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SYNTHETIC C-TERMINAL PEPTIDE OF IL-1 FUNCTIONS AS A BINDING DOMAIN AS WELL AS AN ANTAGONIST FOR THE IL-1 RECEPTOR

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Fragments of IL-1β were chemically synthesized and tested for biological activity as well as binding of radiolabelled peptides to the IL-1 receptor. A peptide from the extreme C-terminal region of IL-1β was found to antagonize intact, native IL-1β in the thymocyte bioassay. In addition, this C-terminal region peptide, when radiolabelled, can function as a ligand for the IL-1 receptor on murine cell lines and effectively compete with intact radiolabelled recombinant IL-1β. © 1987 Academic Press, Inc.

The lymphokine termed Interleukin-1 (IL-1) has been shown to be a family of polypeptides, mediating pleiotropic biological effects. Various roles for these molecules have been demonstrated in rheumatoid arthritis (1), bone resorption (2), fever induction (3), modulation of the central nervous system (4), as well as the immune system (5). Thus, the elucidation and characterization of the diverse target tissues and the receptor for IL-1 become critical in understanding its mode of action as well as devising approaches to study regulation at the receptor level.

It has been reported that within the amino acid sequence of IL-1β, five regions are apparently highly conserved. These regions were termed A through E and comprise the following respective residues: region A, 1-20; region B, 82-92; region C, 150-162; region D 165-186 and region E, 219-240 (6). Because such regions or domains connote a universal function, two regions of the C-terminal portion of IL-1β, based on published cloned sequences, were chosen for synthesis (7,8). The extreme C-terminal end was first selected, and the sequence from amino acid residues 237 to 269 was synthesized and named the Y-33-S peptide. A second peptide consisting of residues 172 to 196 was made and termed the C-26-L peptide. An additional residue of cysteine was added to the N-terminus of the C-26-L peptide to facilitate chemical conjugation

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for subsequent sera production. In this report the biological and binding activities of these two peptides were investigated.

MATERIALS AND METHODS

Peptides and Recombinant IL-1β The peptides were custom synthesized by Peninsula Laboratories Inc., Belmont, CA 94002 and generously provided by E.I. Dupont de Nemours and Co, 500 S. Ridgeway Ave., Glenolden, Pa. Recombinant IL-1β was obtained from Cistron Biotechnology, Pine Brook, N.J.

Semi-purified IL-1 LPS-stimulated human monocyte supernatants were concentrated 10-fold with an Amicon filtration unit with a YM-10 membrane and applied onto a MONO Q column (Pharmacia, FPLC) and the run-through fractions pooled, lyophilized and assayed for biological activity.

Thymocyte Assay The bioassay for IL-1 was performed as described elsewhere (9).

Radiolabelling of Peptides and rIL-1β Both peptides which contain a single tyrosine residue were radiolabelled with ^{125}INa (Amersham) and chloramine-T as previously described (10), and rIL-1β was similarly labelled with ^{125}INa and chloramine-T as previously described (11). Specific activities achieved were 30-50 μCi/μg.

Cell Lines Both murine cell lines, EL-4 and L1210, were originally obtained from the ATCC, Parklawn Drive, Rockville, MD. and were maintained in RPMI 1640 with 10% fetal calf serum with antibiotics and L-glutamine.

Radioreceptor assay The assay was performed as described previously (11). Briefly, cells were washed three times with PBS containing 0.1% BSA and adjusted to 1 x 10^8 viable cells per ml. Added to 1.5 ml microfuge tubes were either 100 μl of buffer or competitor, 500 nM for Y-33-S-peptide or 200 nM for rIL-1. Then 50 μl of radiolabelled Y-33-S-peptide, C-26-L-peptide, or rIL-1 (100,000 to 300,000 CPM) was added next. Binding was initiated by addition of 100 μl of cells (1 x 10^7) and rotated for indicated time and temperature. Bound ligand was separated from free ligand by removing 75 μl aliquots and layering over a mixture of bis(2-ethyl-hexyl) phthalate and dibutyl phthalate oil (Kodak) and centrifuging for one minute at 4°C in a Beckman microfuge B to pellet the cells. The pellet which contained bound radiolabelled ligand to cells was cut off and counted in a gamma counter.

Cross-linking of Peptides or rIL-1β Radiolabelled peptides or rIL-1β were allowed to associate to 1 x 10^7 viable L1210 cells as described above for 2 to 4 hours at 4°C. The cells were washed twice with PBS and resuspended in 50 μl of plain PBS. Added to this was 2 μl of 50 mg/ml DSS or DST freshly dissolved in DMSO (Pierce Chemical, Co.) The cells were incubated at 4°C for 1 hour with rotation. The cells were washed twice with plain PBS and resuspended in 100 μl of PBS containing 1% TX-100 and 50 mM PMSF to solubilize the cells. After 5 minutes the cells were then centrifuged for 15 minutes in a Beckman Microfuge B to pellet debris and insoluble material. The supernatants containing the solubilized receptor were either prepared for gel electrophoresis or were frozen at -70°C.

Gel Electrophoresis The samples for electrophoresis were prepared by diluting 1:1 in sample preparation buffer and boiled for 2 minutes. Pre-stained molecular weight standards were prepared also in a similar fashion (BRL, Gaithersburg, MD). 8% polyacrylamide gels with a 3% stacker were prepared and run as described by Laemlli (12). After electrophoresis the gel was fixed, dried and KODAK XAR film was exposed to the gel at -70°C. The film was usually exposed for 10-12 days and then developed.

RESULTS

The peptides were first tested for biological activity in the thymocyte assay which is the most commonly used bioassay for IL-1. As shown in Figure no. 1, when Y-33-S was added in increasing concentrations to a fixed concentration of semi-purified IL-1-β, the Y-33-S peptide consistently inhibited the biological activity of native IL-1-β in a dose dependent manner. When the C-26-L peptide was tested in a similar fashion, no reproducible, dose dependent inhibition of native IL-1-β was observed with the concentrations tested (data not shown). No biological activity was observed when either of these two peptides were added alone in this assay. These results suggested that Y-33-S but not C-26-L may function as an IL-1 antagonist. The specificity of this response was tested by adding peptides unrelated to the IL-1 assay. Substance P, β-endorphin, vasoactive intestinal peptide and neurotensin at 4 μg/ml, did not inhibit the ability of semi-purified IL-1β to stimulate incorporation of ^{3}H thymidine (data not shown).

The peptides were radiolabelled with ^{125}INa and the binding properties of these radiolabelled peptides were investigated in radioreceptor binding studies. Previously, it was reported that ^{125}I-IL-1β bound to EL-4 cells with a Kd of 1.96 ($\pm$0.8) nM with

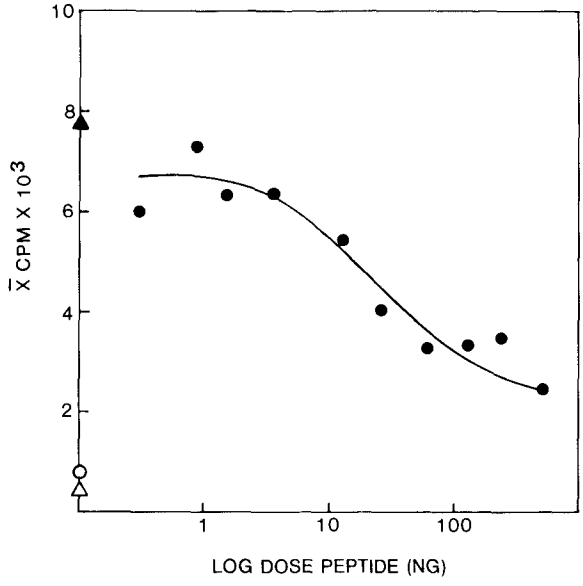

Figure 1. Activity of Y-33-S peptide in the thymocyte assay. Increasing concentrations of Y-33-S peptide (●–●) were added to the assay in the presence of 12 units semi-purified human IL-1β. IL-1β alone (▲–▲), Y-33-S alone (○–○) and no additions (△–△).

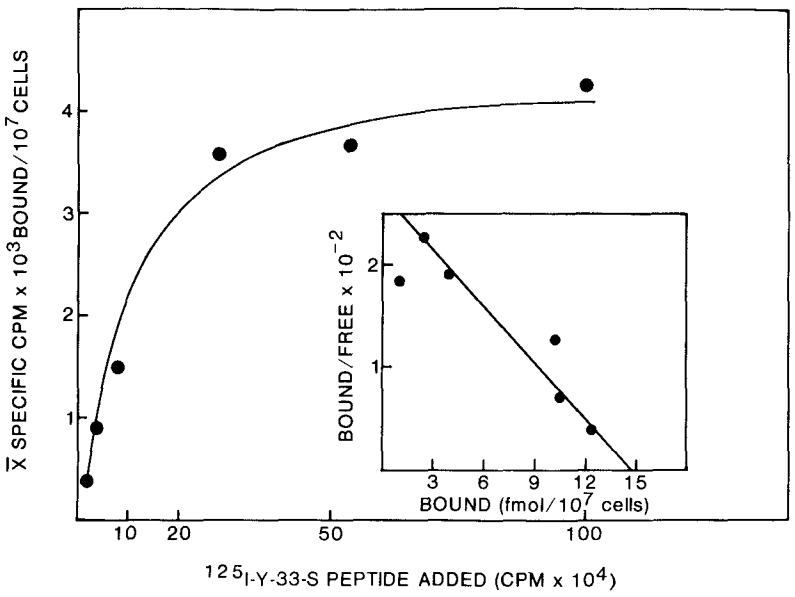

Figure 2. Concentration dependence of ^{125}I-Y-33-S peptide specific binding to 1 x 10^7 EL-4 cells at 4°C. Inset shows the data plotted according to Scatchard. These data yielded a K_d of 2 nM and a B_{max} of 890 binding sites per cell.

200-1100 binding sites per cell (13,14). Here, ^{125}I-Y-33-S but not ^{125}I-C-26-L specifically bound to 1x10^7 EL-4 cells and equilibrium was achieved at 4-6 hours at 4°C (data not shown). Figure 2 shows that EL-4 cells bound ^{125}I-Y-33-S in a specific and saturable manner. The inset panel shows the data according to Scatchard (15). These data indicate that ^{125}I-Y-33-S binds to EL-4 cells with a Kd of 2.0 nM and there are 890 specific high affinity sites per cell.

Other murine lymphoid lines were screened for IL-1 receptors. The murine T-cell, L1210, specifically bound more ^{125}I-rIL-1 than EL-4. Thus, additional studies were performed using L1210. When either ^{125}I-rIL-1β or ^{125}I-Y-33-S associated to 1x10^7 L1210 at room temperature, equilibrium was achieved at 45 to 60 minutes at 25°C (data not shown). Figure 3A shows that ^{125}I-rIL-1 bound with high affinity to L1210 cells. Intact rIL1β inhibited the binding of ^{125}I-rIL-1β best, followed by Y-33-S and C-26-L. The respective IC_{50} values are 6nM, 60nM and 700nM. Similarly, ^{125}I-Y-33-S bound with high affinity to L1210. Competition studies indicated that the order of potency was rIL-1>Y-33-S>C-26-L (Figure 3B). In this case the respective IC_{50}'s were 20nM, 40nM and 200nM. Thus, the pharmacology of binding to L1210 is similar regardless whether radiolabelled rIL-1 or Y-33-S was used as the ligand.

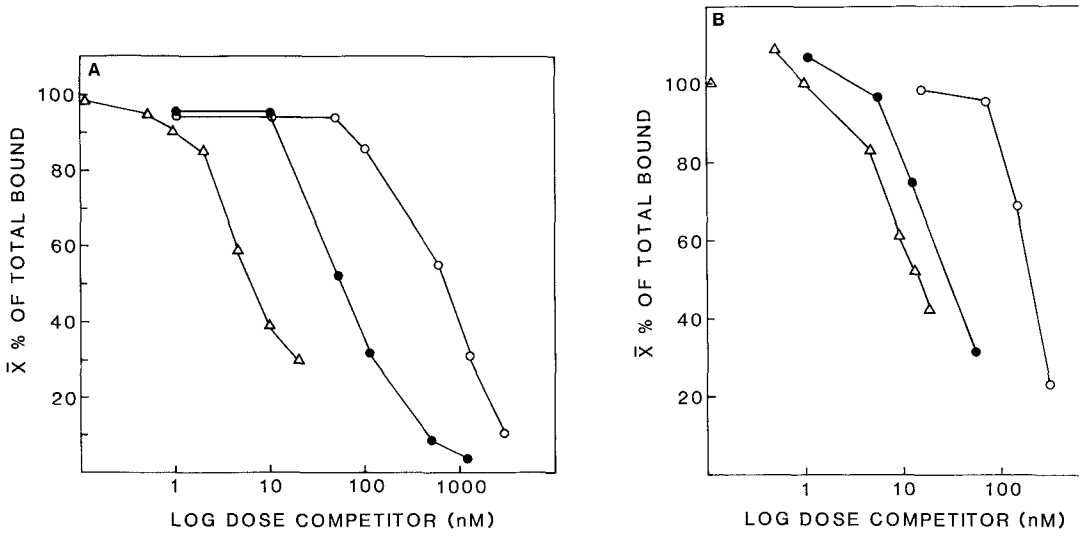

Figure 3A. Competition of Binding. The amount of ^{125}I-rIL-1β bound to 1 x 10^7 L1210 cells was determined as a function of unlabelled rIL-1β (△—△), Y-33-S (●—●) or C-26-L (○—○).

Figure 3B. Competition of binding. The amount of ^{125}I-Y-33-S bound to 1 x 10^7 L1210 cells was determined as a function of unlabelled rIL-1β (△—△), Y-33-S (●—●) or C-26-L (○—○).

The biochemical nature of this IL-1 binding site was next investigated. Previously it was reported that cross-linking of IL-1β to EL-4 or 3T3 cells yielded a major band in the range of 97 kd molecular weight (13,16). Because the molecular weight of the IL-1β is 17,800 daltons, the molecular weight of the IL-1 receptor is $\approx$ 80 kd. Here, ^{125}I-rIL-1β was cross-linked to L1210 with DSS,and the cells solubilized and extracted with 1% TX-100. The proteins were subjected to SDS-gel electrophoresis, the gel was dried and exposed to KODAK XAR film. Figure 4A shows these results, where in lane 1 a major band was resolved at $\approx$ 97 kd, strongly indicating that this IL1 binding site on L1210 is similar to that described with EL-4 and 3T3. This 97 kd was absent in lane 2 which had excess Y-33-S (500nM), lane 3 which had excess C-26-L (1 μM) and lane 4 which had excess r-IL-1β (200nM). In all gels, the material seen at the top of the gel represents material which did not enter the resolving gel whereas the radioactivity at the bottom of the gel (<43kd) represents radiolabelled rIL-1 which did not fully cross-link.

To further investigate this binding to L1210, ^{125}I-Y-33-S was cross-linked to L1210, extracted with 1% Tx-100 and electrophoresed in a similar manner as above. Figure 4B shows cross-linked ^{125}I-Y-

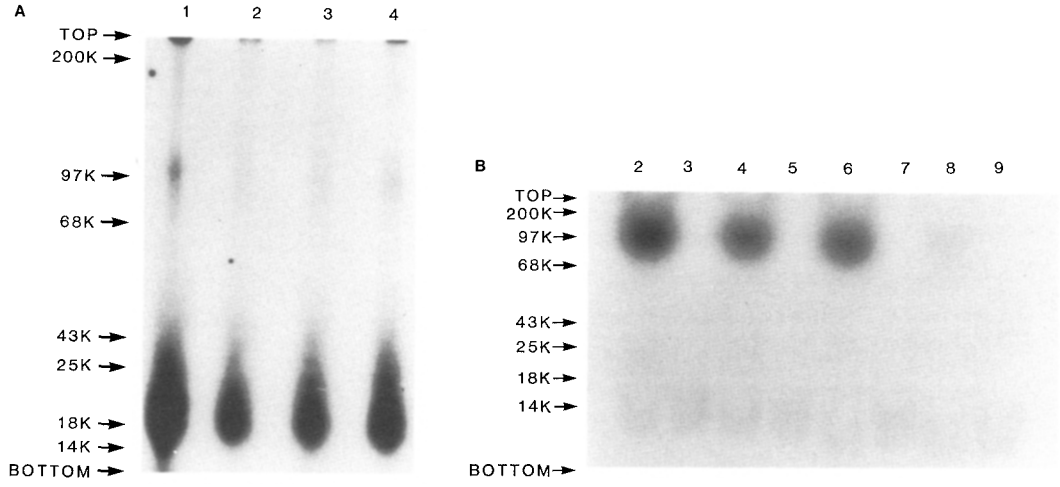

Figure 4A. Autoradiogram of 8% polyacrylamide gel with solubilized L1210 membranes after cross-linking with ^{125}I-rIL-1β. 1x10^7 cells were incubated at 4°C for 3-4 hours with ^{125}I-rIL-1β (≈200,000 CPM) in the presence of no additions (lane 1), 500nM Y-33-S (lane 2), 1μM C-26-L (lane 3) or 200nM rIL-1β (lane 4). These cells were washed of unbound ligand, cross-linked with DSS, solubilized with 1% TX-100 and prepared for gel electrophoresis as described in the methods and material section. The molecular weight standards are myosin H-chain (200 kd), phospholipase B (97.4 kd), bovine serum albumin (68 kd), ovalbumin (43 kd), chymotrysinogen (25.7 kd), β-lactoglobulin (18.4 kd) and lysozyme (14.3 kd).

Figure 4B. Autoradiogram of 10% polyacrylamide gel with solubilized L1210 membranes after cross-linking of ^{125}I-Y-33-S. 1x10^7 cells were incubated at 4°C for 3-4 hours with ^{125}I-Y-33-S (≈200,000 CPM) in the presence of no additions (lanes 2,4,6 and 8) or with 500nM Y-33-S (lanes 3,5,7 and 9). The cells were washed of unbound ligand and crosslinkers added. Lanes 2-3 had DSS at a final concentration of 100μg, while lanes 4-5 were a final concentration of 1 mg. Lanes 6-7 had DST at a final concentration of 100μg and lanes 8-9 were a final concentration of 1mg. These cell preparations were solubilized and prepared for gel electrophoresis as described in materials and methods. The molecular weight standards used are the same as in figure 4A.

33-S yielded a major band at ≈ 80-85 kd. Similar results were obtained using the cross-linkers DSS or DST. If one subtracts the molecular weight of the Y-33-S peptide (3558), the molecular weight of the IL-1 receptor is ≈ 76-82 kd. Thus, regardless if one cross links ^{125}I-rIL-1 or ^{125}I-Y-33-S the molecular weight of the IL-1 receptor is similar.

DISCUSSION

It is generally accepted that for polypeptide hormones, distinct domains may exist which are responsible for binding activity and/or biological activity. The 27 amino acid hormone secretin is a potent agonist which stimulates enzyme and electrolyte secretion from the exocrine pancreas whereas secretin

5-27 functions as an antagonist. Similarly, the 28 amino acid VIP stimulates amylase release from pancreatic acini whereas VIP^{10-28} is an antagonist. Thus, when secretin and VIP bind to their respective receptors the N-terminus is essential for biological activity whereas much of the binding domain is present in the C-terminal (17-19). Similarly, Rosenwasser et al. (20) utilizing deletion mutants of IL-1β demonstrated that the absence of this Y-33-S peptide (the E-region) leads to a decrease of the biological activity. These results suggests that the C-terminal region of IL-1β is important for biological activity and probably represents a binding domain. Further, the biologically active region was shown to be towards the N-terminal of IL-1β (the C and D regions) which does not overlap with the E-region containing the Y-33-S sequence.

This prompted us to synthesize 2 fragments of IL-1β and investigate the biological activity of these fragments. The Y-33-S and C-26-L peptide had no biological activity in the mouse thymocyte assay themselves, however, Y-33-S, but not the C-26-L peptide antagonized the uptake of ^{3}H-thymidine caused by r-IL-1β. In particular, Y-33-S bound with high affinity to EL-4 (Kd=2nM) and to a single class of sites (890/cell). Similarly, the Y-33-S peptide bound with high affinity (IC_{50}=20-60nm) to L-1210 whereas r-IL1β and C-26-L were approximately 1-order of magnitude more or less potent, respectively. While the thymocyte assay is the most commonly used bioassay for IL-1, other assays do exist. These are the fibroblast proliferation assay (21), induction of collagenase from synovial cells (1), induction of Interleukin-2 from T-helper cells (22) and fever induction (3). The Y-33-S and C-26-L peptides are currently being tested in these assays.

The use of Y-33-S as a ligand is a novel result and suggests a careful screening of other lymphokines and growth factors in terms of structure-function relationships. The experiments with EL-4 do indicate that similar values for the Kd and B_{max} may be obtained to those published even though only a fragment of the intact molecule is used. The competition data are also consistent with the idea that the Y-33-S peptide represents one of the binding domains for IL-1 and the cross-linking data demonstrate biochemically that Y-33-S and intact IL-1 are interacting with the same site. Thus, the Y-33-S peptide may serve as a good IL-1 receptor probe.

The use of synthetic peptides such as Y-33-S from a larger molecule such as IL-1 presents some unique opportunities because such peptides can be made in copious quantities at a reasonable cost without having to purify the native molecule from either

natural or recombinant sources. The Y-33-S peptide may lead to a better understanding of the structure-function relationships of IL-1β to its receptor.

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