A Granulopoiesis Inhibitor Partially Purified from Large-Scale Serum-Free Cultures of Porcine Leukocytes

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A method is described for the partial purification of a granulomonopoiesis inhibitor derived from 200 l-batches equivalent to 1 kg or $2 \cdot 10^{12}$ leukocytes of porcine blood. Serum-free conditioned media were concentrated and separated via ammonium sulfate precipitation, acetone partitioning, ultrafiltration and gel chromatography. The active substance revealed a molecular mass of 500–700 Da and was not thiol-dependent, thus contrasting it with previously-reported granulopoiesis inhibitors. Inhibition of colony formation in an in vitro myeloid stem cell assay using various colony-stimulating factors showed that mature granulocytes and monocytes were equally affected. Apparent lack of cytotoxicity was suggested using a new reversibility test.

Introduction

Endogenous granulopoiesis inhibitors (GIs) have been characterized as penta- and decapeptides, respectively [1, 2], partly complying with the definition of chalones as cell-line specific, reversibly-acting and non-toxic inhibitors of cell proliferation [3]. Previous studies suggested that GIs must be minute trace components in biological fluids such as cell conditioned media [4]. Therefore, requisites for closer characterisation of GIs must include large-scale isolation procedures of inhibitor producer-cells and of the biologically active solutes present in their culture supernatant solutions. We have recently found that leukocytes biotechnologically isolated from porcine blood upon incubation exude GI activity in addition to numerous other cytokines [5]. Due to its relatively high granulocyte concentration, porcine blood seemed to be a suitable starting material practical for scaling up.

Materials and Methods

Porcine leukocyte and conditioned media

Leukocytes from porcine blood and media were prepared as described in detail elsewhere [5]. In short, leukocytes from a daily batch of 200 l porcine blood containing 10 mM sodium citrate and 0.1% w/v methyl cellulose MC 25 (Fluka, Buchs, Switzerland) were separated from the bulk of plasma, thrombocytes and erythrocytes at $1 \times g$. After collection of leukocytes by sedimentation at $400 \times g$ for 10 min at 10 °C, traces of contaminating blood cells other than leukocytes were removed by a brief hypotonic shock in 0.2% NaCl. The remaining viable leukocyte suspension was washed twice in 1.5 mM phosphate-buffered physiological (150 mM) saline containing 1 mM cysteine, pH 7.40 (PBSC). The leukocyte mixture (about 60% granulocytes) was suspended at a density of $10^7 - 10^8$ cells/ml in a fully synthetic, chemically-defined serum-free medium [6]. Its 94 components included 50 nM of the lectin mitogen Concanavalin A (CON) [5]. The cells were incubated in this medium for 40 h at 37 °C at constant pH (7.2), D-glucose (5 mM) and oxygen (0.2 mM) concentration. CON-free medium is otherwise identical and termed REF.

Cell culture supernatant solutions and initial fractionation

A culture supernatant solution (conditioned medium) was obtained by centrifugation at $400 \times g$ for 10 min at 4 °C. The supernatant was again centrifuged at $10000 \times g$ for 30 min at 4 °C.

This clear supernatant served as a crude starting material and was immediately subjected to the following purification steps after the phosphate con-

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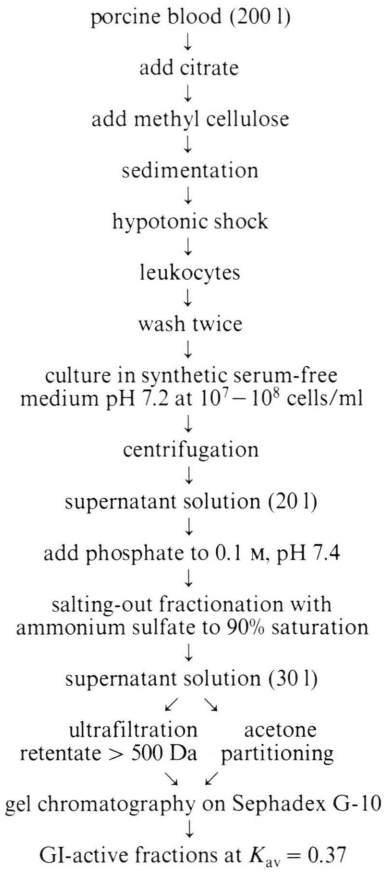

Fig. 1. Schematic large-scale preparation of crude granulopoiesis inhibitor (GI) from cultured porcine leukocytes.

centration had been adjusted to 0.1 M at pH 7.4. An established procedure for the separation and isolation of other cytokines of leukocytes was followed [5]; for a summary see Fig. 1. This comprised a salting out fractionation of the supernatant at various ammonium sulfate (AS) saturation degrees; *i.e.* 0–35%, 35–45%, 45–90% and 90% (3.6 M) AS. The three salt-insoluble precipitate fractions and the salt-soluble (fourth) fraction (90% AS saturation) obtained were screened for GI activity by dissolving portions in PBSC and then desalting by ultrafiltration through membranes with a nominal molecular mass retention capacity of 500 Da (UM 05, Amicon Corp.). Osmolalities were adjusted to about 290 mOsm with PBSC. Since the major portion of GI activity was found in the salt-soluble fourth fraction, this fraction was used for further GI purification. From a daily 200 l blood batch, 30 l of this AS containing fourth fraction were obtained.

Acetone partitioning

To remove salts from the fourth fraction, it was flash-evaporated and air-dried at 40 °C. About 700 g residue (mainly AS) were obtained per liter. An equivalent of 1 l acetone containing 0.5 mM 2-mercaptoethanol (2-ME) was added and stirred at 40 °C for 30 min. After centrifugation ($4000 \times g$, 30 min, 4 °C) the acetone was removed by flash evaporation and the oily residue extracted with 20 ml H_2O, 0.5 mM 2-ME and 0.05% w/v TWEEN 80. Alternatively, the fourth fraction was diluted with four volumes of PBSC, passed through a UM 05 membrane and washed repeatedly with PBSC until AS could no longer be detected using $BaCl_2$. The retentate could thereby be concentrated 300 times.

Gel chromatography

Salt-free GI concentrates were chromatographed at 4 °C on Sephadex G-10 columns (size range from 13×900 mm to 70×1400 mm). The mobile phase was 150 mM NaCl containing 1 mM Na/K-phosphate, 0.05% w/v TWEEN 80 and 0.5 mM 2-ME, pH 7.20. Active fractions were obtained at $K_{av} = 0.37$ ($V_e/V_o = 1.3$) and at $K_{av} = 0.0$ ($V_e/V_o = 1.0$), with V_o and V_t as determined using bovine serum albumin and 4 M NaCl, respectively. Due to instability even at − 20 °C dilute fractions were each immediately concentrated by ultrafiltration on UM 05 membranes as described above or lyophilized. Protein was determined according to [7].

Biological assays

Tests for inhibition and stimulation of colony growth using murine bone marrow cells or PHA-stimulated human lymphocytes from peripheral blood were carried out as described [8–10]. Granulocyte/macrophage colony stimulating factor (CSF) was prepared from mouse lung conditioned medium according to [11]. The controls, consisting of 0.15 M NaCl or chromatography elution buffer were made 0.5 mM in 2-ME as were the samples to be tested. Assays consisting of a total volume of 300 µl accomodated up to 100 µl of sample.

Test for reversibility of GI action

The procedure has been described elsewhere in detail [12]. Briefly, target cells, sample or control substance and all remaining assay constituents were

mixed, drawn into the capillaries and incubated for the contact periods indicated in Fig. 5. Thereafter, the agar gels were slowly flushed out in unchanged form into 35 mm petri dishes and incubated for 5 min in 3 ml wash solution having the same concentration of all assay constituents [8] with saline in place of the sample. The agar gels were redrawn into new capillaries and incubated as usual for 7 days at 37 °C in a 7.5% CO_2 atmosphere. Control gels containing samples, which were not washed, were also incubated 7 days to demonstrate the maximum degree of inhibition. The inhibitory dosages were adjusted to give a relative inhibition of 75% (ID_{75}). Cell viability was checked throughout by using the trypan blue exclusion test.

Morphological analyses

After 7 days of incubation the agar gels were flushed out onto microscope slides, dried, fixed with methanol and stained according to Giemsa.

Results

Leukocytes and conditioned media

200 l of porcine blood yielded about 1 kg or 2×10^{12} leukocytes consisting of 58% neutrophils, 2.5% eosinophils, 0.5% basophils, 7% monocytes and 32% lymphocytes. Average initial viability was about 98%, recovery about 50% of the leukocytes originally present. One half of the conditioned medium contained Concanavalin A (CON) as cellular activator, the other half served as reference (REF). After the 40 h period of culture around 95% of the cells remained viable, as measured by trypan blue exclusion, and functionally active as measured in terms of chemostatic and chemokinetic migration [5] elicited by isolated humoral effectors [13].

Salting-out fractionation with ammonium sulfate

Ammonium sulfate (AS) itself was found to exhibit in the granulocyte agar colony test an ID_{50} of 1.5 mM (0.02% w/v) and in the lymphocyte agar colony test and ID_{50} of 11 mM (0.15% w/v). Samples were desalted (see below) until sulfate could no longer be detected as $BaSO_4$. Of all the supernatants and precipitates tested by dose-response, only the supernatant of the 90% AS saturation showed a non-toxic GI activity after extensive salting. However, considerable stimulation of murine and human bone marrow cells was found by the 45–90% precipitate [14].

Desalting

The viscosity of the 90% AS supernatant made it necessary to dilute the solutions to about 25% AS

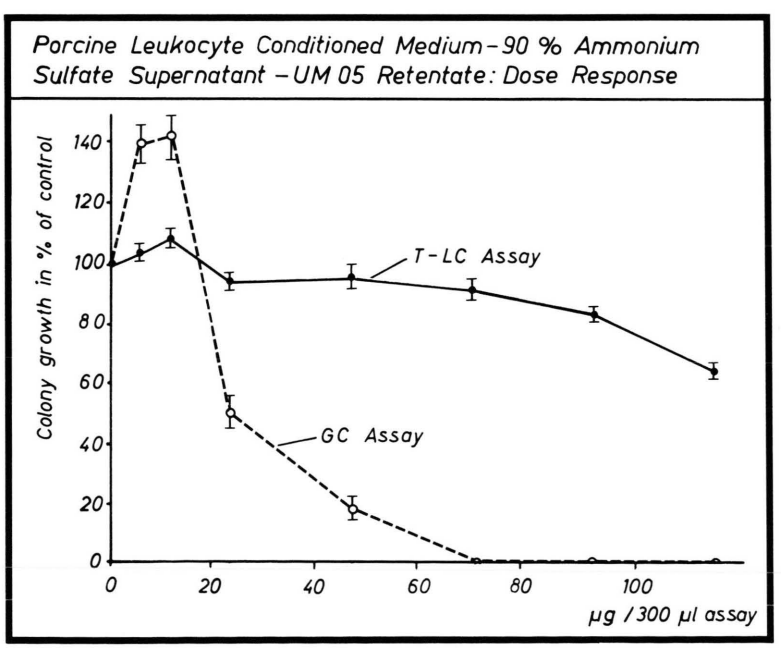

Fig. 2. Dose-response relationship for a UM 05 retentate derived from the 90% ammonium sulfate supernatant of porcine leukocyte conditioned medium. 1 µg sample contained 570 ng of protein.

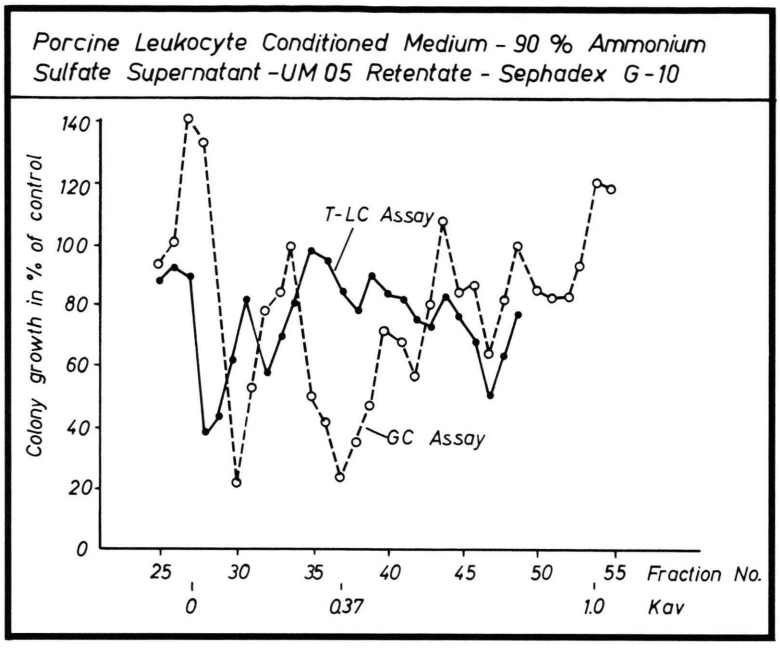

Fig. 3. Sephadex G-10 chromatography of a UM 05 retentate derived from the 90% ammonium sulfate supernatant of porcine leukocyte conditioned medium.

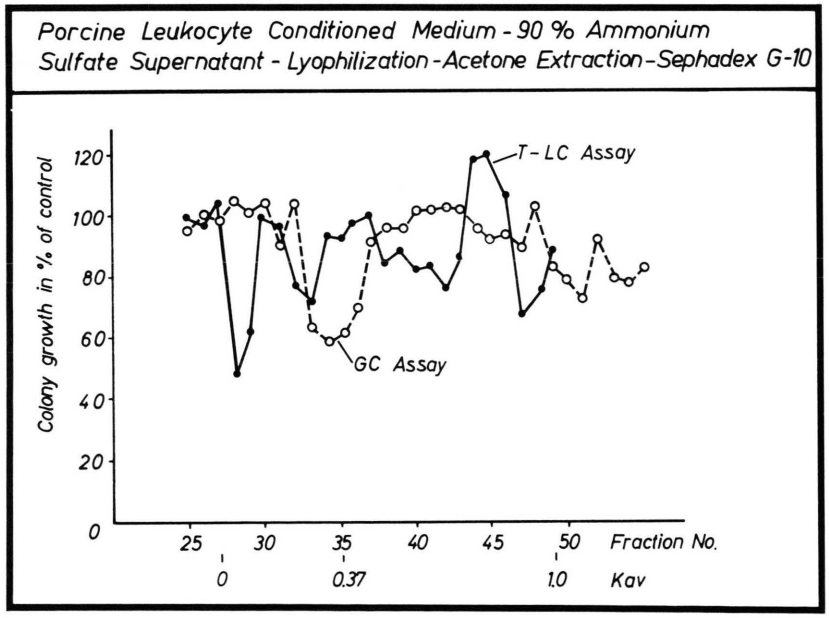

Fig. 4. Sephadex G-10 chromatography of an acetone extract derived from the 90% ammonium sulfate supernatant of porcine leukocyte conditioned medium.

saturation. Using ultrafiltration with the UM 05 membrane to desalt, GI activity was consistently found in the retentate, which was washed until free of AS and subsequently concentrated to 1/300 of its original volume. At this stage the content of components having molecular masses larger than 500 Da amounted to at least 3.2 g per kg of cultured leukocytes. The dose-response curve for GI activity of such a UM 05 retentate is given in Fig. 2.

In all GI preparations derived from conditioned REF- and CON-media, retentates obtained from REF-cultures were found to be less active (up to 50%) than those from CON-cultures. However, in contrast to samples from REF-cultures, those from

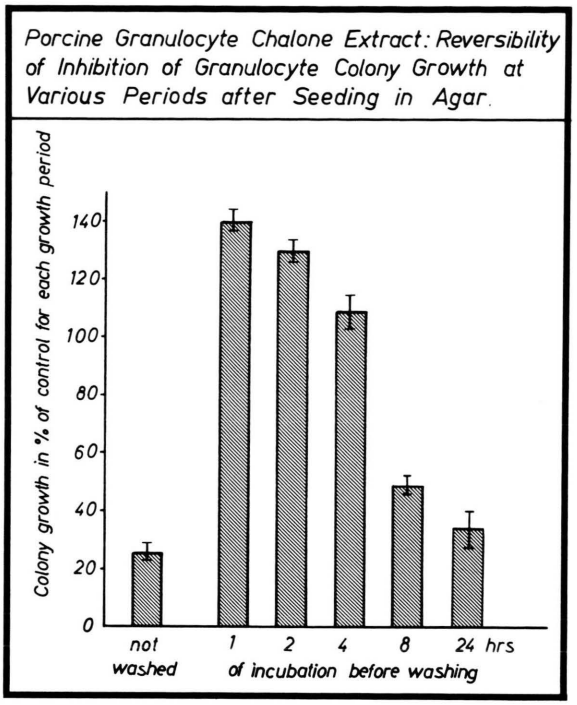

Fig. 5. Reversibility of inhibition of a granulopoiesis inhibitor ($K_{av} = 0.37$-pool) at various contact periods after seeding with murine bone marrow cells in agar.

CON-cultures contained proliferation inhibitory activity for lymphocytes as well.

Lyophilized retentates stored at 4 °C retained their activity indefinitely. The same was true for retentates containing at least 2 mg/ml protein frozen at − 20 °C. 0.5 mM 2-mercaptoethanol (2-ME) or 0.5 mM dithiothreitol (DTE) added before storage and/or testing had no appreciable effect on the inhibitory activity.

In one process, acetone was used to extract GI activity from freeze-dried residues of the 90% AS supernatant. Thereby most of the salts and lipids could be removed from the original solution. GI activity was found to be present in the acetone-soluble portion.

Gel chromatography

On Sephadex G-10 UM 05-retentates yielded significant GI fractions at $K_{av} = 0.37$ and occasionally (in 9 of 21 fractionations) also at $K_{av} = 0.1$ and 0.7 (Figs. 3 and 4). The latter 2 peaks were not detectable in fractions of acetone-extracted samples (Fig. 4). Moreover, stimulatory activity, indicated in Figs. 2 and 3, was removed by acetone treatment (Fig. 4). Yet Sephadex G-10 chromatography proved to be unsatisfactory for desalting the 90% AS supernatant. This salt did not elute like NaCl at $K_{av} = 1.0$, but from $K_{av} = 0.2$ in a broad range, thus overlapping with the GI activity.

Collection of the active pool around $K_{av} = 0.37$ provided a further purification of about 20-fold. All active fractions were stainable with ninhydrine and chlorotoluidine. The $K_{av} = 0.37$-pool was immediately concentrated by ultrafiltration on UM 05 membranes or lyophilized.

Stability

The GI activity of the $K_{av} = 0.37$-pool was found to be labile and even upon storage at − 20 °C could not be preserved indefinitely. The addition of 2-ME or DTE did not enhance its activity nor could lost activity be restored by pre-incubation with 0.5 mM 2-ME for 2 h at room temperature prior to testing as reported previously [15]. The activity in fresh fractions did not decrease upon contact to air or even pure oxygen. Heating to 56 °C for 3 min or to 90 °C for 5 min also had no apparent effect. Concentration by ultrafiltration on UM 05 membranes and sterile filtrations through cellulose acetate filters caused up to 20% loss of activity.

Reversibility test of GI action

Bone marrow cells in agar cultures were treated for various periods between 1 and 24 h with GI (ID_{75}), then washed and incubated for 7 days to allow colony formation. Fig. 5 shows that the inhibition of cell proliferation normally caused by GI could be reversed during exposure periods of up to 4 h. Under experimental conditions described in detail elsewhere [12], 90 − 95% of the inhibition could be abolished. At this time cell viability was at least 95%. When the agar contents were not washed free of GI material, the expected inhibition of 75% was seen instead.

Morphological analyses

The colonies remaining after applications of inhibitory doses (ID_{35} to ID_{75}) in the test assays showed that approximately the same ratios of polymorphonuclear to mononuclear cells were present as

in the control assays. (Average number of colonies per assay 23, average number of cells per colony 78).

Discussion

Porcine leukocytes proved to be an efficient source for large-scale production, preparation and further purification of an endogenous GI activity. Use of a chemically-defined, serum-free medium enabled a suitable conditioning of leukocytes [5, 6]. Moreover, it eliminated the need to subsequently remove foreign proteins. But we cannot support a previous finding [16] that granulocytes used for the isolation of such inhibitory factors were best conditioned in pure saline for 5 h. Salting-out fractionation with AS was a reliable method for initial separation of salt-soluble GI material [5, 6, 14], but necessitated extensive desalting prior to testing. The processes described for AS removal proved to be efficient and extendable to technical levels [5]. $BaCl_2$ was a convenient indicator for AS presence, since the detection limit of $BaSO_4$ is as low as 9.5 μM as compared with the toxicity of AS (ID_{50} in the granulocyte assay: 1.5 mM).

Sephadex G-10 chromatography separated stimulatory and up to 3 GI factors (Fig. 3). Of the latter only the peak at $K_{av} = 0.37$ (fraction No. 37) was found to be reproducible, a fraction also discovered in other tissues and species with similar cell-line specificity [15, 17, 20]. The inhibitor at $K_{av} = 0.1$ (fraction No. 30) may represent an aggregate form of fraction $K_{av} = 0.37$ as described elsewhere [20], or a real hitherto unknown, even more unstable inhibitor.

The GI material routinely found at $K_{av} = 0.37$ may be assumed to have a hydrodynamic equivalent to a molecular mass of less than 700 Da. This agrees with previous estimates for a human GI [1]. Assuming about 600 Da, the ID_{50} of 1.2 μg protein of the $K_{av} = 0.37$-pool in a 300 μl assay (Fig. 2) represented an ID_{50} of about 6 μM for this partially purified inhibitor, the chemical nature of which is yet to be elucidated.

Upon concentration it was often noted that the activity at $K_{av} = 0.37$ reappeared at higher molecular masses, probably due to aggregation. Concentrates with > 1 mg protein/ml or lyophilisates could not be kept stable in organic solvents as experienced with murine tumor extracts [18]. Nor could lost activity be reconstituted by 2-ME as was the case with bovine granulocyte extracts [17], or using human leukocyte extracts [15]. Attempts to stabilise the active G-10 pool by adding other, individual fractions of the chromatogram on Sephadex G-10 or mixtures thereof, were unsuccessful.

Stimulatory factors apparent at a $K_{av} = 0$ (Fig. 3) indicated molecular masses higher than 700 Da, possibly identical with, or similar to, colony stimulating factors (CSF) formed by leukocytes in culture concommitantly with GI [14]. These were separated from GI by salting-out fractionation at 45 – 90% AS saturation. The factors may also be responsible for the stimulation seen in Figs. 2, 3 and 5.

At the stage of purification described here, the presence of minor toxic compounds cannot be excluded. However the following findings contradict a mere cytotoxic inhibition by the peak at $K_{av} = 0.37$:

(1) Dividing T-lymphocytes are not affected (Fig. 2); (2) cell viability is not reduced after at least 24 h of incubation and (3) the inhibition is reversible for at least 4 h (Fig. 5), in contrast to the effect caused by a series of defined cytotoxic substances that show a greater degree of inhibition, also appearing at earlier times (Kastner and Maurer, in preparation). After about 5 h a lasting inhibition by GI occurs, in agreement with previous findings suggesting a "point of no return" [19].

Paukovits and Laerum [1] recently isolated to homogeneity a hemoregulatory peptide in their search for a granulocyte chalone. The chemically-synthesized peptide, however, revealed in vitro and in vivo effects at variance with those originally postulated for chalones. Increasing evidence suggests that granulopoiesis is regulated by several inhibitors acting at the various levels in the hematopoietic hierarchy [21[. In support of this is Balazs' [2] and our finding that inhibitors lacking thiol character may also be involved. This warrants further isolation of all possible inhibitors concerned and elucidation of their modes of action and interaction.

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