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PARTIAL PURIFICATION AND CHARACTERIZATION OF A GUANYLATE CYCLASE INHIBITOR WITH CYTOTOXIC PROPERTIES FROM THE BITTER MELON (MOMORDICA CHARANTIA)

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Summary - A crude preparation from the bitter melon (Momordica charantia) was reported to both inhibit guanylate cyclase activity from various tissues and to prevent growth of concanavalin A-stimulated (con A) rat splenic lymphocytes. We further report that this crude preparation inhibits tritiated thymidine incorporation into con A-stimulated normal human lymphocytes but does not affect cell viability. In contrast, at the same concentrations, the crude preparation is cytotoxic to several human lymphoblastoid cell lines. In an effort to determine if both the guanylate cyclase inhibitory activity and the cytotoxic activity occur within the same molecular species we have partially purified and characterized the "factor". Following isolation both cytotoxic and guanylate cyclase inhibitory activities coeluted as a single, trypsin-sensitive peak.

Introduction - The bitter melon plant (Momordica charantia) contains many lectins which, while they are not toxic to animals in situ, will inhibit protein synthesis in vitro (1-3). Veseley et al. (4) and Claflin et al. (5) reported that an aqueous extract of the ripe fruit contained an inhibitor of guanylate cyclase. The crude preparation blocked both basal enzyme activity and enzyme activation by a chemical carcinogen. This extract also blocked con A-induced incorporation of tritiated thymidine into rat lymphocyte DNA (5).

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The abbreviations used are: Cyclic AMP, cyclic adenosine 3',5'-monophosphate; cyclic GMP, cyclic guanosine 3',5'-monophosphate; con A, concanavalin A.

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DNA histograms demonstrated that the extract inhibited at the G_2+M phase of the cell cycle. It was uncertain whether the same molecular species was responsible for both the anti-tumor growth effects and the guanylate cyclase inhibitory effects. More recently we reported that addition of this crude preparation to human lymphocytes prevented con A-stimulated thymidine incorporation into DNA and an induced change in a cyclic AMP phosphodiesterase. These effects were reversible and could be partially prevented by the addition of exogenous 8-bromo cyclic GMP to the human lymphocytes (6).

In the present investigation we report a partial purification of a factor from the bitter melon that inhibits guanylate cyclase activity and prevents growth of both normal and leukemic human lymphocytes. At the same concentrations this partially purified factor kills lymphoblastoid cells in a dose-dependent manner while not affecting the viability of normal lymphocytes.

Methods - Preparation of the Bitter Melon Factor - Bitter melon (Momordica charantia, also called balsam pear) was obtained from markets or grown locally and prepared initially by a modification of Claflin et al. (5). Bitter melons were homogenized in cold (4°C) phosphate-buffered saline (PBS), filtered through two layers of cheesecloth, then through Whatman #1 filter paper. The filtrate was centrifuged at 10,000 rpm (Sorval SS-34) for 30 minutes, then precipitated for 30 minutes at 4°C with 50% saturated ammonium sulphate. The pellet from this preparation (10,000 rpm for 10 minutes) was redissolved in PBS and concentrated by Amicon ultrafiltration (UM-2). This preparation, referred to herein as the "crude preparation", was stored frozen or used in subsequent purification procedures. The preparation was unstable and lost activity after 6 months of storage at 0°C. The concentrated preparation was added to a G-200 column (92 ml bed volume) which was equilibrated and eluted with PBS. The activity peak, as measured by a cell viability assay, was then concentrated by Amicon ultrafiltration and electrophoresed using a modification of Laemmli et al. (7). This method substitutes 0.1% Nonidet P-40 for sodium dodecyl sulphate. The gels were run (12 x 0.3 cm) at 2 mA per gel and 4°C. The gels were then sliced (3 mm) and eluted overnight in 1.0 ml of PBS. Each eluted fraction was tested for cytotoxicity.

Cell Culturing and Assay Procedures - Normal human lymphocytes were isolated from venous blood of healthy donors as described previously (6,8). Lymphocyte yields were approximately 800 x 10^6 cells per 500 ml of normal blood. Cells were greater than 95% viable as judged by trypan blue exclusion. All cells were cultured in RPMI 1640 media (Gibco) plus 20% fetal calf serum. Normal blasts were prepared by culturing normal human lymphocytes with sterile con A (25 µg/ml) for 24-48 hours. Blast status was determined microscopically and by measuring tritiated thymidine incorporation (methyl-^{3}H thymidine 90 Ci/mmole, ICN) into DNA (cpm incorporated into 10% trichloroacetic acid precipitable material). The cell lines used were: IM9 (B-cell, human myeloma), MOLT-4 (T-cell, human acute lymphocytic leukemia). Cell growth and viability were measured by ^{3}H-thymidine incorporation into DNA and by trypan blue exclusion, respectively.

Enzyme Assays - Guanylate cyclase was assayed by the method of Garbers and Murad (9). Assay mixtures contained 0.25 mM GTP, 12.5 mM $MnCl_2$, crude guanylate cyclase, and 0.2 ml of buffer containing 100 mM triethanolamine at pH 7.9, 25 mM theophylline, and 900,000 cpm ^{3}H-GTP. After incubation at 37°C for 10-30 minutes the assay mixture was precipitated with Zn acetate and $NaCO_3$, frozen and thawed, and then centrifuged. The product was separated on PEI-cellulose as described (9). The cyclic GMP radioimmunoassay was as described in Brooker et al. (10) using ^{125}I-tracer purchased from NEN and antibody kindly provided by Dr. Ferid Murad, Department of Clinical Pharmacology, University of Virginia.

Results - The crude bitter melon preparation decreased tritiated thymidine incorporation into DNA in both normal human lymphocytes and lymphoblastoid cell lines (data not shown). However, at the concentration used, only the lymphoblastoid cell lines were killed. Furthermore, growth inhibition of normal, con A-stimulated lymphocytes was fully reversible (data not shown). As indicated in Figure 1, the killing of lymphoblastoid cell lines was dose-dependent. Much higher concentrations of crude preparation (greater than 3-fold) were needed to kill normal cells. It was apparent that the B-cell line (IM9) was more sensitive to the crude preparation than was the T-cell line (MOLT-4).

Because normal unstimulated lymphocytes are considered to be quiescent, it was necessary to determine the effects of the crude preparation on dividing cells of normal lymphocytic origin. Normal lymphocytes were transformed to

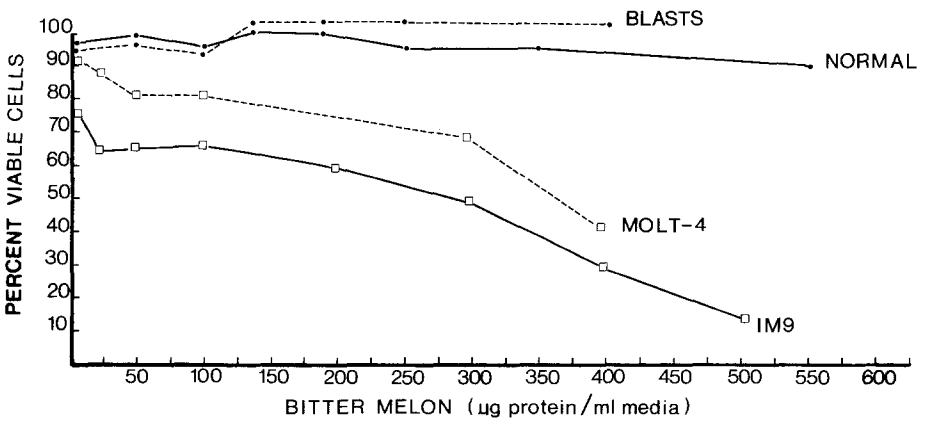

Figure 1 - Response of Human Lymphocytes to Increasing Doses of Crude Bitter Melon - Cells (10 x 10^6) were cultured for 24 hours in RPMI 1640 media plus 20% fetal calf serum. Source of cells and bitter melon preparation are described in the text. Viability was measured by trypan blue exclusion. Results are those of samples assayed in duplicate.

blasts by incubation with con A for 24 hours. As indicated, though the blasts were also dividing cells, the crude preparation did not affect them. At the concentrations used, only the lymphoblastoid cell lines were affected.

At low concentrations of the crude preparation (50 µg protein or less) all cells remained greater than 95% viable for 24 hours (see IM9 curve, Figure 1). Therefore, the lower concentrations were used to determine effects on cellular cyclic GMP levels while avoiding the cytotoxic effects. This concentration of crude preparation lowered cyclic GMP levels in cells from a leukemic donor and from a lymphoblastoid cell line (IM9), whereas no change was seen in the cyclic GMP levels of normal cells (Table 1) or in the

TABLE 1

EFFECT OF CRUDE BITTER MELON EXTRACT ON GUANYLATE CYCLASE ACTIVITY AND CYCLIC GMP LEVELS

Sample	Cyclic GMP Levels[1] (fmoles/10^8 Cells)
Leukemic Cells	2300
Leukemic Cells + Bitter Melon	640
Normal Cells	7.6 ± 0.3 (3)
Normal Cells + Bitter Melon	8.7 ± 0.5 (3)
IM9 Cells	390 ± 90 (4)
IM9 Cells + Bitter Melon	75 ± 14 (4)
Sample	**Guanylate Cyclase Activity[2] (per cent of control)**
IM9 Cells	100%
IM9 Cells + Bitter Melon	43%
Normal Cells	100%
Normal Cells + Bitter Melon	93%

[1] Leukemic cells were obtained from a single donor with acute lymphocytic leukemia (B-cell). Cells (1 x 10^8) were incubated with bitter melon (50 µg protein per ml media) for 24 hours and assayed for cyclic GMP by radioimmunoassay. In all cases cells were greater than 90% viable after the incubation. (Numbers in parentheses indicate the number of samples assayed and are ± S.E.M.)

[2] Guanylate cyclase activity was measured by separation of products as described in the text. Cells (1 x 10^8) were homogenized in assay buffer and assayed for 20 minutes at 37°C with or without the addition of the crude bitter melon preparation (50 µg protein/ml media). No change was noted in the guanylate cyclase activity from normal lymphocytes at this concentration of crude preparation. Samples were assayed in duplicate.

cyclic AMP levels from all cells studied (data not shown). When added to a cell homogenate (Table 1) the preparation inhibited guanylate cyclase activity by 57%; adenylate cyclase activity was not affected (data not shown).

The crude preparation did not cause cellular leakage of cyclic GMP in that the level of cyclic GMP in the media remained unaltered. This crude preparation had no phosphodiesterase activity and did not inhibit any form of phosphodiesterase from rat liver or lymphocytes. Although the preparation did have trace amounts of cyclic GMP binding activity, it did not inhibit either cyclic AMP or cyclic GMP binding by lymphocytes or rat liver. No cyclic AMP binding activity was observed (11). The preparation did contain low levels of both adenylate and guanylate cyclase activities.

In an attempt to characterize a single molecular species with both cytotoxic effects and guanylate cyclase inhibitory activity, we sequentially purified the crude preparation by elution from a G-200 column followed by electrophoresis on a native gel. Figure 2 illustrates a typical activity profile when the crude preparation is eluted, in PBS, from a Sephadex G-200 column. Two major peaks of activity are shown. Using molecular weight

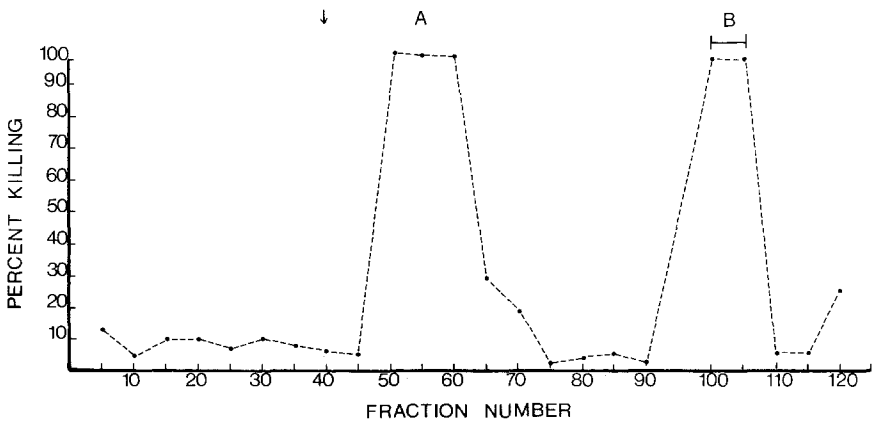

Figure 2 - Elution of the Bitter Melon Cytotoxic Factor from a G-200 Column - One mg in 1.0 ml of crude bitter melon was eluted from a G-200 column as described in the text. Fractions were collected (0.5 ml) and aliquots (0.10 ml) were assayed with approximately 1 x 10^7 cells for 24 hours. Methods for measuring cell viability (o——o) are as described in the text. Bars indicate fractions which were pooled for subsequent analysis. Per cent killing is expressed as a per cent of a control sample to which an equal volume of PBS was added. The void volume is indicated by the arrow. Peak B was used for electrophoresis as shown in Figure 3.

standards, we estimated that the killing activity of peak B eluted with an approximate molecular weight of 70,000. It should be emphasized that cytotoxic activity was found in many fractions throughout the isolation procedure. These activities were very potent, were not trypsin sensitive, and did not affect guanylate cyclase activity. Only the 70K peak exhibited guanylate cyclase inhibitory effects and was, therefore, used in further studies. This peak was pooled (see bars), concentrated by Amicon ultrafiltration, and further purified by using a native polyacrylamide gel system (Figure 3). Two cytotoxic activity peaks were noted but staining of parallel-run gels indicated that many other bands were present. Each peak was pooled and assayed for effects on cellular cyclic GMP levels (Table 2). Only the first peak decreased cyclic GMP levels in the IM9 cell line. This activity peak also inhibited guanylate cyclase activity (300 µl inhibited IM9 guanylate cyclase activity by 50%). Both the cytotoxic activity and guanylate cyclase inhibitory activity were lost after a 10 minute incubation with 0.1 µg trypsin (Sigma, E.C. No. 3.4.31.4) at 25°C.

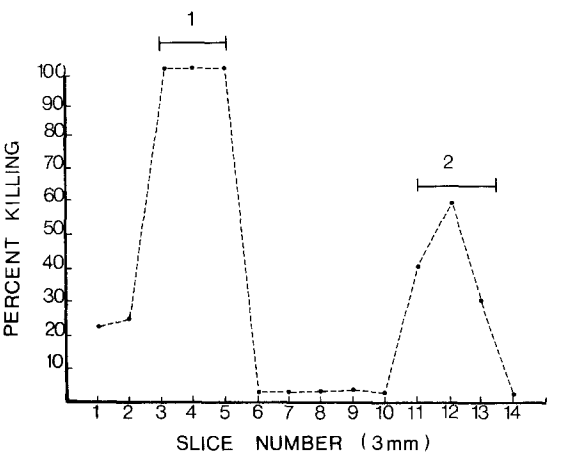

Figure 3 - Separation of the Bitter Melon Factor on Native Polyacrylamide Gels - The entire activity peak from Figure 2 (peak B) was pooled, concentrated, and applied, in sucrose, to the native gels (46 µg protein). Electrophoresis was at 4°C and 2 mA per gel for 14 hours. Slices (3 mm) were eluted in PBS (2 ml) and eluates (pooled every other fraction) were assayed for cell killing using 1 x 10^7 IM9 cells for 24 hours. Incubation conditions are as described in Figure 2. Bars indicate which fractions were pooled. Per cent killing is expressed as a per cent of a control sample to which an equal volume of PBS was added. A sliced gel, with no sample added, had no effect on cell viability.

TABLE 2

EFFECTS OF "PURIFIED" BITTER MELON FACTOR ON CYCLIC GMP LEVELS

Sample	Cyclic GMP Levels (fmoles/10^8 cells)	Per cent of Control
Control[1]	230 ± 75 (5)	100%
+ Peak # 1	52 ± 12 (5)	22%
+ Peak # 2	300 ± 57 (3)	130%

[1] Aliquots from each pooled peak (300 µl) were added to 1 x 10^8 IM9 cells and incubated for 24 hours. Cyclic GMP levels were determined by radioimmunoassay. Cell viability was not affected when aliquots were used at this concentration. PBS was added in place of bitter melon in the control sample. Protein was not measurable in the peaks eluted from the gels. However, iodination of the sample did reveal that a labelled band was present within these peaks. (Numbers in parentheses are total samples assayed. Values are ± SEM.) A plain sliced gel, without the addition of the bitter melon factor, had no effect on cyclic GMP levels.

Discussion - Changes in cyclic GMP levels have been implicated as a cause of cell proliferation, DNA and RNA synthesis, and possibly malignant transformation (12-19). Thus, any compound that affects cyclic GMP levels may be useful in providing insight into the mechanism of action of this nucleotide.

Modulation of guanylate cyclase activity, and thus of cyclic GMP, has been extensively studied (20-23,25). Several types of chemical carcinogens including nitrosamine, diepoxides, and hydrazine stimulate guanylate cyclase activity, whereas alpha-halo ethers, aromatic amines, and some azo dyes and aflatoxins produce a striking inhibition of guanylate cyclase (25). Most of these compounds, it is believed, act on the guanylate cyclase enzyme through changes in the redox state of the preparation or through free radical formation (for an excellent review, see reference 24).

The factor described in this report is a potent inhibitor of guanylate cyclase. Furthermore, it is the only factor we have found to be preferentially cytotoxic for human lymphoblastoid cells (vs. normal human lymphocytes). Though it is cytotoxic to lymphoblastoid cell lines, we have observed other peaks of potent killing activity during our purification procedures. Some of the "anti-tumor growth activity" observed by Claflin et al. (5) may be due to these other factors. Some may belong to the category of lectins found in this plant (1-3).

The particular factor reported herein may act by inhibiting guanylate cyclase activity and lowering cellular cyclic GMP levels, thereby preventing cell growth or causing cell death. The factor must be purified before its mechanism of action on lymphoblastoid cells can be determined.

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