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# EFFECTS OF MONOCLONAL ANTIBODIES RAISED AGAINST THE COMMON ACUTE LYMPHOBLASTIC LEUKEMIA ANTIGEN ON ENDOPEPTIDASE-24.11 ACTIVITY

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**Abstract**—The common acute lymphoblastic leukemia antigen (CALLA, CD10) has been identified as neutral endopeptidase-24.11 (NEP), a mammalian ectoenzyme involved in the inactivation of regulatory peptides, such as the enkephalins and atrial natriuretic peptide. Twenty monoclonal antibodies directed against the human antigen, were tested for their ability to inhibit the enzymatic activity of the human and rat peptidases expressed by cell lines. Six anti-CALLA antibodies were found to inhibit 50% or more of the hydrolysis of D-Ala$^2$-leucine enkephalin by the neutral endopeptidase present on the human leukemic cell line Reh6 and, to a lesser extent, the hydrolysis of atrial natriuretic peptide. This may indicate that their binding may affect regions of the active site more important for the dipeptidylcarboxypeptidase activity of the enzyme. Only four antibodies cross-reacted with the peptidase from the rat epithelial cell line Rat2, as shown by membrane immunofluorescence, and these also partially inhibited enzyme activity. No antibody was able to inhibit completely the activity of the human and rat enzymes and all the active antibodies appeared to behave as non-competitive inhibitors of substrate cleavage. These monoclonal antibodies could be used in mapping studies of NEP.

The common acute lymphoblastic leukemia antigen (CALLA‡, CD10) is a membrane glycoprotein identified on subpopulations of immature B-lymphoid cells [1, 2], malignant lymphoid cells of T- and B-lymphocyte lineage [3, 4], neutrophils [5], bone marrow stroma cells and also on non-hematopoietic tumors [6, 7] and renal epithelium fibroblasts [8, 9]. The presence of CALLA on leukemia cells of patients with acute leukemia, blastic crisis of chronic myelocytic leukemia and some non-Hodgkins lymphomas has made it a useful marker for the diagnosis of these diseases.

The cloning and sequencing of the cDNA of human kidney CALLA [10] showed that its deduced amino acid sequence is identical to that of human NEP (EC 3. 4. 24. 11) [11]. NEP is a zinc-containing proteolytic ectoenzyme whose sequence is highly conserved in rabbit [12], rat [13] and human [11]. It is mainly located in kidney [14] but also found discretely distributed in the brain and in several other peripheral tissues [15, 16]. The enzyme hydrolyses a variety of biologically active peptides such as enkephalins in the brain [17] and the circulating atrial natriuretic peptide in the periphery [18], and NEP inhibitors are currently undergoing clinical trials to evaluate their potential as analgesic or antihypertensive agents (review in Ref. 19). Recent studies have shown that, as expected, CALLA and CALLA-bearing cells have an NEP-like activity [20, 21]. The role of the enzyme on lymphoblastic cells or in B-cell differentiation is however still unknown.

Since the discovery of CALLA, many CD10 mAbs have been developed for diagnostic use and cellular studies [22, 23]. Some have also been used for *ex vivo* treatment of bone marrow and *in vivo* passive serotherapy and one mAb, linked to ricin A chain, has been demonstrated to disrupt the growth of a CALLA+ cell line *in vitro* (review in Refs 23 and 24). However, as these antibodies were raised against a protein of no known function there have been no reports on their effects on NEP activity. We have therefore looked at the effects of 20 of these CD10 mAbs on the enzyme expressed on the human leukemic cell line Reh6 [25]. The cross-reactivity of these antibodies, prepared against the human antigen, with NEP present on the Rat2 renal epithelial cell line [26] has also been examined. Of the 20 CD10 mAbs tested, none inhibited completely the activity of the human and rat peptidases and the degree of inhibition was found to vary with the substrate used. The antibodies which altered NEP activity to any substantial degree were found to be non-competitive inhibitors.

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‡ Abbreviations: CALLA, common acute lymphoblastic leukemia antigen: NEP, neutral endopeptidase-24.11; CD10 mAb, anti-CALLA monoclonal antibody; hANP, human atrial natriuretic peptide; thiorphan, *N*-[(*R*,*S*)-2-mercaptomethyl-1-oxo-3-phenylpropyl]glycine.

Table 1. Characteristics and reactivity of the CD10 monoclonal antibodies in membrane immunofluorescence towards NEP on the human leukemic B-cell line, Reh6 and the Rat2 renal epithelial cell line

| Antibody | Isotypes | Membrane fluorescence Human NEP | | Rat NEP |
|---|---|---|---|---|
| 55 | γ2a | + | (6400) | − |
| 79 | γ2a | + | (12800) | − |
| K14 | γ2b | + | (25600) | − |
| K50 | γ3 | + | (3200) | + |
| ALB2 | γ2a | + | (6400) | − |
| ALB1 | γ1 | + | (6400) | − |
| VIL-A1 | μ | + | (6400) | − |
| PHM6 | γ2b | + | (3200) | + |
| BA3 | γ2b | + | (3200) | + |
| 24.1 | γ3 | + | (800) | − |
| SS-2/36 | γ1 | + | (3200) | − |
| S-8 | γ | + | (1600) | − |
| M-L57 | γ1 | + | (800) | − |
| J13 | μ | + | (25600) | − |
| J5 | γ2a | + | (800) | − |
| NL-5 | γ2a | + | (3200) | − |
| CLB-CALLA1 | γ2a | + | (3200) | − |
| W8E7 | γ2a | + | (3200) | + |
| NU-N1 | γ1 | + | (400) | − |
| NU-N2 | γ2b | + | (1600) | − |

All antibodies were as ascites, except ALB1, NL-1 and W8E7 which were purified immunoglobulins. The values indicated in the brackets are the reciprocal of the last saturating dilution of each mAb sample for $2 \times 10^5$ cells. The absolute mAb concentration for ALB1 and ALB2 was 0.8 μg/mL.

## MATERIALS AND METHODS

*Materials.* [$^3$H]D-Ala$^2$-leucine enkephalin (51 Ci/mmol) was purchased from the CEA (Saclay, France) and D-Ala$^2$-leucine enkephalin from Bachem (Switzerland). Captopril was a generous gift of Servier Laboratoires (France) and bestatin was from Roger Bellon Laboratoires (France). The NEP inhibitor thiorphan was synthesized as described previously [27]. hANP was from Neosystem (France). All other products were purchased from Sigma-France.

*Antibodies.* The 20 CD10 mAbs were provided during the Third Leukocyte Differentiation Antigen Workshop (Oxford, 1985). The origin of the different mAbs is indicated in Ref. 28. Two of these mAbs (ALB1 and ALB2) were produced in our laboratory [29] and are commercially available from Immunotech. W8E7 can be purchased from Beeton-Dickinson (Division Diagnostic) and J5 from Coulter Corporation, Hialeah, FL, U.S.A. All mAbs used in this study were of mouse origin and their isotype is indicated in Table 1. They were produced against whole leukemic cells. In order to determine the concentration of specific antibody in each sample, they were submitted to serial half dilutions and each dilution was tested on Reh6 cells. The presence of antibody on the cells was detected using FITC-coupled goat anti-mouse F(ab)'2 (Immunotech S.A., Marseille, France). To 50 μL of $2 \times 10^5$ Reh6 cells were added 50 μL of the successive dilutions. After 30 min incubation at 4°, the cells were washed and incubated with the secondary reagent at a saturating concentration. The cells were washed and fixed in phosphate buffered saline (Mérieux Institute, Lyon, France) containing 1% formaldehyde. The amount of fluorescence on the surface of the cells was determined by flow cytometry (Coulter Profile II Analyser), and the last saturating dilution of each mAb sample was calculated.

*Cell cultures.* The human leukemic B-cell line Reh6 was cultured at 37° in RPMI medium (Gibco/BRL, France) supplemented with 10% fetal calf serum and the renal epithelial cell line Rat2 in MEM (Gibco) supplemented with 10% fetal calf serum.

*Membrane preparations.* Confluent Rat2 cells (25 million cells/flask) were dissociated for 3 min with a solution of trypsin–EDTA (Gibco) and diluted in an excess of MEM. Membranes from Reh6 and Rat2 cell lines were prepared by washing the cells in 50 mM Tris–HCl pH 7.4 and centrifuging at 100,000 *g* for 1 hr at 4°.

*Incubation of NEP with antibodies and measurement of* [$^3$H]D-*Ala$^2$-leucine enkephalin degradation.* Antibodies were initially diluted 50-fold in 50 nM Tris–HCl pH 7.4. Dilutions of the membrane preparations in the same buffer were calculated in order to obtain similar concentrations of protein (1–2 mg/mL) and enzymatic activities that hydrolysed less than 10% of the total substrate in 25 min. Ten microlitres of these membrane preparations, corresponding to $2 \times 10^5$ cells, were incubated with 10 μL of the diluted antibodies at 4° overnight. Enzymatic activity was then measured using [$^3$H]D-Ala$^2$-leucine enkephalin as substrate in a total volume of 100 μL 50 mM Tris–HCl pH 7.4. The enzyme–antibody solutions were preincubated for 15 min at 25° with 10 μM bestatin and 1 μM captopril to prevent hydrolysis of the substrate by aminopeptidases and angiotensin converting enzyme. [$^3$H]D-Ala$^2$-leucine enkephalin (20 nM) was then added and the reaction was stopped after 25 min incubation at 25° by addition of 10 μL 0.5 M HCl. The tritiated metabolite [$^3$H]tyrosyl-D-alanyl-glycine was separated on Porapak Q (Waters Associates) as described previously [30]. Ten microlitres of the membrane preparation, incubated overnight without antibody, in a final volume of 20 μL Tris buffer and treated as described above was taken as a control.

*Determination of inhibition constants.* $IC_{50}$ values were calculated by preincubating the enzyme–antibody solutions for 15 min with 10 μM bestatin and 1 μM captopril and increasing concentrations of thiorphan before addition of the substrate.

*Steady-state kinetics.* The $K_m$ and $V_{max}$ values for D-Ala$^2$-leucine enkephalin were determined from Lineweaver–Burk plots, with substrate concentrations ranging from 3.125 to 800 μM, and 20 nM [$^3$H]D-Ala$^2$-leucine enkephalin included as a tracer.

*Influence of CD10 mAbs on hANP degradation by NEP.* Twenty microlitres of an enzyme–antibody solution were incubated at 37° for 45 min with 3 μL of a 500-μM hANP solution in the presence of 10 μM bestatin and 1 μM captopril. The metabolites were then separated and hANP metabolism quantified by reverse phase HPLC (Shimadzu) with a C18 nucleosil column, using a gradient of acetonitrile from 5 to 45% in 0.07% trifluoroacetic acid in 20 min, as described previously [31].

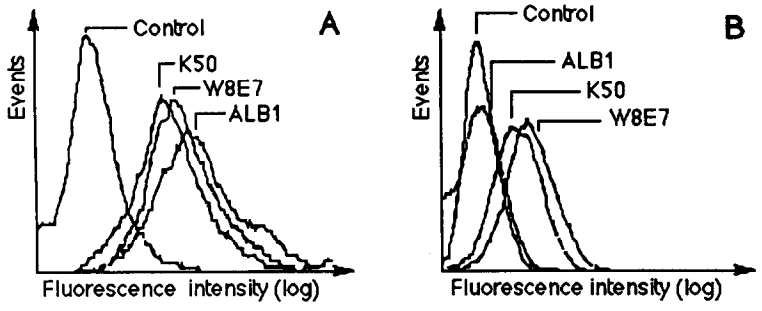


Fig. 1. CD10 immunofluorescence on the human leukemic B-cell line Reh6 (A) and on the rat renal epithelial cell line Rat2 (B). Three mAbs are shown as examples; the mAbs K50 and W8E7 bind to the human and rat NEP located on the surface of the cells whereas the epitope recognized by the mAb ALB1 is present only on the human cell line.

*Membrane immunofluorescence.* Indirect immunofluorescence was performed according to a method described previously [32] and analysed by flow cytometry on an ATC-3000 (Odam-Brucker, Wissenburg, France).

*Protein determination.* Protein concentrations were determined by the method of Bradford [33] with bovine serum albumin as standard.

## RESULTS

### *Membrane immunofluorescence*

As shown in Table 1, immunoglobulins of $\gamma 1$, $\gamma 2a$, $\gamma 2b$ or $\gamma 3$ and $\mu$ isotypes were tested. The reactivity of the CD10 mAbs was verified by membrane immunofluorescence experiments against the Reh6 membrane antigen. All the 20 mAbs bound to the Reh6 cells, indicating that the conditions used were appropriate for enzyme–antibody recognition (Table 1 and Fig. 1). The maximum dilution of antibody which still saturated the enzyme in the Reh6 membranes was also determined. This varied from 1:400 for NU-N1 to 1:25,600 for K14 (Table 1). Subsequently all mAbs were used at a 1:100 final dilution for inhibitor studies. Four of the mAbs (K50, PHM6, BA3 and W8E7) also reacted against the rat cell line Rat2, indicating the presence of cross-reacting epitopes between the two species (Table 1 and Fig. 1).

Table 2. Inhibition (%) of [$^3$H]D-Ala$^2$-leucine enkephalin and hANP degradation by human lymphocyte Reh6 CALLA and Rat2 renal epithelial NEP in the presence of CD10 monoclonal antibodies

| Antibodies | Inhibition (%) of leucine enkephalin hydrolysis | | Inhibition (%) of hANP hydrolysis | |
|---|---|---|---|---|
| | Human NEP | Rat NEP | Human NEP | Rat NEP |
| 55 | 40 | 10 | NT | NT |
| 79 | 40 | 10 | NT | NT |
| k14 | 30 | 10 | NT | NT |
| k50 | 60 | 50 | 25 | 5 |
| ALB2 | 65 | 15 | 20 | NT |
| ALB1 | 30 | 10 | NT | NT |
| VIL-A1 | 45 | 10 | NT | NT |
| PHM6 | 55 | 35 | 25 | 15 |
| BA3 | 35 | 30 | NT | 30 |
| 24.1 | 45 | 5 | NT | NT |
| SS-2/36 | 45 | 0 | NT | NT |
| S-8 | 30 | 10 | NT | NT |
| M-L57 | 20 | 5 | NT | NT |
| J13 | 40 | 5 | NT | NT |
| J5 | 15 | 5 | NT | NT |
| NL-1 | 20 | 0 | NT | NT |
| CLB-CALLA1 | 55 | 10 | 25 | 10 |
| W8E7 | 55 | 20 | 10 | 10 |
| NU-N1 | 50 | 0 | 10 | 0 |
| NU-N2 | 5 | 0 | 0 | 0 |

mAbs were used at a final dilution of 1:100. Incubations of the membrane preparations with mAbs and measurement of enzymatic activity were as described in Materials and Methods. Membrane preparations incubated with Tris buffer alone were taken as a control. The data are the mean values of three independent experiments performed in duplicate and are given as +10%. NT, not tested.

Table 3. Effects of monoclonal antibodies on steady-state kinetic parameters of D-Ala² leucine enkephalin for Reh6 NEP

| Antibody | Reh6 NEP $K_m$ (μM) | Reh6 NEP $V_{max}$ (pmol/min/ng) |
|---|---|---|
| None | 130.1 ± 18.1 | 0.180 ± 0.04 |
| ALB2 | 50.3 ± 13.0 | 0.060 ± 0.02 |
| K50 | 65.0 ± 14 | 0.075 ± 0.012 |
| PHM6 | 138.3 ± 13 | 0.083 ± 0.011 |
| CLB/CALLA | 68.5 ± 0.7 | 0.080 ± 0.02 |
| W8E7 | 60.0 ± 9 | 0.070 ± 0.014 |
| NUN1 | 73 ± 14.1 | 0.075 ± 0.007 |
| NUN2 | 125.0 ± 20 | 0.190 ± 0.05 |

Antibodies were used at a final dilution of 1:100 and the incubations carried out at 20° overnight. Values are means ± SE.

*Effects of CD10 mAbs on enzymatic activity of membrane-bound NEP*

Six of the antibodies (K50, ALB2, PHM6, CLB-CALLA1, W8E7 and NU-N1) were found to inhibit significantly (≥50%) the cleavage of the Gly³-Phe⁴ bond of the pentapeptide [³H]D-Ala²-leucine enkephalin by NEP present on Reh6 membrane preparations (Table 2). The most potent antibodies were ALB2 and K50 which gave a 65 and 60% inhibition, respectively. Ten other antibodies (55, 79, K14, ALB1, VIL-A1, BA3, 24.1, SS-2/36, S-8 and J13) had an intermediate effect of between 30 and 40%. M-L57 and NL-1 gave a 20% inhibition. J5, which has been used in a number of *in vivo* and *in vitro* studies (review in Ref. 23) inhibited substrate degradation by only 15%. No significant inhibition was observed with NU-N2. ALB2 at a final dilution of 1:100 had no effect on the $IC_{50}$ of the NEP inhibitor thiorphan which was 9 ± 5 nM for controls and 12 ± 5 nM in the presence of the antibody.

The four cross-reacting CD10 mAbs (K50, PHM6, BA3 and W8E7) partially inhibited the Rat2 enzyme, the most potent being mAb K50 with 50% inhibition (Table 2). As expected, the 16 remaining mAbs were unable to inhibit significantly the degradation of [³H]D-Ala²-leucine enkephalin by the rat enzyme.

The most potent inhibitory antibodies were also tested for their effects on the degradation of a larger substrate, hANP. This peptide, which has the amino acid sequence:

SLRRSSCFGGRMDRIGAQSGLGCNSFRY,

is cleaved by NEP at the Cys⁷-Phe⁸ bond, within the disulfide ring formed by the two cysteine residues [18]. As shown in Table 2, the antibodies had less effect on the hydrolysis of hANP than on the hydrolyis of [³H]D-Ala²-leucine enkephalin. ALB2 and K50, for example, only inhibited hANP degradation by 25 and 20%, respectively.

*Effects of CD10 mAbs on steady-state kinetic parameters*

The $K_m$ and $V_{max}$ values of D-Ala²-leucine enkephalin for Reh6 NEP were measured in the presence or absence of a 100-fold dilution of the inhibitory mAbs. The results are shown in Table 3. All the mAbs tested were found to decrease the $K_m$ and $V_{max}$ approximately 2-fold, except for mAb PHM6 which had no effect on the $K_m$ for Reh6 NEP but decreased the $V_{max}$ value 2-fold. NU-N2, in agreement with the results in Table 2, did not modify the steady-state kinetic parameters of the peptidase. The Lineweaver–Burk plots of the native and inhibited enzyme showed that the lines intersected below the abscissa, indicating a mixed non-competitive inhibition (Fig. 2).

## DISCUSSION

NEP/CALLA is anchored in the lipid bilayer of the cell plasma membrane at its N-terminal and has its active site exposed at the cell surface [34]. The CD10 mAbs used in this study were produced against whole leukemic cells and should therefore recognise the extracellular C-terminal domain which contains the active site of the enzyme.

Only one antibody (NU-N2) had no effect on the hydrolysis of [³H]D-Ala²-leucine enkephalin and hANP by human NEP although it is able to interact with the enzyme, as shown by immunofluorescence. Any conformational modification expected to occur when this antibody binds to the protein does not therefore induce important structural changes in its active site. The 19 other CD10 mAbs were found to inhibit the enzyme to a greater or lesser extent, six of them inhibiting [³H]D-Ala²-leucine enkephalin hydrolysis by more than 50%. The mAb 24-1, which

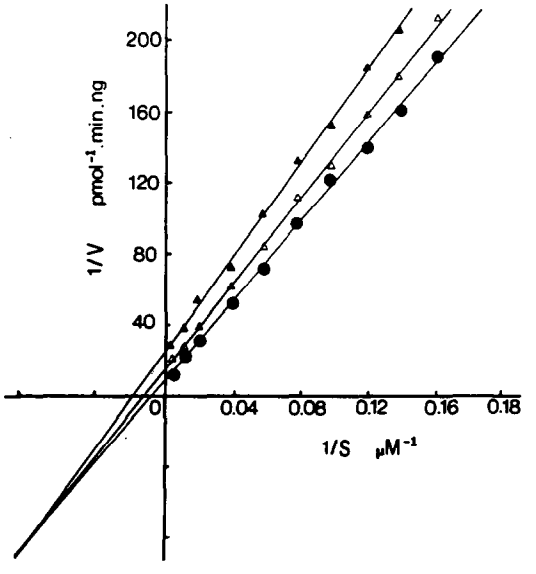


Fig. 2. Lineweaver–Burk plot of the hydrolysis of D-Ala²-leucine enkephalin by human NEP in the presence or absence of mAbs NU-N1 and mAb ALB2. Human Reh6 NEP was incubated in Tris buffer alone (●) or buffer containing mAb NU-N1 (△) or mAb ALB2 (▲) for 4 hr at 20°. The initial rate of D-Ala²-leucine enkephalin degradation ($V$) was determined for substrate concentrations ($S$) ranging from 3.125 to 800 μM. The Lineweaver–Burk plot ($1/V$ against $1/S$) shows that the three lines intersect above the abscissa. Similar plots were obtained with the other CD10 mAbs tested (Table 3).

competes with J5 for binding to the surface molecule [35], gave 45% inhibition whereas J5 gave only 15% inhibition, suggesting that the two mAbs probably recognize two different but spatially close epitopes. No CD10 mAb was found to inhibit completely the degradation of the pentapeptide by the human enzyme and therefore the antibodies probably do not recognize the active site itself. In addition their effects on enzymatic activity are probably not due to a steric hindrance between the bound mAb and the substrate, as the hydrolysis of the 28-residue cyclic hANP was less affected than that of the pentapeptide. It seems more likely that antibody binding causes a slight transconformational change in the active site of the enzyme. This is supported by the steady-state kinetics showing that the mAbs tested are non-competitive inhibitors of NEP. Furthermore, the most potent inhibitory mAb, ALB2, did not influence significantly the $IC_{50}$ value of thiorphan. This molecule has a strong nanomolar affinity for NEP, due to its mercapto group which strongly chelates the zinc atom present in the active site [27], and thus its binding is less likely to be perturbed by minor changes in the active site than that of the peptide substrates, which have micromolar affinities.

NEP, present on neutrophil membranes, has been proposed to inhibit the chemotactic response of these cells by cleaving the chemotactic peptide, f-Met-Leu-Phe [36]. Studies carried out with mAb BA3 have shown that it also reduces *in vitro* the chemotactic response of neutrophils by 20% [37]. Although this work was carried out before the co-identity of CALLA and NEP was established, it has since been hypothesized that BA-3 binds to an epitope distinct from the active site and induces a conformational change which affects substrate degradation [23]. This is borne out by the present results where it was found that BA3 inhibited the hydrolysis of [$^3$H]D-Ala$^2$-leucine enkephalin by only 35%.

In general, the mAbs were more efficient in inhibiting the degradation of [$^3$H]D-Ala$^2$-leucine enkephalin than that of hANP. A number of explanations are possible, but it is interesting that with the former substrate NEP acts as a dipeptidylcarboxypeptidase and with the latter as an endopeptidase. For dipeptidylcarboxypeptidase activity it is known that an interaction between the C-terminal free carboxyl group of the substrate and an enzyme residue, recently proposed to be Arg$^{102}$ [38, 39], is important for substrate binding. This interaction is strongly reduced when the enzyme acts as an endopeptidase. It is possible therefore that the binding of some, or all, of the inhibitory antibodies, causes conformational changes in the vicinity of Arg$^{102}$.

Despite the 94% homology between the primary sequences of rat and human NEP [11], only four out of 20 CD10 mAbs cross-reacted. Thus, only four of the 20 epitopes seem to be common in the rat and human enzyme. The most likely explanation is a difference in the degree of glycosylation of the two enzymes. Although both enzymes have five putative N-glycosylation sites in common, rat NEP has an additional sixth site [11]. However, the existence of some cross-reactive epitopes could be used advantageously for initial *in vivo* studies in rats before using CD10 mAbs as vectors of drug targeting [23, 24]. The large number of monoclonal antibodies raised against CALLA can now be used for structural and conformational studies of NEP.

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