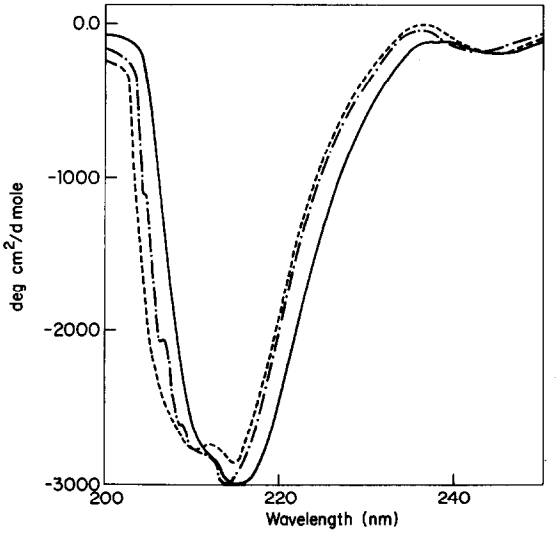

Fig.1. Circular dichroism spectra of recombinant IL-1. Three recombinant proteins: IL-1β (——), Clone-18 (– – –) and Glu-4 (-·-·-) were examined.

It has been shown that IL-1 might be an effective antitumor agent against certain tumor targets [8,23,27]. It is interesting to note that the biological potency of IL-1 as measured by an in vitro assay is reflected in in vivo antitumor activity. Table 2 depicts the efficacy of different rIL-1 muteins on a B16 melanoma tumor in C57BL/6 mice [23]. The ED_{50}, the dose that yields 50% inhibition of tumor growth, corresponds well with the in vitro activity. Clone 18 has a lower ED_{50} value indicative of its greater potency. Glu-4, however, showed no inhibition or toxicity at 10 μg/mouse per day.

All the recombinant clones described in this communication are produced in a similar fashion. The expression system and purification procedure of each clone are identical. Thus, it is possible that the variation in bioactivity is a result of the protein's secondary and tertiary structure. It is surprising that analyses of CD spectra reveal no detectable difference in the secondary structure of the various rIL-1s tested (fig.1). From a CD scan

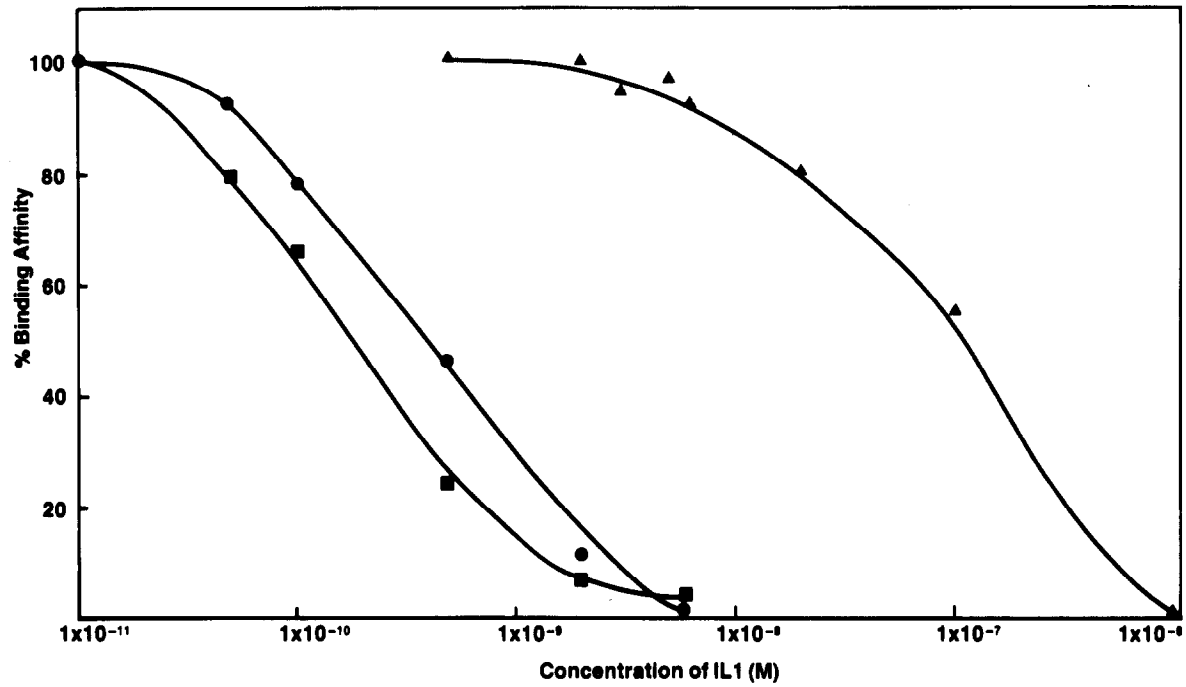

Fig.2. The receptor binding assay of ^{125}I-labeled IL-1 to EL4/9 cells. EL4/9 cells (2×10^7 cells per ml) were incubated with ^{125}I-labeled IL-1 (0.2–1 nM) and varying concentrations of unlabeled IL-1 or IL-1 analogs IL-1β (●), Clone-18 (■) and Glu-4 (▲) for 3 h at 37°C.

of heat inactivated IL-1 (not shown) a rearrangement in secondary structure indicative of increased β-sheet formation was observed. We concluded that the differences in activity of these recombinant proteins are not due to denaturation of the protein but, rather, are attributed by changes in folding of the polypeptides.

Table 2

Anti-tumor effect of rIL-1 mutant proteins on B16 melanoma

Clone tested	ED_{50}[a] (μg/mouse per day)
IL-1β	3.3
Clone-18	1.8
Glu-4	NE[b]

[a] Dose response curves were generated for each recombinant protein by calculation of tumor mass [23] and the ED_{50} value was the dose that yielded 50% inhibition of tumor growth from two independent experiments

[b] NE, not effective, Glu-4 gave no inhibition or toxicity at 10 μg/mouse per day

In summary, we utilized site specific mutagenesis to engineer a series of recombinant IL-1s which have various degrees of bioactivity. Manipulation of the amino-terminal sequence of IL-1 generated recombinant proteins that showed increased or decreased bioactivity as compared to native IL-1. Generally speaking, the terminal regions of proteins are less likely to be involved in the active center [24]; nevertheless, we have shown that the arginine at the amino-end of the IL-1 molecule has a direct impact on configuration and bioactivity. The mutant clones described in this communication should prove of great value in examining the structure-function relationship of IL-1 and lead to the synthesis of agonists or antagonists for IL-1.

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