Isolation and Synthesis of a Hemoregulatory Peptide

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A peptide was isolated in pure form from human leukocytes which strongly inhibits the proliferation of immature meyloid cells *in vitro* (committed stem cells). Structural investigations yielded *p*Glu-Asp or Glu-Asp or Glu-Cys-Lys-OH as the probable sequence of this peptide. The Glu^2,Asp^3-analog, prepared synthetically, displayed similar activities and when applied *in vivo* showed effects on the hemopoietic system ranging from an inhibition of pluripotent and committed stem cells to variations in the bone marrow proliferation and alterations in peripheral blood counts.

It has been reported [1] that leukocyte extracts are capable of inhibiting the proliferation of immature myelopoietic cells and this phenomenon has been brought into conjunction with models of growth regulation by feedback inhibition [2]. We wish now to report about the purification, structure and biological properties of a peptide mediating such inhibitory effects and about properties of an analogous synthetic peptide. Detailed accounts of experimental procedures and results as well as an extensive documentation of the biological experiments will be published elsewhere.

Table I shows – in a schematic way – the sequentially applied methods which were used for the preparation of the active peptide. This peptide (Granulopoiesis Inhibiting Factor, GIF) selectively acts upon myelopoietic proliferation *in vitro* (Table II). When injected *in vivo* into mice at a relatively low dose level a selective inhibition in the myelopoietic system is seen (committed stem cells and later morphologically recognizeable cells) whereas other lineages are not significantly affected. This material is chemically homogeneous after dansylation and/or [^{3}H]carboxymethylation on a) four different systems of thin layer chromatography and b) thin layer and paper electrophoresis at pH = 1.9 and 6.5. From the electrophoretic and chromatographic behaviour of this peptide its functional groups could be identified qualitatively and quantitatively as being a) a single primary aminogroup which is not the N-terminal group, b) a single thiol group and c) three carboxyl groups in addition to a pyroglutamyl residue identified earlier by enzymatic methods [3]. These results are consistent with earlier findings [4] and are corroborated by the identification of Asp, Glu, Cys and Lys in dansylated hydrolysates of GIF.

Partial acid hydrolysis of [^{3}H]carboxymethylated GIF yielded the peptide [^{3}H]CM-Cys-Lys-OH, the sequence of which was determined by [^{14}C]dansylation and total hydrolysis [5]. The absence of Glu from this peptide identified it as the C-terminal part of GIF, the N-terminal part being then *p*Glu-(Asp/Glu)-(Asp/Glu)-OH in accordance with the distribution of functional groups in the molecule. The structure of GIF as determined in this way also gives an explanation for the proteolytic stability of this peptide [6].

Based on arguments concerning the relative ease of formation of the C-terminal Cys-Lys peptide we have synthesized several of the possible candidate peptides and wish to report the results obtained with the peptide *p*Glu-Glu-Asp-Cys-Lys-OH (code HP5b). This peptide has been synthesized by the active ester coupling method using *p*-nitrophenyl and N-hydroxysuccinimide esters of BOC-protected aminoacid derivatives. Benzyl type substituents were used for protecting side chain functional groups during synthesis. After each coupling step the product was purified by chromatography on Sephadex LH-20. The final deprotection was achieved with hydrogen bromide in anhydrous trifluoroacetic acid and the product was purified by gel- and ion exchange chromatography.

HP5b, when acting *in vitro* on cultures of normal hemopoietic cells shows a strong dose dependent inhibition of myeloid colony formation whereas the formation of erythropoietic colonies was not affected. The same preferential action was observed with permanent cell lines of myeloid and non-myeloid origin. When HP5b was applied *in vivo* (Fig. 1, Table III), we observed a highly complex pattern of effects dominated by a pronounced inhibition of stem cells committed for myelopoiesis

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Table 1. Methods used for the purification of Granulopoiesis Inhibiting Factor (GIF).

Step No.	Starting Material from step No.	Method	Factions/eluates obtained	Yield
1	human leukocytes (30 g)	ultrafiltration (YM-10) of crude conditioned medium	a) retentate b) filtrate	n. d.
2	1 b	anion exchange (AG 1 × 2, formate)	a) 0.1 M triethylamine b) H_2O c) 1 M HCOOH	1.24 g
3	2 c	anion exchange (AG 1 × 2, formate)	a) 0.1 M triethylamine b) H_2O c) 0.1 M HCOOH d) 1 M HCOOH	1.22 g
4	3 c	thiopropyl-sepharose 6 B	a) phosphate buffer b) ammonium acetate c) –"– + 50 mM 2-ME	0.8 mg
5	4 c	Sephadex G-10	a) front region b) fractions around $V_e/V_0 = 1.37$ c) later fractions	0.6 mg < 10 µg 0.2 mg
6	5 b	cation exchange (AG 50 × 4, H^+)	a) bound at pH = 4.1 b) unbound at pH = 4.1	< 10 µg < 10 µg
7	6 b final product			

Table II. Effects of purified GIF on *in vitro* colony formation and thymidine incorporation of myeloid and nonmyeloid hemic cells.

Dilution factor	Colony formation		Thymidine incorporation	
	Myeloid (CFU-C)	Erythroid (CFU-E)	Bone marrow	Thymus
	Percent of control			
1 : 13	0	–	–	–
1 : 50	0	–	23.0 ± 3.1	78.4 ± 3.5
1 : 130	0	–	–	–
1 : 250	–	–	23.9 ± 0.7	84.2 ± 2.0
1 : 500	–	–	26.3 ± 1.7	79.7 ± 2.0
1 : 650	69.0 ± 8.3	105.0 ± 5.8	–	–
1 : 2500	–	–	94.7 ± 4.6	99.4 ± 6.0

* The final product from Table I, step 7 (less than 10 µg) was dissolved in 1 ml of water. The relative "concentration" of this solution was set at 1 and the dilution factors given in the table are expressed relative to this solution.

(CFU-c) followed by prolonged reductions of the more mature granulocytic cells in the bone marrow, and accompanied by pronounced variations and temporary increases in the erythropoietic lineage. These effects may be the result of the transient depression of pluripotent stem cells (CFU-s) as observed after multiple or prolonged application of the peptide. The effects further extend to variations in the peripheral blood which again are dominated by a prolonged reduction of granulocyte numbers accompanied by transient changes in the lymphocyte population. HP5b seems to act more strongly in certain dose ranges (ca. 10^{-5} M) as compared to others (ca. 10^{-8} M). Very high doses (above 10^{-4} M) as well as long term continuous application abolish the effects on some of the populations investigated.

Table III. Hematological effects *in vivo* of synthetic hemoregulatory peptide HP5b. All values are given as percentages of the respective control and were calculated as cells per femur on the basis of the total cell counts per femur. Peripheral blood values are expressed per mm^3 and given as percentage of the control values. An aterisk indicates statistical significance.

	1×10^{-5} M	9×10^{-5} M (9 days)		10^{-5} M continuous infusion for 6 days			idem for 19 days
day no.	1	10	16	6	13	19	19
total cell number	105	89	–	96	89	–	93
CFU-S	107	48 *	–	74 *	163 *	151 *	85 *
CFU-C	83 *	73 *	102	23 *	–	175 *	71 *
myelopoietic cells							
total	82	86 *	73 *	116	100	101	88
proliferative	89	100	62 *	102	89	92	112
non-proliferative	72 *	72 *	96	135 *	109	115	66 *
erythropoietic cells	98	89	114	66 *	67 *	117	99
S-phase cells	85 *	88 *	89	–	–	–	–
labeled cells							
myeloid	75 *	–	41 *	–	–	–	–
erythroid	68 *	–	170 *	–	–	–	–
peripheral blood cells							
granulocytes	54	64	118	58 *	60 *	82	191 *
monocytes	33 *	83	146	100	62 *	91	307 *
lymphocytes	95	106	97	164 *	126 *	91	157 *
total WBC	79	94	103	122 *	87 *	88	188 *

* The final product from Table I, step 7 (less than 10 µg) was dissolved in 1 ml of water. The relative "concentration" of this solution was set at 1 and the dilution factors given in the table are expressed relative to this solution.

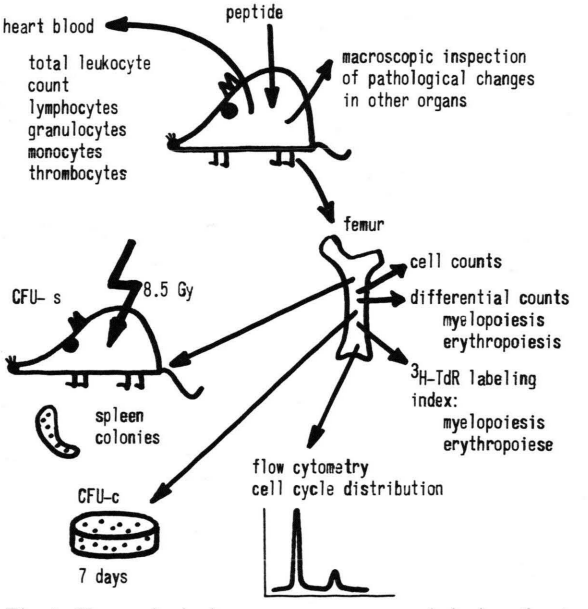

Fig. 1. Hematological parameters measured during the *in vivo* application of the synthetic peptide HP5b.

All hematological effects observed were reversible and no macroscopic pathological changes were observed in the other organs of the animals. Investigations on the influence of HP5b on the circadian rhythmicity of the bone marrow show that the gross inhibitory action comes about by the prevention of the normal proliferative wave during the first part of the day. The results of preliminary survival experiments with mice indicate that HP5b might be effective as a protector against the myelotoxic effects of certain cytostatic drugs. HP5b pretreated mice show a doubled median survival time when treated with lethal doses of cytosine arabinoside, which may be the result of the reversible antiproliferative action of the peptide at the stem cell level [7].

Although the studies leading to the preparation of HP5b originated from work on the socalled granulocytic chalone, this concept appears too narrow to be able to account for the complexity of the phenomena induced by this peptide *in vivo*.

Acknowledgements

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Erratum

N. Yamamoto, J. Schneider, Y. Hinuma, and G. Hunsmann, Adult T-Cell Leukemia-Associated Antigen (ATLA): Detection of a Glycoprotein in Cell- and Virus-Free Supernatant.

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The second sentence on the top right of page 731 should read: In addition, Yamamoto and Hinuma [9] detected four [^{35}S]methionine-labelled polypeptides of 70 k, 53 k, 36 k, and 24 k with ATL patient sera in ATLV producing MT-2 cells [6].

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