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Hemoregulatory Peptide pGlu-Glu-Asp-Cys-Lys: A New Synthetic Derivative for Avoiding Dimerization and Loss of Inhibitory Activity

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SUMMARY

The hemoregulatory peptide (pGlu-Glu-Asp-Cys-Lys, pEEDCK) is a potent inhibitor of stem cell recruitment, which is a major source of hematological complications after cytostatic tumor therapy. By preventing recruitment, pEEDCK can keep hemopoietic stem cells in their normal nonproliferative state and in this way prevent damage by certain cell cycle-specific cytostatic drugs. pEEDCK could play a role as hemoprotector in tumor chemotherapy. As a thiol-containing peptide, pEEDCK is highly sensitive to oxidation, resulting in the formation of a dimer. Although monomeric pEEDCK is a strong inhibitor of colony-forming units-granulocyte/macrophage (CFU-GM) clonal growth, the dimer was previously found to enhance colony-stimulating factor-triggered CFU-GM colony formation. It seemed, thus, necessary to find methods that avoid undesired dimerization reactions. A solid phase strategy for pEEDCK synthesis is presented. The primary synthetic product, *S-tert*-butyl-sulfenyl-pEEDCK, was purified and stored with the thiol-protecting group remaining attached. Conversion to active monomeric pEEDCK was achieved by reductive treatment *in situ* before application and removal of *tert*-butyl-mercaptane *in vacuo*. The activation reagent (dithioerythritol) prevented reoxidation also in culture media, where unprotected peptide was oxidized rapidly ($t_{1/2}$ < 13 min). The purified synthetic peptide was found to be a potent inhibitor of CFU-GM colony formation ($IC_{50} = 1.1 \times 10^{-12}$ M) *in vitro*. It was also found to inhibit colony formation of some leukemic cell lines (HL-60, RAJI) although at much higher concentrations (10^{-8} to 10^{-9} M). Friend leukemia cells were not inhibited in the dose range where CFU-GM were sensitive.

The hemoregulatory peptide pEEDCK (1) is a potent inhibitor of hemopoietic cell proliferation *in vitro* and *in vivo*. *In vitro* it specifically inhibits CFU-GM colony formation at picomolar concentrations (1–3). It antagonizes the stimulating activity of GM-CSF (3, 4) and interleukin-3 (5) and seems to synergize with other inhibitors like tumor necrosis factor-α and interferon-γ (5). pEEDCK inhibition is mediated by protease-sensitive membrane receptors (6–8) on the target cells. Experiments with highly purified hemopoietic progenitor cells (5) indicate that pEEDCK acts *in vitro* directly on its target cells without involvement of accessory cells. Application of pEEDCK *in vivo* reduces the femoral CFU-GM and CFU-S content (1, 3, 4), leading to corresponding alterations in the other compartments of the murine hematopoietic system (9). Toxicity was excluded as the cause of these inhibitory effects *in vitro* and *in vivo* (4). pEEDCK is not species specific (2, 4, 6), being equally active on human and rodent hemopoietic cells.

pEEDCK is of interest as a protector of the hemopoietic system against damage by cytostatic drugs (10). Hemopoietic stem cells (CFU-S), most of which are normally not in active proliferation, enter the cell cycle after cytostatic drug treatment. These proliferating stem cells are then highly sensitive to further drug applications, eventually leading to severe and long lasting damage to the hemopoietic system. We have shown previously (10) that pEEDCK can prevent this stem cell recruitment and, by keeping CFU-S in a noncycling state, renders them less sensitive to cycle-specific cytostatic drugs. As we have demonstrated, this can be used to protect mice against the myelotoxicity of high dose cytosine arabinoside treatment (3, 10).

As an acidic, thiol-containing peptide, pEEDCK presents some unique problems during synthesis and handling. Most earlier procedures for the synthesis of pEEDCK have resulted in preparations with low specific activity ($IC_{50} \sim 10^{-9}$ M) (1, 4, 11), which could only be purified with considerable difficulty to give a specific activity of $IC_{50} < 4 \times 10^{-12}$ M. Most of the

ABBREVIATIONS: pEEDCK, hemoregulatory peptide, pyroGlu-Glu-Asp-Cys-Lys; CFU-GM, colony-forming units-granulocyte/macrophage; DABCAM, 4-dimethylaminoazobenzene-4′-carboxamidomethyl; DABIA, 4-dimethylaminoazobenzene-4′-iodoacetamide; FMOC, fluorenylmethyloxycarbonyl; GM-CSF, colony-stimulating factor-granulocyte/macrophage; IC_{50}, concentration leading to 50% inhibition; IMDM, Iscove's modified Dulbecco's medium; NBT, nitroblue tetrazolium [2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-diphenylene)-ditetrazolium chloride]; PBS, phosphate-buffered saline (0.01 M Na-phosphate buffer, pH 7.4, in 0.9% NaCl); DTE, dithioerythritol; HPLC, high pressure liquid chromatography; CFU-S, colony-forming unit-spleen.

difficulties encountered during synthesis of pEEDCK are related to the arrangement of functional groups in the sequence and to the oxidation sensitivity of the thiol group.

Oxidation of pEEDCK in the absence of other thiol-containing compounds leads to the formation of a symmetric dimer. Synthetically prepared pEEDCK dimer was found (12) to be a potent enhancer of GM-CSF-induced CFU-GM colony growth and *in vivo* application leads to increases in early and late hematopoietic populations in normal (13) and cytostatic compound-treated mice (14).

It is obvious that the opposite biological effects of monomer and dimer demand the use of preparations that are free from contaminating reduced or oxidized peptide, respectively. This paper describes improved synthetic and handling procedures that were designed in view of these problems. We also present data on the stability of pEEDCK in culture media, obtained by a novel technique for the analytical detection and quantification of this peptide. We further present data concerning the action of pEEDCK monomer and dimer on the proliferation and differentiation of normal CFU-GM and leukemic cells.

Media and Chemicals

Amino acid derivatives and other chemicals for peptide synthesis were obtained from Bachem (Bubendorf, Switzerland) or Novabiochem (Läufelfingen, Switzerland). DABIA was from Fluka (Buchs, Switzerland). Benzidine, all-*trans*-retinoic acid, and 12-*O*-tetradecanoyl-phorbol-13-acetate were from Sigma (Taufkirchen, FRG). Cell culture media (Dulbecco's modified Eagle medium, α minimum essential medium, RPMI 1640 medium) and media components were obtained from GIBCO (Paisley, Scotland) or from PAA Laboratories (Gallneukirchen, Austria). Sera were from Boehringer (Vienna, Austria) or Seromed (Vienna, Austria). Only pretested batches were used for the experiments. All other chemicals and solvents were of analytical grade or better and were obtained from Merck (Darmstadt, FRG) and Promochem (Wesel, FRG).

Hemoregulatory Peptide pEEDCK

The hemoregulatory peptide, which was designated HP5b in previous publications, is here called pEEDCK, using the one-letter codes of amino acids for sequence-related nomenclature. Two batches of pEEDCK monomer, prepared by different synthetic strategies, were used in these experiments.

Batch pEEDCK-OSL. Some experiments were performed with a purified synthetic preparation of pEEDCK that was provided by Nycomed (Oslo).

Batch pEEDCK-VIE. Most other experiments were performed using synthetic peptide prepared by a strategy providing optimal protection against oxidation of the thiol group. In essence, the oxidation-insensitive mixed disulfide of pEEDCK with *tert*-butyl mercaptane was prepared, and the disulfide was only reduced immediately before the peptide was used. Details of the synthesis and activation procedures are given below.

Synthesis of pEEDCK-VIE

(ε-*tert*-Butyloxycarbonyl)-L-lysyl-*p*-alkoxybenzylalcohol resin (I). One gram of FMOC-(ε-*tert*-butyloxycarbonyl)-L-lysyl-*p*-alkoxybenzylalcohol resin (0.44 mmol of lysine) was placed in a synthesis vessel constructed according to Stewart and Young (15) and was washed twice with dichloromethane and twice with dimethylformamide. The FMOC group was then removed to yield **I** by shaking for 20 min with dimethylformamide/piperidine (1:1). After being washed twice with dioxane/water (2:1), twice with dimethylformamide, and twice with dichloromethane, the resin was suspended in 3 ml of dichloromethane. The presence of **I** was checked by the reaction of free amino groups with ninhydrin, according to the method of Kaiser *et al.* (16).

FMOC-(*S*-*tert*-butylsulfenyl)-L-cysteinyl-(ε-*tert*-butyloxycarbonyl)-L-lysyl-*p*-alkoxybenzylalcohol resin (II). One and one half mmoles (647 mg) of FMOC-(*S*-*tert*-butylsulfenyl)-L-cysteine were dissolved in 3 ml of dichloromethane and cooled to 0° in an ice bath, and an ice-cold solution of 0.75 mmol (155 mg) of dicyclohexylcarbodiimide in dichloromethane was added. After 1 hr at 0°, the dicyclohexylurea was filtered off and the solution of di-(FMOC-(*S*-*tert*-butylsulfenyl)-L-cysteinyl)-anhydride was immediately added to a suspension of **I** in 3 ml of dichloromethane. The progress of the coupling reaction was checked repeatedly by reaction of aliquots with ninhydrin (16). When no free amino groups could be detected (after 1–3 hr), the resin was washed twice with dimethylformamide and twice with dichloromethane to give **II**.

FMOC-(β-*tert*-butyl)-L-aspartyl-(*S*-*tert*-butylsulfenyl)-L-cysteinyl-(ε-*tert*-butyloxycarbonyl)-L-lysyl-*p*-alkoxybenzylalcohol resin (III). The free amino base of **II** was prepared by reaction with dimethylformamide/piperidine and washing, as described above. In a separate vessel, 1.5 mmol (617 mg) of FMOC-(β-*tert*-butyl)-L-aspartic acid were dissolved in 3 ml of dichloromethane/dimethylformamide (3:1), cooled to 0°, and converted to the symmetric anhydride by reaction with 0.75 mmol (155 mg) of dicyclohexylcarbodiimide, as described above. The anhydride solution was immediately added to the suspension of **II** base. After 45 min, the ninhydrin reaction had become negative, and the resin was washed twice with dimethylformamide and twice with dichloromethane to give **III**.

FMOC-(γ-*tert*-butyl)-L-glutamyl-(β-*tert*-butyl)-L-aspartyl-(*S*-*tert*-butylsulfenyl)-L-cysteinyl-(ε-*tert*-butyloxycarbonyl)-L-lysyl-*p*-alkoxybenzylalcohol resin (IV). **IV** was prepared as described above for **III**, using 1.5 mmol (638 mg) of FMOC-(γ-*tert*-butyl)-L-glutamic acid instead of the aspartic acid derivative.

Pyroglutamyl-(γ-*tert*-butyl)-L-glutamyl-(β-*tert*-butyl)-L-aspartyl-(*S*-*tert*-butylsulfenyl)-L-cysteinyl-(ε-*tert*-butyloxycarbonyl)-L-lysyl-*p*-alkoxybenzylalcohol resin (V). The FMOC group was removed from **IV** as described in detail above. After the resin was washed with dioxan/water twice, dimethylformamide (twice), and ethyl acetate/dimethyl sulfoxide (3:2), a solution of 1.5 mmol (505 mg) of pyroglutamic acid pentachlorophenyl ester in 5 ml of ethyl acetate/dimethyl sulfoxide (3:2) was added. After 15 min, 50 μl of triethylamine were added. When the ninhydrin reaction had become negative (25 min), the resin was washed with ethyl acetate/dimethyl sulfoxide (3:2) (twice), dimethylformamide (twice), and dichloromethane (twice) to give **V**.

Pyroglutamyl-L-glutamyl-L-aspartyl-(*S*-*tert*-butylsulfenyl)-L-cysteinyl-L-lysine (VI). **V** was suspended in a mixture of 8 ml of anhydrous trifluoroacetic acid and 2 ml of anisole. After 1 hr, the mixture was filtered and the resin was washed with 10 ml of anhydrous trifluoroacetic acid. The combined filtrates were evaporated *in vacuo* to about 5 ml and added dropwise to 50 ml of dry diethyl ether that was precooled to −20°. After precipitation was complete, crude **VI** was obtained by centrifugation in a spark-free centrifuge and dried *in vacuo*. The yield was 412 mg of the trifluoroacetate salt of **VI** (116% of theoretical yield).

Purification of VI

The material designated pEEDCK-VIE in this paper was purified from a 50-mg aliquot of crude **VI** by reverse phase chromatography on a Merck-Lobar C_8 column (10 × 240 mm), employing a gradient from 0 to 20% acetonitrile in 0.1% trifluoroacetic acid. Only the largest peak (of three) contained material that gave the characteristic smell of *tert*-butyl-mercaptane after treatment with DTE. The corresponding fractions were combined and lyophilized, giving 33.4 mg of pure **VI**.

Alternative Synthetic Strategies

In some earlier preparations of pEEDCK-VIE, the free amino base of **II** was synthesized as follows, using procedures described by Birr *et al.* (17).

α,α-Dimethyl-3,5-dimethoxy-benzyloxycarbonyl-(*S-tert*-butylsulfenyl)-L-cysteine symmetric anhydride (VII). One gram of α,α-dimethyl-3,5-dimethoxy-benzyloxycarbonyl-(*S-tert*-butylsulfenyl)-L-cysteine·dicyclohexylamine salt (obtained from Serva, Heidelberg, FRG) was suspended in 50 ml of ice-cold water and acidified to pH 3.0 with 1 M citric acid. The mixture was extracted twice with ice-cold peroxide-free diethyl ether and the organic phase was dried over Na_2SO_4 and evaporated *in vacuo* to a small volume. Free α,α-dimethyl-3,5-dimethoxy-benzyloxycarbonyl-(*S-tert*-butylsulfenyl)-L-cysteine was precipitated with petroleum ether, centrifuged, and dried *in vacuo*. The residue was dissolved in 1.5 ml of dichloromethane, cooled to 0°, and added to an ice-cold solution of 0.75 mmol (155 mg) of dicyclohexylcarbodiimide in 1 ml of dichloromethane. After 1 hr, a solution of **VII** was obtained by filtration.

α,α-Dimethyl-3,5-dimethoxy-benzyloxycarbonyl-(*S-tert*-butylsulfenyl)-L-cysteinyl-(ε-*tert*-butyloxycarbonyl)-L-lysyl-*p*-alkoxybenzylalcohol resin (VIII). One gram of FMOC-(ε-*tert*-butyloxycarbonyl)-L-lysyl-*p*-alkoxybenzylalcohol resin (0.44 mmol of lysine) was converted to the free base as described above. After the washing steps, the resin was suspended in dichloromethane and the solution of **VII** was added. After 120 min, the ninhydrin test had become negative, and the resin was washed twice with dimethylformamide and dichloromethane.

The free amino base of **II** was prepared from **VIII** as follows. To the suspension of **VIII** in dichloromethane was added 5% trifluoroacetic acid in dichloromethane. After 15 min at room temperature, the resin was washed twice with dichloromethane and neutralized by two treatments with triethylamine/dichloromethane (1:9). Excess base was removed by washing (six times) with dichloromethane to give the free amino base of **II**. The synthesis was then continued in the same way as described above.

Storage and Activation of pEEDCK-VIE

The peptide was stored as lyophilized powder or a 1 mg/ml aqueous solution at −30°, with the sulfur protection still attached. No losses were observed during storage over more than 2 years. Immediately before use, the sulfur protection was removed by treatment with a 10-fold molar excess of DTE in 0.5 M triethylamine in 20% methanol and drying *in vacuo* (4–6 Pa). *t*-Butylmercaptane is sufficiently volatile to be removed under these conditions. In detail, the peptide solution (10 μg = 14.4×10^{-9} mol) in 10 μl of water was evaporated to dryness *in vacuo* at room temperature. The residue was dissolved in a solution of 22 μg (14.4×10^{-8} mol) of DTE in 0.5 M triethylamine in 20% methanol. After 30 min at 37°, the mixture was dried *in vacuo* until the characteristic odor of *tert*-butyl-mercaptane was no longer detectable, dissolved in the appropriate medium, and filtered through a 0.22-μm filter (Millipore). For determination of dose-response curves, dilutions were made into RPMI 1640 medium. Control experiments showed that, at the peptide dilutions used, DTE and trifluoroacetate (the counter ion of the amino group after purification of the peptide) had no influence on CFU-GM colony formation.

Synthesis of the pEEDCK Dimer

Solid phase synthesis of the symmetrical pEEDCK dimer was performed in the same way as described above for the VIE monomer, with the single exception that for thiol protection the acetamidomethyl group was utilized. The crude synthetic product obtained after acidolytic deprotection (the acetamidomethyl group remains attached to cysteine) was purified by gel chromatography on Sephadex G-10 and showed a single peak in reverse phase HPLC. The dimer was obtained by simultaneous sulfur deprotection and oxidation with iodine in methanolic solution, as described by Stewart and Young (15). In detail, 95.1 mg of lyophilized acetamidomethyl-peptide (0.141 mmol) from the pooled Sephadex G-10 fractions were dissolved in 1.75 ml of methanol, and 0.141 mmol (35.7 mg) of iodine in 300 μl of methanol was added. After 1 hr, the mixture was cooled to 0° in an ice bath and a solution of Na-thiosulfate was added until the color of iodine disappeared. The solvent was then evaporated *in vacuo* and the residue was chromatographed on a Sephadex G-10 column (3.5 × 50 cm) with 0.1% trifluoroacetic acid. The first peak was pooled and lyophilized. The yield was 26.8 mg. Analytical reverse phase HPLC indicated a homogeneous product.

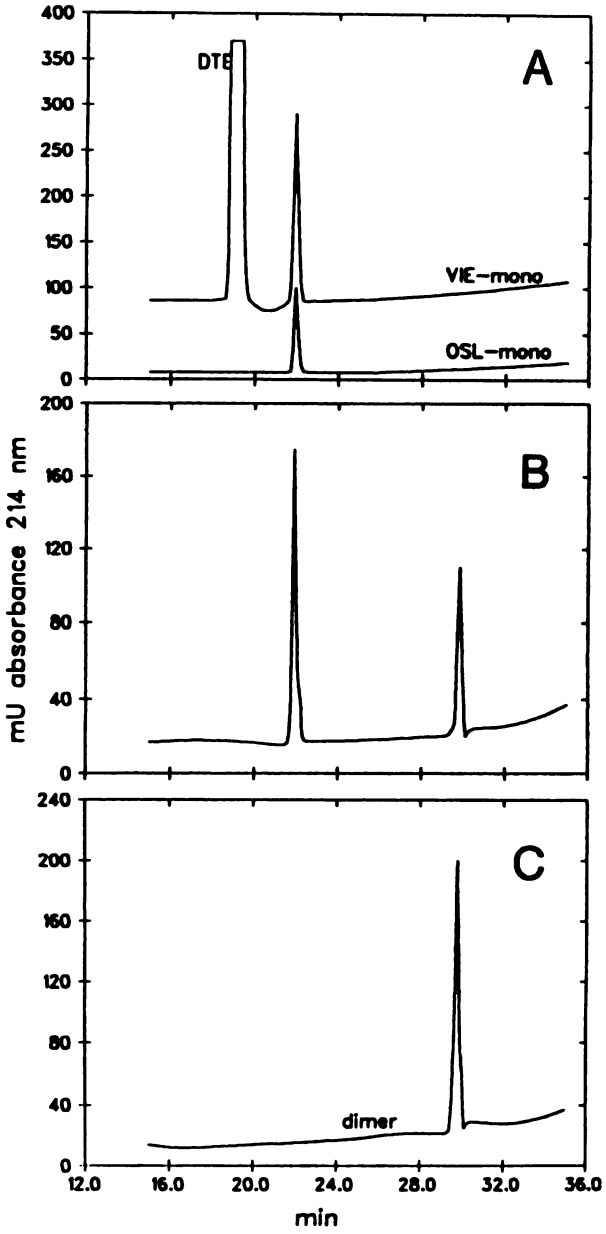

Fig. 1. Reverse phase HPLC of pEEDCK monomer and dimer. A, Batches pEEDCK-VIE (synthesized as given in Materials and Methods) and pEEDCK-OSL. B, pEEDCK-OSL treated with a stream of oxygen for 1 min. C, Synthetic dimer (see Materials and Methods). Chromatographic conditions: Beckman-Altex ODS column, 5 μm, 4.6 × 250 mm; 0.7 ml/min; 0.1% trifluoroacetic acid with a linear acetonitrile gradient (0% at 0 min to 10% at 40 min); detection at 214 nm.

Analytical Methods

The purity and identity of peptide batches VIE and OSL were checked by reverse phase HPLC. Chromatography was performed on a Beckman-Altex C_{18} column (5 μm, 4.6 × 250 mm), using a 0–10% acetonitrile gradient (40 min) in 0.1% trifluoroacetic acid (flow rate, 0.7 ml/min) and monitoring at 214 nm. Alternatively, the peptides were derivatized by a modification of the method of Chang *et al.* (18),

by reaction with DABIA and HPLC analysis, as described below for the stability experiments.

Analytical ion exchange chromatography was performed with the underivatized peptides (protected and deprotected pEEDCK-VIE and pEEDCK-OSL) on a Pharmacia Mono-Q column, with a gradient from 0 to 0.5 M NaCl in 20 mM Tris·HCl buffer, pH 7.0, and monitoring at 214 nm.

Stability Investigations

Ten micrograms of pEEDCK (VIE or OSL) dissolved in 10 μl of water were added to 40 μl of the medium to be investigated and incubated at 37° for the times indicated. At the respective time points, aliquots of 5 μl were taken and added to a mixture of 15 μl of DABIA in dimethylformamide (1 mg/ml) and 15 μl of 0.5 M triethylamine in 20% aqueous methanol. After 1 hr at room temperature in the dark, the reaction mixture was acidified with 1 μl of concentrated trifluoroacetic acid (color change of the azo-dye from yellow to purple). Two hundred microliters of water were added and the solution was filtered (0.45 μm; Millipore SJHV004NS) before 50 μl (corresponding to 351 pmol of pEEDCK at $t = 0$ hr) were applied to a Merck glass-Cartouche column (C_{18}, 5 μm, 3 × 30 mm) and chromatographed with a 5-min gradient of acetonitrile into 10 mM sodium acetate buffer, pH 7.0, containing 2% dimethylformamide. The effluent was monitored at 436 nm. Peak areas were calibrated against the amount of peptide applied to the column, giving a linear relationship in the relevant dose range.

For incubation in the presence of GM-CSF, 5 units of recombinant growth factor (Genzyme, Boston) were added to the phosphate buffer and the same procedure was followed as above. When the stability of pEEDCK was measured in the presence of cells, the following procedures were used. (a) Fresh femoral bone marrow cells (2.5×10^6) from female BALB/c mice were suspended in 80 μl of isotonic phosphate buffer (10 mM Na-phosphate buffer, pH 7.4, containing 140 mM NaCl). Twenty micrograms of pEEDCK in 20 μl of the same buffer were added and the mixture was incubated at 37° for the times indicated. At the selected time points, aliquots of 5 μl were taken, derivatized with DABIA, and analyzed as described above. (b) HL-60 cells from a logarithmic phase culture were washed with isotonic phosphate buffer (see above) and resuspended in the same buffer. Then, 2.5×10^6 cells in 40 μl of buffer and 10 μg of pEEDCK in the same buffer were mixed, incubated, derivatized with DABIA, and analyzed as outlined above.

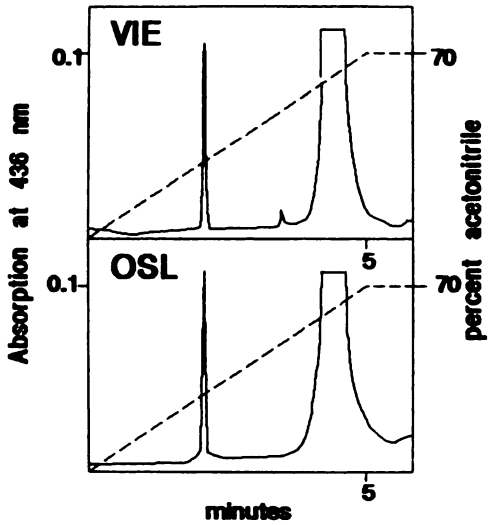

Fig. 2. Reverse phase HPLC of pEEDCK (batches OSL and VIE, the latter treated with DTE) after reaction with DABIA. Chromatographic conditions: Merck glass-Cartouche C_{18} column, 5 μm, 3 × 30 mm; 10 mM sodium phosphate, pH 7.0, with 2% dimethylformamide; acetonitrile gradient as indicated; 1 ml/min.

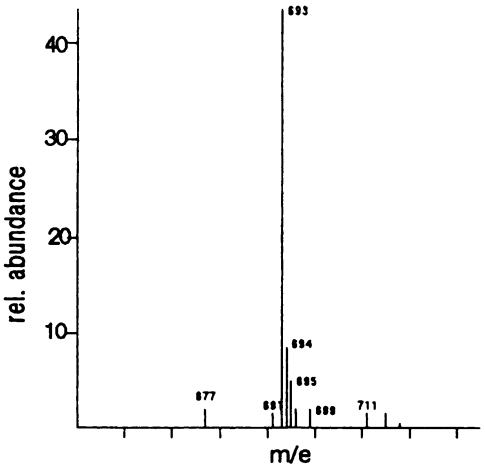

Fig. 3. Fast atom bombardment mass spectrum of **VI**, *S-tert*-butylsulfenyl-pEEDCK. Only the region of the molecular ion (*m/z* 693) is shown at 10× amplification. The base peak is *m/z* 45.

Culture of CFU-GM *In Vitro*

The effect of pEEDCK on murine myeloid progenitors was assayed by a modification of the methylcellulose technique of Worton *et al.* (19), as we have described in detail previously (20). In short, 2.5×10^5 bone marrow cells from 8–12-week-old female BALB/c mice were incubated with the material to be tested in 20–50 μl of RPMI 1640 medium containing 10^{-5} M $CaNa_2EDTA$ (7). After 1 hr at 37°, the cell suspension was diluted with cloning medium to give the final culture conditions as follows: 0.8% methylcellulose (4000 cps) and 20% horse serum in α minimum essential medium, with 5×10^4 bone marrow cells/ml. Mouse lung conditioned medium (21) or recombinant murine GM-CSF (5×10^7 units/mg; Genzyme, Boston, MA) was used for inducing CFU-GM growth. The pEEDCK concentrations given in this paper are the concentrations at preincubation (before dilution into cloning medium). One-milliliter aliquots were placed into 35-mm Petri dishes (bacteriological type; Falcon no. 1008) and incubated for 5–7 days at 37° in 5% CO_2 in air (fully humidified). Aggregates of more than 50 cells were scored as colonies by counting under an inverted microscope.

Cell Lines

HL-60, Raji, and Friend leukemia cells were propagated in the cell culture department of the Institute for Tumorbiology-Cancer Research. HL-60 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 10% horse serum. The cells were diluted twice weekly with fresh medium in order to keep them exponentially growing. At appropriate intervals, they were checked for differentiation ability by treatment with retinoic acid, as described previously (22). When this ability declined, either they were centrifuged over Lymphoprep (Nycomed, Oslo) or a new culture was started from a liquid nitrogen storage pool of early passages. Raji and Friend leukemia cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and otherwise treated in the same way. Immediately before use, dead cells were removed by centrifugation over a layer of Lymphoprep (Nycomed, Oslo). For clonal growth, the same technique was used as for CFU-GM but no GM-CSF was added to the cultures. One thousand cells/dish were plated and the colonies were counted after 14 days (HL-60) or 8 days (Raji, Friend).

Differentiation Experiments

The cell lines (HL-60 and Friend) were cultured under standard conditions (see above) for 4 days in the presence of various concentrations of pEEDCK or other known inducers of differentiation; 1 μM retinoic acid, 160 mM dimethyl sulfoxide, or 1 nM 12-*O*-tetradecanoylphorbol-13-acetate were used as positive controls. The percentage of

cells with functionally differentiated characteristics were determined by a modification of the NBT assay for HL-60 cells, as described previously (22), or the benzidine/H_2O_2 assay (23) for Friend leukemia cells.

Results

Identity and purity of pEEDCK. The material synthesized by the procedures described above (VI, *tert*-butyl-sulfenyl-pEEDCK-VIE) was homogeneous in analytical reverse phase HPLC, before and after (Fig. 1A) reduction with DTE. Analytical ion exchange chromatography on a Pharmacia Mono-Q column also gave a single symmetric peak. The amino acid composition was determined by the ion exchange/ninhydrin procedure, giving (mol of amino acid/mol of peptide) Asp, 1.00; Glu, 2.00; and Lys, 1.00. Cysteine was not detected in this routine system, due to its presence as the mixed disulfide with *tert*-butyl-mercaptane, which survives hydrolysis and elutes at a different position. No attempt was made to identify the *S-tert*-butylsulfenyl-cysteine peak. Amino acid analysis was also performed after DTE reduction (using the same analytical technique), giving Asp, 0.98; Glu, 2.00; Cys, 0.97; and Lys, 1.00.

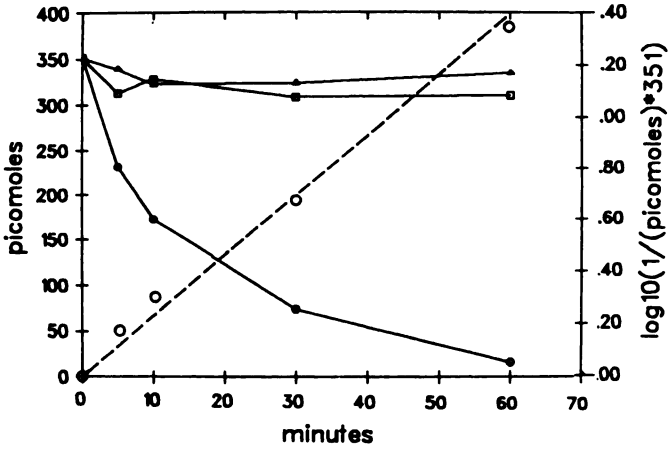

Fig. 4. Stability of pEEDCK in PBS and RPMI 1640 medium at 37°. — — (*left ordinate*), the time course of pEEDCK disappearance, as followed by reaction with DABIA and HPLC as in Fig. 2. – – –, the regression line of the OSL/RPMI values transformed according to the equation for first-order kinetics (*right ordinate*). ○, pEEDCK-OSL in RPMI 1640; □, pEEDCK-OSL in PBS; ▲, pEEDCK-VIE in RPMI 1640.

TABLE 1

Stability of pEEDCK monomer under various conditions

pEEDCK was incubated (37°) in the media with or without other ingredients (for details, see text) and the pEEDCK concentration was measured by the DABIA/HPLC method at selected time points. DTE in the solutions containing pEEDCK-VIE comes from the activation reaction (removal of *tert*-butyl-sulfenyl group) before the use of the peptide.

Peptide[a]	Medium	Other ingredients	Half-life
			min
pEEDCK-VIE	RPMI	DTE[b]	>1000
pEEDCK-VIE	IMDM	DTE[b]	>1000
pEEDCK-VIE	PBS	DTE[b]	>1000
pEEDCK-OSL	RPMI		12.8[c]
pEEDCK-OSL	IMDM		11.4[c]
pEEDCK-OSL	RPMI	2-ME[b,d]	28.9[c]
pEEDCK-OSL	RPMI	EDTA[b]	36.5[c]
pEEDCK-OSL	PBS		487
pEEDCK-OSL	RPMI	DTE[b]	>1000
pEEDCK-OSL	PBS	Bone marrow	460
pEEDCK-OSL	PBS	HL-60	470
pEEDCK-OSL	PBS	rGM-CSF[e]	180

[a] 10 μg/50 μl = 3×10^{-4} M.
[b] 3×10^{-3} M.
[c] First-order kinetics.
[d] 2-ME, 2-mercaptoethanol.
[e] rGM-CSF, recombinant GM-CSF.

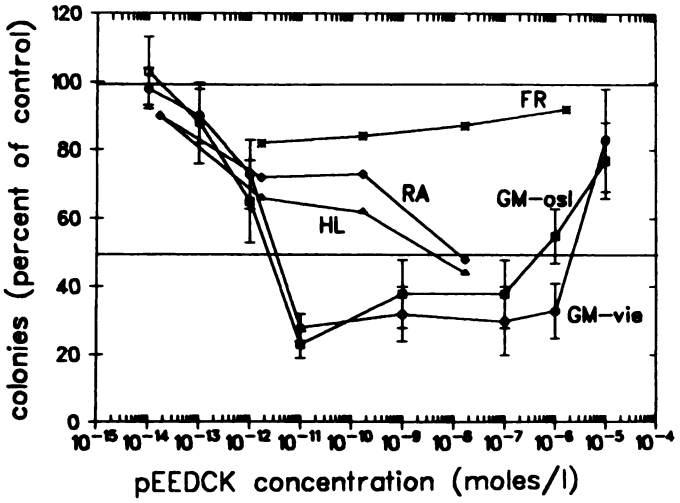

Fig. 5. Dose-response curves of monomeric pEEDCK on normal and malignant hemopoietic cells. *GM-osl*, pEEDCK-OSL on normal murine CFU-GM; *GM-vie*, pEEDCK-VIE on normal murine CFU-GM; *HL*, pEEDCK-VIE on HL-60 cells; *RA*, pEEDCK-VIE on Raji cells; *FR*, pEEDCK-VIE on Friend leukemia cells. The cells (in PBS) were exposed to the indicated concentrations of pEEDCK for 1 hr at 37° before plating.

TABLE 2

Effects of pEEDCK on differentiation and cloning ability of transformed cells

Cells were cultured for 4 days in the presence of pEEDCK or other inducers as indicated. Then, the percentage of cells giving a positive NBT or benzidine reaction was determined. The values given are the ranges observed in three to five repeated experiments. Four-day cells were also cloned in methylcellulose. Percentages of colony counts are given relative to cells from untreated control cultures. Numbers in parentheses are numbers of independent experiments.

	HL-60		Friend	
	NBT-positive cells (5)	Colonies (3)	Benzidine-positive cells (4)	Colonies (3)
	%	% *of control*	%	% *of control*
Control	3.5–8	100[a]	3–7	100[b]
pEEDCK				
1.67×10^{-6} M	4–6	77–95	5–8	83–100
1.67×10^{-8} M	4–7	75–152	3–4.5	100–107
1.67×10^{-10} M	4–7	95–116	2.5–7	82–100
DMSO,[c] 160 × 10^{-3} M	27–51	0	29–49	0–18
RA, 10^{-6} M	25–40	0	NT	NT
TPA, 10^{-9} M	29–39	0	NT	NT

[a] Control, 304 ± 13 colonies/1000 cells.
[b] Control, 410 ± 20 colonies/1000 cells.
[c] DMSO, dimethylsulfoxide; RA, all-*trans*-retinoic acid; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; NT, not tested.

Both monomer preparations (pEEDCK-OSL and pEEDCK-VIE after removal of the thiol-protecting group) showed identical retention times in reverse phase HPLC (Fig. 1A). Keeping the unprotected OSL monomer under atmospheric conditions or repeated freezing and thawing of the preparation led to the rapid appearance of a second peak in the chromatograms (Fig. 1B), which represents the dimer of pEEDCK, its elution position being identical to that of synthetically prepared dimer (Fig. 1C). No immediate dimer formation was observed when DTE-treated pEEDCK-VIE (10-fold molar excess of DTE still pres-

TABLE 3

Effect of pEEDCK dimer on growth and differentiation of HL-60 cells

The dimer was added daily, at the concentrations indicated, during the first 4 days of liquid culture. On day 6 the cell number, cloning efficiency, and percentage of NBT-positive cells were determined. The dimer did not change the growth rate during the logarithmic phase. Retinoic acid was added once on day 0.

Concentration	Cells × 10^{-6}/ml on day 6	Colonies from 10^3 day 6 cells	NBT-positive cells
M			%
0	1.9	233	9.7
10^{-11}	2.6	231	9.5
10^{-7}	2.7	239	4.8
Retinoic acid (1 μM)	0.67	4	26.4

ent) was kept under atmospheric conditions. When the peptides (pEEDCK-OSL and DTE-treated pEEDCK-VIE) were derivatized at their thiol groups by reaction with DABIA and chromatographed on a Merck glass-Cartouche column (C_{18}, 3 × 30 mm), as described above, a single peak was observed, which eluted at identical retention times for both preparations (Fig. 2).

Mass spectrometric identification. The fast atom bombardment mass spectrum (Fig. 3) of **VI** (*tert*-butyl-sulfenyl-pEEDCK-VIE) showed a $(M + 1)^+$ molecular ion at m/z 693.1, in good agreement with the calculated M_r of 692.2512. This is further supported by the presence of m/z 636, indicating the loss of a *tert*-butyl group from the molecule. On the high mass side of the molecular ion, the characteristic isotope peaks due to the presence of two sulfur atoms are clearly discernible. Fragments with m/z 112, 241, 356, and 547 confirm the sequence pyroGlu-Glu-Asp-(*S-tert*-butyl-sulfenyl)-Cys-Lys, which was the aim of the synthesis. A low intensity ion at m/z 711 (693 + 18) indicated the presence of trace amounts of ring-opened Glu-Glu-Asp-(*S-tert*-butylsulfenyl)-Cys-Lys, which, when deliberately synthesized, was found to be biologically inactive (after reduction with DTE). Thin layer chromatographic examination showed that N-terminal glutamic acid was introduced into the synthesis as a trace impurity of the pyroglutamic acid pentachlorophenyl ester used in the last coupling step (**V**) of the synthesis. The amount of this peptide in the final synthetic product pEEDCK-VIE was apparently too small to be detected by HPLC methods.

Stability of the peptide. It was important to protect the peptide preparation against oxidation. When the *tert*-butylsulfenyl group was still attached, the peptide (pEEDCK-VIE) was completely stable with respect to oxidation. The stability of preparations with a free thiol function under conditions relevant for bioassay was checked by incubation in the test medium at 37°. The disappearance of deprotected pEEDCK-VIE and unprotected pEEDCK-OSL was measured in PBS, as well as in RPMI medium and IMDM. At various times, aliquots of the incubation mixture were derivatized with DABIA and analyzed by HPLC, measuring the area under the DABCAM-pEEDCK peak. Quantitative evaluation according to first-order kinetics (Fig. 4) gave the results summarized in Table 1. Pure unprotected pEEDCK-OSL was oxidized rapidly in RPMI medium (k = 0.054 min^{-1}, $t_{½}$ = 12.8 min) and in IMDM (k = 0.061 min^{-1}, $t_{½}$ = 11.4 min). The addition of a 10-fold molar amount of $CaNa_2EDTA$ (8) improved the situation somewhat (k = 0.019 min^{-1}, $t_{½}$ = 36.5 min). A similar stabilization of pEEDCK-OSL was seen with a 10-fold molar excess of 2-mercaptoethanol (k = 0.024 min^{-1}, $t_{½}$ = 28.9 min). In simple buffer solutions like PBS, unprotected pEEDCK-OSL was much more stable, even in the absence of EDTA or 2-mercaptoethanol ($t_{½}$ = 487 min). The kinetics, however, did not follow first- or second-order laws. The half-life time was determined directly from the concentration versus time curve. On the other hand, pEEDCK-VIE, after activation with a 10-fold molar excess of DTE, which was also present in the incubation mixture, was much more stable, giving $t_{½}$ values above 1000 min even in RPMI medium and IMDM. The same level of stability could be attained with pEEDCK-OSL when DTE was added to the incubation mixture. HPLC analysis of underivatized pEEDCK-OSL, however, showed that practically all samples contained measurable amounts of dimer already at the start of the experiment. This was reduced by DTE treatment, but even with the highly sensitive DABIA method it was impossible to determine whether and when the last traces of dimer had disappeared. We have found earlier that the $(pEEDCK)_2$ dimer was active far below the picomolar range (12). In contrast to this, the *S-tert*-butyl-sulfenyl derivative, which is the storage form of pEEDCK-VIE, is biologically inactive, and any chemically undetectable traces surviving the DTE treatment will, thus, not interfere with biological experiments.

Based on these observations, all solutions in RPMI medium were only prepared with the stabilized batch pEEDCK-VIE, whereas batch pEEDCK-OSL was only used in simple salt solutions and buffers, with 2-mercaptoethanol added as described previously (4). The relatively large amounts of DTE present in the activation mixtures of pEEDCK-VIE do not interfere with the biological experiments performed with the peptide. For such uses, pEEDCK is diluted usually to below 10^{-8} M, leading to a DTE concentration of less than 10^{-7} M. Control experiments showed that DTE did not influence CFU-GM growth or other parameters studied at concentrations below 10^{-5} M.

The stability of pEEDCK-OSL was also measured in the presence of bone marrow or HL-60 myeloid leukemia cells. The peptide (without EDTA or 2-mercaptoethanol) was incubated at 37° with 2.5 × 10^6 cells suspended in 0.1 ml of PBS, and samples were analyzed for their pEEDCK content by the DABIA method described above. The observation times were extended to several hours. pEEDCK concentration decreased with time, reaching 50% of the initial value at 490 min in pure PBS, at 460 min in the presence of murine bone marrow cells, and at 470 min in the presence of HL-60 cells. Mathematical evaluation showed that the rate of pEEDCK disappearance did not follow simple first- or second-order kinetics.

When pEEDCK-OSL (without EDTA or 2-mercaptoethanol) was incubated in PBS with 5 units/ml recombinant murine GM-CSF, the 50% value was reached after 180 min. Also in this case the process did not follow simple kinetic equations. The bovine serum albumin present in the GM-CSF preparation as a carrier protein left the disappearance rate unchanged from the value for pure PBS (approximately 490 min).

Inhibition of murine CFU-GM *in vitro*. The biological activity of both peptide preparations (pEEDCK-OSL and DTE-treated pEEDCK-VIE) was checked by their inhibition of clonal growth of murine CFU-GM *in vitro*. Both peptides showed identical dose-response curves (Fig. 5), in accordance

with their chemical identity (Fig. 1A, Fig. 2). Significantly reduced colony numbers were observed at concentrations higher than 10^{-13} M. The IC_{50} was calculated by the method of Spearman-Kärber (as described in Ref. 24), giving, for pEEDCK-VIE, $IC_{50} = 1.16 \times 10^{-12}$ M, 95% confidence interval = 0.91–1.47×10^{-12} M, and, for pEEDCK-OSL, $IC_{50} = 1.01 \times 10^{-12}$ M, 95% confidence interval = 0.80–1.01×10^{-12} M. The dose-response curve showed a broad minimum between 10^{-11} and 10^{-6} M, with maximal inhibition up to 75%. Above 10^{-5} M, pEEDCK did not inhibit colony growth. When pEEDCK-VIE with the *tert*-butyl-sulfenyl group still attached to cysteine was assayed on CFU-GM colony growth, no significant differences were observed throughout the whole concentration range (10^{-14} to 10^{-5} M).

Effect of pEEDCK monomer on clonal growth of transformed cells *in vitro*. The effects of pEEDCK-VIE (DTE treated) on the cell lines HL-60 (myeloid), Raji (T lymphoid), and Friend (erythroid) were determined. The preincubation technique was used as described earlier (20). Fig. 5 gives the results obtained, showing that HL-60 and Raji cells were strongly inhibited at submicromolar peptide concentrations. However, their sensitivity was considerably lower than that of nontransformed CFU-GM. The following IC_{50} values were found: CFU-GM, 1.16×10^{-12}; HL-60, 4×10^{-9}; and Raji, 1×10^{-9} M. Friend leukemia cells were not significantly inhibited in this concentration range.

Effect of pEEDCK monomer and dimer on differentiation of transformed cells. Differentiation in HL-60 and Friend cells was induced by dimethyl sulfoxide, retinoic acid, and 12-*O*-tetradecanoyl-phorbol-13-acetate, and the effects were compared with those produced by various concentrations of pEEDCK. Differentiation was checked by the NBT reaction (for HL-60 cells) or by the benzidine reaction (Friend cells). In parallel, the capacity for clonal growth was determined. Table 2 gives the results, which show that pEEDCK did not induce differentiation in HL-60 or Friend cells under conditions where these cells responded to the standard inducers (retinoic acid, dimethyl sulfoxide, and 12-*O*-tetradecanoyl-phorbol-13-acetate). The cells neither showed increased functional markers nor lost their ability for clonal growth.

The dimer was also tested for its effect on growth and differentiation of HL-60 cells (Table 3). Although $(pEEDCK)_2$ permitted the cells to reach higher plateau densities (as exemplified by the cell counts on day 6), the growth rate during the logarithmic phase (days 2–4) was not changed, as compared with untreated cultures. Day 6 cells showed normal cloning efficiency, and the percentage of NBT-positive cells was not increased over the spontaneous level in control cultures.

Discussion

We have identified a low molecular weight peptide as the CFU-GM-inhibitory compound of human leukocyte or rat bone marrow extracts (25). Purification to homogeneity was achieved from normal human leukocytes (1, 20). The sequence pyroGlu-(Asp or Glu)-(Asp or Glu)-Cys-Lys was determined by radiochemical and electrophoretic methods (26). Of the possible chemically synthesized alternatives that correspond to this sequence, only one, pEEDCK, shares the CFU-GM-inhibitory properties with the natural peptide (2, 4, 6), whereas the others were found to be either inactive or toxic.

As a thiol peptide, pEEDCK is rather sensitive to oxidation. It is, however, very important to stabilize the inhibitory pEEDCK monomer preparations against oxidative formation of dimer, because we have previously shown that the pEEDCK dimer is an extremely potent enhancer of GM-CSF-induced colony formation of murine bone marrow cells (12). The limit of significant dimer effects is 2–3 orders of magnitude below the concentration range where the monomer exerts its inhibitory effect. Small impurities of dimer in a monomer preparation may, thus, completely abolish any observable inhibition. Such accidental dimer admixtures may explain the observed occasional absence of inhibitory effect (27) or even reversal to stimulation. We have found (Ref. 7 and Table 1) that the pH-dependent dimerization of pEEDCK could be retarded by complexing catalytic metal ions with EDTA, but this did not provide complete protection against oxidation and of course did not prevent the formation of dimer during storage and manipulation before the protective agent is added. Eriksen *et al.* (8) have synthesized the dimer by solution methods. Monomeric pEEDCK-OSL was prepared from the dimer by reduction with dithiothreitol. Although this proved to be a valuable method for large scale synthesis of pEEDCK, it may be difficult to eliminate the last (chemically undetectable, but still biologically active) traces of residual dimer surviving the reduction step. This, together with traces of dimer formed during storage and handling, may represent a possible reason for the sometimes unsatisfactory inhibition of CFU-GM growth *in vitro* that we have observed with pEEDCK-OSL (27).

The present paper describes an alternative approach to the stability problem. pEEDCK was synthesized and stored as the Cys-(*tert*-butylsulfenyl) derivative, the asymmetric disulfide with *tert*-butyl-mercaptane. Immediately before use, the pEEDCK monomer was generated *in situ* from this stable derivative by reduction with a large molar excess of DTE and evaporation *in vacuo* of the volatile *tert*-butyl-mercaptane. The thermodynamics of the DTE reaction as well as additional shifting of the equilibrium to the right by removal (by evaporation) of one of the reaction products ensure quantitative yields of fully reduced monomer. The characteristic smell of mercaptanes makes the human nose an exquisitely sensitive detector for the completeness of this reaction. HPLC checks indicated that less than 0.01% (detection limit) of the protected peptide remained unreacted after 30 min at 37°. In addition, we have found that the thiol-protected *S-tert*-butylsulfenyl-pEEDCK itself was biologically inactive in the relevant concentration range. The formation of pEEDCK dimer after the activation reaction was prevented by the excess of DTE present in the reaction mixture. Control experiments have shown that dilution of the activation mixture to the concentrations required for CFU-GM experiments also diluted the DTE to levels that were not inhibitory for colony formation. No formation of dimer in these highly diluted final solutions seemed to occur, provided they were used not too long after preparation. Additional safety could be provided by dilution into mercaptoethanol-containing solvents and/or by addition of EDTA to all media (7), but this seemed to be unnecessary with pEEDCK-VIE. Unprotected pEEDCK-OSL was rapidly oxidized in standard cell culture media (RPMI medium and IMDM) with half-life times just above 10 min (Table 1), and EDTA or 2-mercaptoethanol did not improve the situation very much. In pure PBS, its stability was acceptable, even without mercaptoethanol added.

pEEDCK has inhibitory activity on murine and human myeloid progenitors, CFU-GM, *in vitro* (Refs. 2–4 and Fig. 5) at low concentrations (>10^{-13} M). Maximal inhibition was found between 10^{-11} and 10^{-7} M. At still higher concentrations, no inhibitory effect was observed. This highly reproducible effect may point to regulatory phenomena at the receptor level, but experimental evidence for this is not available. Erythroid precursors burst-forming units- and colony-forming units-erythroid (2), as well as lymphocyte colony formation,[1] are not inhibited at doses where CFU-GM are sensitive. Although CFU-GM dose-response curves like those in Fig. 5 are observed in many experiments, we have also noticed that often the inhibition is small or even absent (27). Because our stability experiments allowed the selection of culture conditions excluding, for example, partial dimerization of pEEDCK, other biological variables of the culture system seem to cause these effects. The use of low passage HL-60 cells as a target population resulted in much better reproducibility, suggesting the variability of the *ex vivo* bone marrow cells as a possible reason. Because the *in vivo* effects of pEEDCK were always highly reproducible, the effect may also be related to the nature of the stimulator used for culturing CFU-GM.

Our results with transformed hemopoietic cells (Fig. 5) showed that pEEDCK inhibited the myeloid cell line HL-60 and also the lymphoid Raji cell line, whereas the erythroid Friend leukemia cells were unaffected. Our HL-60 results are apparently in contrast to those of Foa *et al.* (28), who have reported that pEEDCK did not inhibit their HL-60 cultures. A possible explanation may be the use of different subclones at different passage numbers. We have observed that, upon prolonged passaging, the HL-60 cells progressively lost their sensitivity to the peptide, as they also lost their differentiation capacity in response to retinoic acid (22). Inhibition of colony formation may reflect direct proliferation inhibition but may also be related to the induction of differentiation in these cells, leading to a loss of their ability to proliferate. We have found no evidence for this being the case. pEEDCK had no influence on the differentiation markers tested (Table 2) and also did not reduce the ability of the cells for factor-independent clonal growth. The differences in colony inhibition between these data (Table 2) and those given in Fig. 5 are due to the different techniques used, i.e., the time between peptide application and plating in methylcellulose, which was 1 hr in the standard preincubation assay (Fig. 5) but 4 days in the differentiation experiments. At this time the peptide is presumably completely degraded, and only permanent (e.g., differentiation-related) changes induced in the target cells should be detectable.

Normal bone marrow or HL-60 cells do not actively oxidize pEEDCK. Its half-life time was not changed by incubation with these cells (Table 1). We have previously shown (6, 7) that bone marrow cells bind the hemoregulatory peptide through membrane receptors, but the peptide concentrations used in the stability experiments described here were far too high to be changed by receptor binding. It is interesting to note that the rate of pEEDCK disappearance was almost tripled in the presence of purified recombinant GM-CSF (Table 1). The molecular basis of this phenomenon and its possible implications for the regulation of myelopoiesis are presently under investigation.

We have shown (3, 10) that properly timed application of monomeric pEEDCK can protect the hematopoietic system against the toxic side effects of cytostatic drugs like cytosine arabinoside. The use of deliberately oxidized pEEDCK preparations instead of pure monomer not only caused no protective effect but even potentiated the toxicity of the drug (10). For achieving inhibitory/protective effects, it seems, thus, highly important to use completely dimer-free pEEDCK preparations. As we have shown here, this can be achieved by the use of stabilized pEEDCK preparations (mixed disulfide with *tert*-butyl-mercaptane), which are activated *in situ* immediately before application.

Preliminary data (14) show that, on the other hand, the dimer itself may be clinically useful as a postchemotherapy stimulator of hemopoiesis, in a similar way as various recombinant hemopoietic growth factors are being promisingly used in clinical trials. The combined application of the stem cell-protecting pEEDCK monomer plus the postchemotherapy regeneration-stimulating dimer may then result in a highly improved hematological status despite cytotoxic chemotherapy. In an initial series of experiments, we could show (14) that mice treated with several courses of cytosine arabinoside plus a carefully timed monomer/dimer combination did not develop the leukopenia usually seen after treatment with cytostatic drugs.

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