Low Molecular Mass Inhibitors from Calf Thymus Selective for T-Lymphocyte Proliferation

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Endogenous factors preferentially inhibiting T-lymphocyte proliferation were prepared from the acetone precipitate of a 60% ethanol extract from calf thymus and their biochemical properties examined. By ultrafiltration the strongest lymphocyte-selective inhibition was found in the molecular mass range between 1 and 5 kDa. Fast protein liquid chromatography (FPLC) of this fraction on an anion exchange column eluted the lymphocyte inhibitors at 205–265 mM ammonium acetate. Staining procedures following IEF and TLC suggested, that the inhibitors may not be glycoconjugates or amines but small weakly acidic peptides (< 3 kDa).

Introduction

Inhibitors of lymphocyte proliferation can be roughly divided into antigen specific and antigen unspecific factors [1]. Antigen specific factors are in general genetically restricted as to histocompatibility [2]. Antigen unspecific suppressor factors normally act without any genetic restriction. Only the latter therefore could potentially be used in passive immunotherapy, *e.g.* to block the proliferation of autoreactive cells in autoimmunity or Ig E-producing B-lymphocytes in allergies.

The isolation of inhibitors of lymphocyte proliferation was strongly influenced by the chalone concept of Bullough and Lawrence [3] who postulated physiological cell line specific and species unspecific inhibitors of cell proliferation. As *in vivo* a lymphocyte specific, antigen unspecific inhibitor only makes sense at a differentiation stage before the lymphocytes become antigen competent and leave the thymus, it is meaningful to look for such factors in the bone marrow and/or thymus. We have used calf thymus as starting material continuing previous studies [4, 5]. The aim of the present paper is to further characterize the biochemical nature of such inhibitors of lymphocyte proliferation.

Materials and Methods

Extraction of calf thymus

9.5 kg of frozen calf thymus were suspended in 19 l deionized water and stirred overnight at 4 °C. After addition of 42.75 l ethanol the suspension was centrifuged in a sieve centrifuge. 55 l of the supernatant were dripped into 220 l of ice cold acetone so that a well-sedimenting white mass precipitated. After air-drying 44.5 g (0.47%) of an "acetone precipitate" were obtained.

Ultrafiltration

A multi-chamber ultrafiltration system (Schütt, Göttingen) developed by Kinawi [6] was used. It consisted of seven flat 7.5 ml volume chambers separated by the following 90 mm i.d. Diaflo membranes (Amicon, Witten): XM 100, XM 50, YM 30, YM 10, YM 5, YM 2 and YC 05. 250 ml of a 20 mg/ml solution of the acetone precipitate in water (undissolved residue: 4%) were pumped through the chamber system in 5 portions at a rate of 12 ml/h. Eluent: bidist. water. The pooled chamber contents of each range were lyophilized. Yield of the combined fractions: approximately 80%.

Anion exchange chromatography

110 mg of the 1–5 kDa fraction of the acetone precipitate were dissolved in 5.5 ml bidist. water and separated by fast protein liquid chromatography (FPLC) at 2.0 ml/min. on the anion exchange column Mono Q (50 × 5 mm, Pharmacia, Freiburg) in bidist. water. The bound portion was eluted with an ammonium acetate gradient of 26 mol/ml. Before testing the fractions were desalted by twofold lyophilisation, dissolved in bidist. water and adjusted to isotonicity.

Abbreviations: BSA, bovine serum albumin; Con A, Concanavalin A; CSF, colony stimulating factor; FCS, fetal calf serum; FPLC, fast protein liquid chromatography; HPTLC, high performance thin layer chromatography; ID 50, dose giving 50% inhibition; IEF, isoelectric focusing; IL-2, interleukin 2; IMDM, Iscove's modified Dulbecco's medium; PAS, periodic acid Schiff base; PHA, phytohemagglutinin; TLC, thin layer chromatography.

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Thin layer chromatography

Kieselgel 60 coated HPTLC aluminium sheets (Merck, Darmstadt) and *n*-propanol/water (7:3) were used. The spots were visualized with orcine/iron(III)chloride/sulfuric acid [7], ninhydrine or UV-light at 254 or 366 nm.

Isoelectric focusing

Focusing was performed on Servalyt precotes 3–6 and 3–10 with anode- and cathode-solution from Serva (Heidelberg) and a calibration kit from Pharmacia (Freiburg). Conditions were applied as suggested by the manufacturer. PAS-staining was performed as described [8]. Normally 150 µg of sample were applied.

Protein estimation

Protein was estimated with Coomassie blue according to the instructions of the protein assay of Bio-Rad (München) using bacitracin and bovine serum albumin (BSA) as standards [9].

Colony tests

Colony tests in glass capillaries using human mononuclear cells [10] or mouse thymocytes [11] stimulated by PHA and CSF-stimulated mouse bone marrow cells [12] were routinely used to follow the inhibitory activity and to demonstrate cell line specificity. For comparison [^{3}H]thymidine incorporation into mouse thymocytes induced by Con A in the presence of IL-2 was performed. As a control [^{3}H]thymidine incorporation into CSF-activated bone marrow cells was measured.

[^{3}H]thymidine incorporation tests

Induction of mouse thymus Con A blasts in the presence of IL-2 was performed [13] in Iscove's modified Dulbecco's medium (IMDM; Boehringer Mannheim) containing 2 mM glutamine, 2E-5 M 2-mercaptoethanol and 10% heat-inactivated fetal calf serum (Lot-No. 155, Paesel, Frankfurt). 2E5 thymocytes of 6–12 weeks old male C57Bl/6 mice per microwell of a 96 well round bottom microtiter plate were stimulated with 3 µg/ml Con A (Lot No. 910026, Calbiochem-Behring Corp.) and 2.5% IL-2 (Lymphocult T–LF, Biotest, Frankfurt). The wells contained 30 µl 150 mM NaCl ore test substance, which was adjusted to isotonicity and pH 7.2. After 3 days of culture at 37 °C and 5% CO_2/air, the wells were pulsed each with 0.2 µCi [6-^{3}H-]thymidine (spec. act. 20.7 mCi/mg) for 4 h. The cells were transferred to filter paper discs by a cell harvester. The ^{3}H-activity of the dried discs was measured by liquid scintillation spectrometry.

Mouse bone marrow cells were cultured similar to the method of Horak *et al.* [14]. 2.5E5 bone marrow cells/ml from C57Bl/6-mice were cultured in IMDM containing 10% FCS (Paesel), 2 mM glutamine and 5% mouse lung conditioned medium [12]. After 3 days of culture in flat bottom microtiter plates at 5% CO_2/air and 37 °C the cells were pulsed and harvested as described for thymus Con A-blasts.

Results and Discussion

In earlier studies [4, 5] we treated calf thymus with acetone, extracted the dried residue with acetate buffer and subjected the extract to an ultrafiltration followed by gel permeation or anion exchange chromatography. The material with the highest lymphocyte selective inhibitory activity of colony growth (ID 50: 5 µg peptide/ml) had an apparent molecular mass between 1 and 10 kDa and was eluted on DEAE-cellulose by 154–200 mM sodium chloride (ID 50: 3 µg peptide/ml).

For large scale preparation we prefered an extraction procedure similar to that of Patt *et al.* [15], whereby the majority of the ethanol extracted activity could be precipitated and concentrated with an excess of acetone. For further purification by molecular size a new multichamber ultrafiltration system by Kinawi [6] was used. The molecular mass fraction 1–5 kDa contained the strongest selectively inhibiting activity of lymphocyte proliferation in the colony tests (Table I).

With benzalkoniumchloride as a cytotoxic control inhibitor [16] both lymphocyte tests were less sensitive than the granulocyte tests (Table II). This is a favourable prerequisite for the isolation of an endogenous lymphocyte selective inhibitor. Caution is necessary for performing only colony tests, because sodium azide for instance but not benzalkoniumchloride would fake a lymphocyte selective inhibitor.

Parallel measurement of viability and [^{3}H]thymidine incorporation into mouse thymocytes stimu-

Table I. Yields and ID 50-values of ultrafiltration fractions of the acetone precipitate in different test systems.

Substance	Yield [%]	ID 50 [μg/ml]				
		Human PHA-blasts	Mouse thymocytes + IL-2 and PHA		Mouse bone marrow + CSF	
		Colony test	Colony test	[^{3}H]thymidine incorp.	Colony test	[^{3}H]thymidine incorp.
acetone precipit.	100.0	110	580	580	900	1700
ultrafiltr. fraction (kDa)						
>100	1.3	800	290	120	300	250
100–50	2.8	410	270	920	>2000	>2000
50–30	1.8	–	300	330	390	1000
30–10	3.5	280	1200	670	>2000	no inh.
10–5	5.7	300	560	1100	940	no inh.
5–1	7.4	230	210	900	>2000	1900
1–0.5	11.9	320	360	1000	1050	2000
<0.5	65.7	88	490	1600	620	no inh.
5–1 (large scale)	–	230	190	440	1000	600

lated by Con A showed, that the 1–5 kDa fraction did not significantly decrease the viability at a dose showing 50% inhibition (Fig. 1).

When this fraction was further analyzed by fast protein liquid chromatography (FPLC; Fig. 2) it was bound to the anion exchange column (Mono Q) in bidist. water. The strongest selectively lymphocyte inhibiting activity was then eluted at an ammonium acetate concentration between 205 and 265 mmol/l which corresponded to fraction numbers 12–14 (Fig. 3). The characterization of these low molecular mass substances was facilitated best by TLC. The most purified and most active fraction 13 did not show any fluorescence at 366 nm and only 3 very weakly fluorescence quenching spots at 254 nm. It did not contain glycoconjugates as shown by orcine staining but a strong colour reaction with ninhydrine (Fig. 4).

After isoelectric focusing the FPLC-fraction 13 could not be stained with Coomassie blue R-250. As this fraction reacted strongly with ninhydrine, it did not contain peptides with a molecular mass > 3 kDa, the lower molecular mass limit for staining of peptides with Coomassie blue [17]. Moreover the focused fraction 1–5 kDa did not stain with the periodic acid Schiff base reagent.

Table II. IDs 50 of control inhibitors in colony and [^{3}H]thymidine incorporation tests.

Test system	ID 50 [μmol/l]		
	Sodium azide	Benzalkonium chloride	Ammonium acetate
Colony formation			
Human PHA-blasts	22	2.4	4700
CSF-induced mouse bone marrow	160	0.8	–
[^{3}H]thymidine incorp.			
Con A-induced thymocytes	350	1.4	9900
CSF-induced mouse bone marrow	340	0.4	–

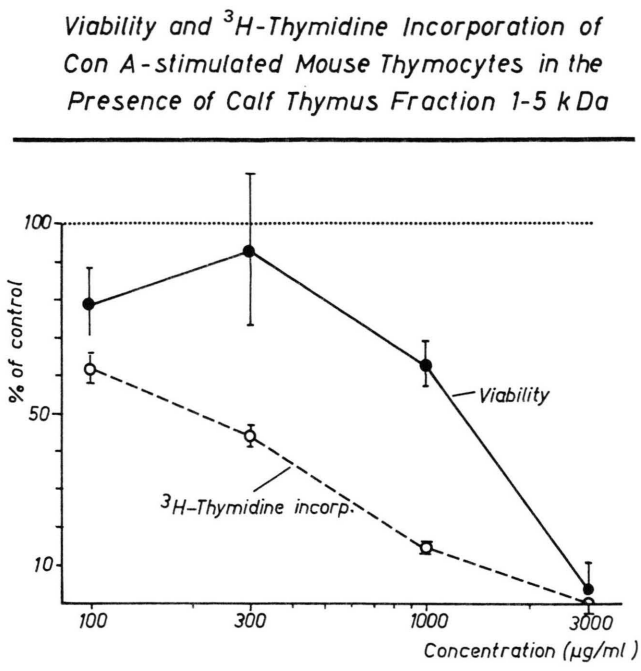

Fig. 1. Correlation of viability and [^{3}H]thymidine incorporation (+/− S.D.) of Con A-stimulated mouse thymocytes in the presence of the 1–5 kDa fraction of the acetone precipitate. Viable cells were stained with fluorescein diacetate, the nuclei of dead cells with propidium iodide [21].

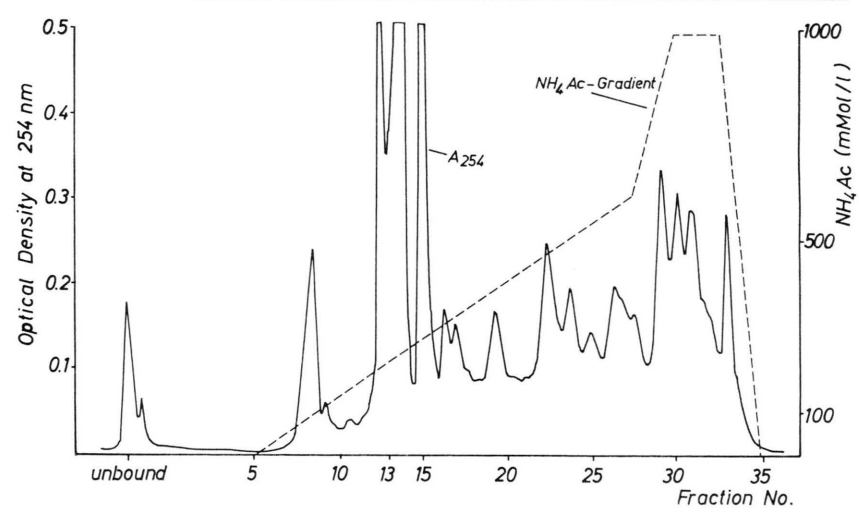

Fig. 2. FPLC of the 1–5 kDa fraction of the acetone precipitate on the anion exchange column Mono Q.

The data suggest that the inhibition of colony formation of human lymphocytes is mediated by slightly acidic peptides with a molecular mass between 1 and 3 kDa. It is different by molecular mass and isoelectric point from facteur thymique serique isomers, which had recently been made responsible in part by Lenfant *et al.* for the inhibiting activities of calf spleen extracts [18] and is not identical with other thymic factors [19, 20].

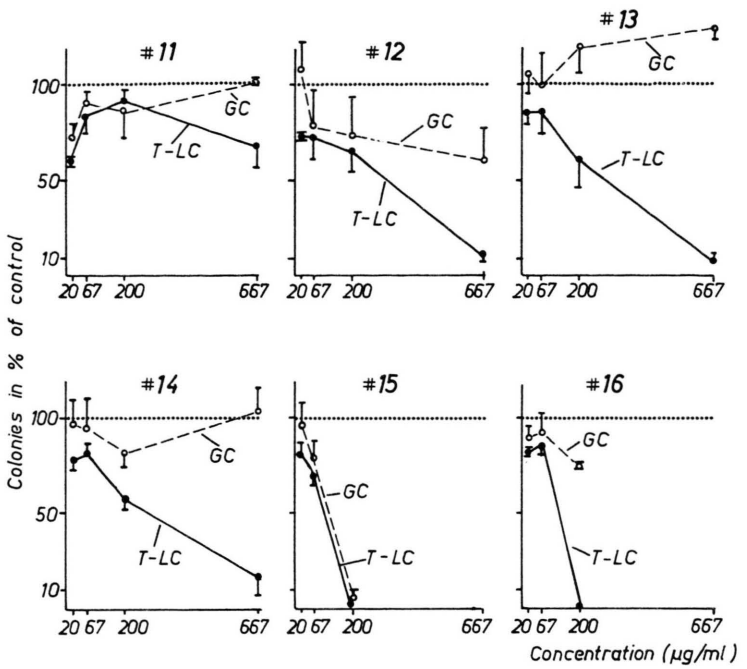

Fig. 3. FPLC of the 1–5 kDa fraction of the acetone precipitate on the anion exchange column Mono Q. Test results of single fractions in the human lymphocyte and mouse granulocyte test.

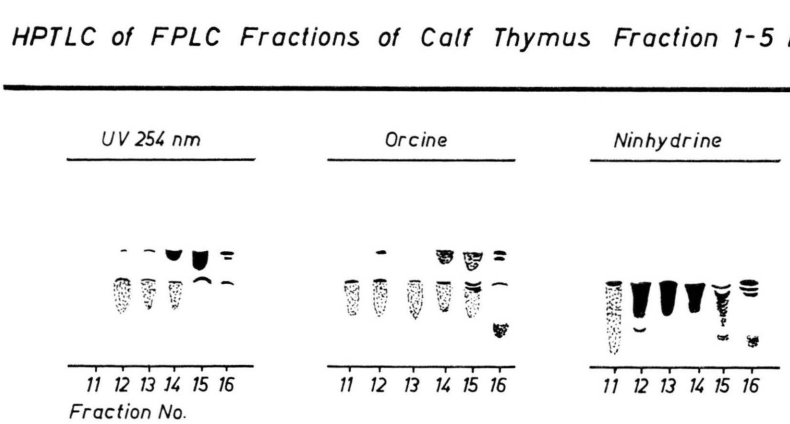

Fig. 4. HPTLC on silica gel in *n*-propanol/water (7:3) of the fractions in Fig. 3.

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