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DIFFERENTIAL CYTOLYSIS OF MURINE SPLEEN, BONE-MARROW AND LEUKEMIA CELLS BY MELITTIN REVEALS DIFFERENCES IN MEMBRANE TOPOGRAPHY

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Summary: L1210 leukemia cells are 2-4 fold more sensitive to the cytolytic effects of melittin, the membrane-active toxin of bee venom, than normal DBA/2 mouse spleen and bone-marrow cells. Lysis of the normal cells was abolished when either 75 mM galactosamine, glucosamine or 100μM β-lactoglobulin was added to the melittin-cell reaction, but lysis of the leukemia cells was unaffected. The amino-groups appeared necessary for blocking melittin-mediated lysis since glucose, galactose and the N-acetyl derivatives were not inhibitory. Bone-marrow cells were more readily protected from lysis than spleen cells. Since melittin-inhibitor complexes were not detected by gel chromatography and the inhibitor could be added to the cell suspension after melittin, the evidence suggests that bone-marrow cells are rich in membrane binding sites for carbohydrates that decrease in mature spleen cells and are virtually absent after neoplastic transformation.

Melittin is an extremely cytolytic polypetide isolated from bee venom (1) and causes inflammatory reactions (2) histamine release from mast cells (3) and rapid lysis of mammalian cells (4). Cytolysis probably results from the interaction of the cationic melittin molecule with both lipid and protein structures at the cell surface (1) although precise mechanisms are not known. We partially characterized some biophysical features of melittin-mediated lysis of DBA/2 mouse cells (5) and found that the lytic properties of melittin could be inhibited by competitive-binding of carbohydrates to sites of melittin interaction. Recognizing that this technique constituted a sensitive probe of cell membrane topography, as defined by melittin-mediated lysis, we evaluated the ability of carbohydrates and β-lactoglobulin to inhibit the lysis of normal DBA/2 spleen (Sp), bone-marrow (BM) cells and lymphoblastic leukemia, L1210. The results indicate that the undifferentiated leukemia is 2-4 fold more sensitive to the lytic effects of melittin than Sp and BM cells. This increased sensitivity probably results from a loss of amino/carbohydrate-binding structures which also bind melittin.

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MATERIALS AND METHODS

DBA/2J female mice were purchased from the Jackson Laboratory. L1210 leukemia cells (a lymphoblastic, null-cell leukemia of DBA/2 origin) were grown as a suspension culture using RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 5 mM L-glutamine and 50 µg/ml gentamicin (all reagents Gibco, Co.) The tumorigenicity and growth properties of this tumor have been described (6).

Melittin (Lot 24F-0197) was purchased from Sigma Chemical Co. and reconstituted to 10 mg/ml in isotonic phosphate buffered saline (PBS), pH 7.2, aliquoted and stored at -20°C. Gel filtration of this preparation on Sephadex G-50 demonstrated a single peak at about 3100 daltons corresponding to the mw of melittin (7) and bioassay for Ca^{++}-stimulated phospholipase-mediated cell lysis proved negative (8). Melittin-β-lactoglobulin complex formation was tested by chromatography of 10 mg melittin through a 115 x 0.5 cm Sephadex G-75 column using PBS, followed by 10 mg β-lactoglobulin, then 10 mg melittin plus 10 mg β-lactoglobulin which had been preincubated for 30 min. Protein was detected by 280 nm scan.

Cytotoxicity and blocking assays were performed using a modified NIH micro technique of trypan blue exclusion (6). Bone-marrow cells were isolated from the hind-limb femurs of several DBA/2J mice. All cells were washed twice in cold PBS and resuspended at 2-4 x 10^6 cells/ml in RPMI 1640 containing 1% FBS. Stock melittin solutions were diluted in the 1% FBS-medium and 50 µl of the cell suspension was added to serially diluted 50 µl melittin solutions, room temperature, for 10 min followed by addition of 10 µl of 0.4% trypan blue dye. Cell viability was scored by counting at least 200 cells and χ^2 analysis was used to determine significant differences between samples of $P<0.05$. Preliminary studies optimized parameters such as cell density, temperature, incubation time and volume. Inhibition of melittin-mediated lysis was measured by adding 50 µl of either an isotonic carbohydrate solution or 300µM β-lactoglobulin in PBS (all reagents purchased from Sigma Chemical Co.) to 50 µl of the cell suspension just before addition of the 50 µl melittin solution. All assays were performed in duplicate, and included both negative-lysis (no melittin) and positive lysis (no inhibitor) controls where appropriate.

RESULTS

The data of Fig. 1 demonstrate the severe cytotytic effect of melittin toward DBA/2 Sp, BM and L1210 cells. In general, L1210 cells were about 2-4 fold more sensitive to lysis than the normal cells. Cell lysis was maximum within 4-5 min and was morphologically characterized by rapid granularity, loss of spherical shape, crenation and intense staining with the trypan blue. The melittin-mediated cytolysis of Sp cells and BM cells was abolished (Fig. 2) when either 75 mM galactosamine ($GalNH_2$) or 75 mM glucosamine ($GluNH_2$) were added to the melittin-cell assay (either prior to the addition of melittin, or within 15-20 sec after addition of melittin). However, L1210 cells were not protected by these amino sugars. The ability of the carbohydrates to block lysis diminished in a concentration-dependent manner (data not shown). The

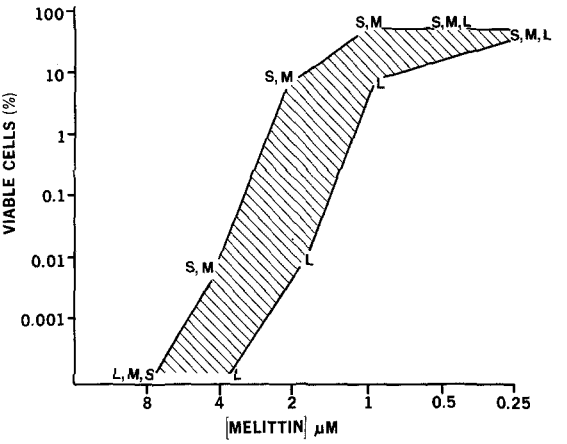

Figure 1: Lysis of DBA/2 mouse spleen (S), bone-marrow (M) and leukemia cells (L) by melittin. The percent viable cells is shown as a function of melittin concentration. Values are represented by the letters, S, M and L and are the representative values of at least duplicates from a minimum of 5 experiments. Washed cells were incubated 10 min with melittin and viability scored by exclusion of vital stain.

inhibition was specific for the amino sugar, since neither galactose, glucose nor their N-acetylated derivatives blocked lysis (see legend, Fig. 2). In addition, the BM cells were protected to a greater extent than Sp cells by both $GalNH_2$ (80±3, 62±3% viability) and $GluNH_2$ (70±2, 56±3% viability).

The addition of 100μM β-lactoglobulin to the BM and Sp cells also protected these cells from lysis by melittin, but did not protect the leukemia

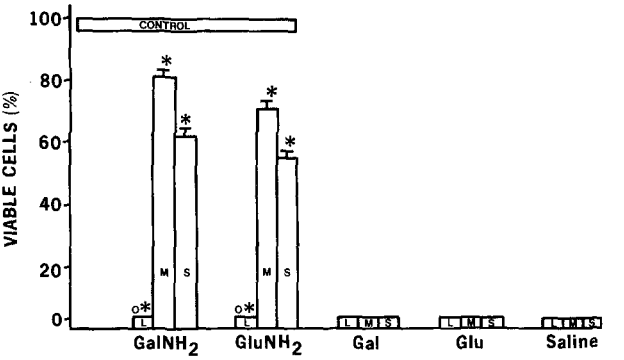

Figure 2: Inhibition of melittin-induced lysis of DBA/2 spleen (S), bone-marrow (M) and leukemia cells (L) by amino-sugars in the presence of 3μM melittin. The percent viable cells is shown after washed cells were combined with either 75 mM galactosamine ($GalNH_2$), glucosamine ($GalNH_2$), galactose (Gal), glucose (Glu) or saline prior to the addition of 3 μM melittin. Vital stain was added after 5 min. Values are mean ± standard error of triplicates. Other non-inhibiting carbohydrates included 2-deoxy-D-glucose, Methyl-D-glucoside, N-Acetyl-D-glucosamine, Methyl-D-galactoside, D and L fucose, D-lactulose, Methyl-D-mannoside, N-Acetyl-D-mannosamine and lactose.

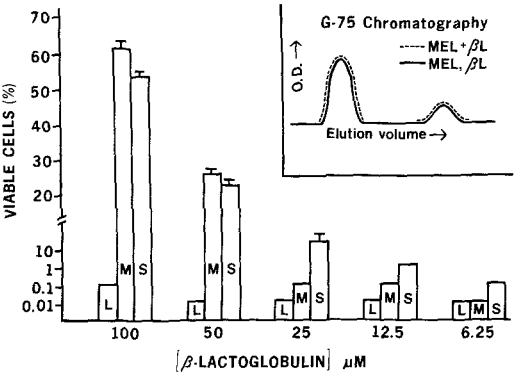

Figure 3: Inhibition of melittin-induced lysis of DBA/2 spleen (S), bone-marrow (M) and leukemia cells (L) by β-lactoglobulin in the presence of 3 μM melittin. The percent viable cells is shown after washed cells were combined with serial dilutions of 100 μM β-lactoglobulin, followed by the addition of 3 μM melittin. Vital stain was added after 5 min. Values are mean ± standard deviation of triplicates. The insert depicts the gel chromatography of either melittin alone, β-lactoglobulin alone (solid line) or melittin combined with β-lactoglobulin (dashed line) through Sephadex G-75 in an attempt to detect melittin-lactoglobulin complex formation. None were detected.

cells. The data of Fig. 3 show the preferential effect of β-lactoglobulin on normal cells. Again, the BM cells were afforded slightly more protection than Sp cells. The insert of Fig. 3 depicts the results obtained from the gel filtration of either 10 mg melittin or 10 mg β-lactoglobulin alone on Sephadex G-75, or the same amounts combined with each other, incubated for 30 min, then chromatographed. The results of each run were identical, i.e., melittin and β-lactoglobulin permeated the gel as essentially free molecules, and were detected as two distinct peaks containing the same amount of protein as if they were added alone. Hence, in agreement with other studies using albumin (9), no melittin-inhibitor complex was detected.

DISCUSSION

Alterations of membrane topography are ubiquitous to neoplastic transformation of cells, resulting in measurable changes in a myriad of biological (10,11), biophysical (12,13) and immunological properties (14,15). The present study demonstrates a subtle and interesting change in undefined structures at the surface of L1210 leukemic cells, as measured by the inhibition of melittin-mediated cell lysis. Two conclusions may be made from the results

presented here. First, carbohydrate and β-lactoglobulin appear to inhibit the lysis of Sp cells and BM cells by competing for common binding sites occupied by melittin. This conclusion is based upon the observations that (a) the inhibitor could be added 15-20 sec after the addition of melittin to the cell lysis assay and still abolish lysis, (b) the failure to detect melittin-lactoglobulin complexes and (c) the lack of any change in the migration of mouse serum proteins, as measured by immunoelectrophoresis, following incubation of serum with melittin (these data have not been presented here, but rather are part of a detailed report on the nature of melittin-mediated lysis, J. Killion and J. Dunn, submitted). Inhibition of lysis clearly depended upon the availability of the reactive amino group, suggesting that the membrane binding site recognizes the NH_2 group at C-2 of the carbohydrate, which seems reasonable in light of the abundance of such groups on the hydrophilic region of melittin (16), a 26 amino-acid cationic polypetide. It is also concluded that neoplastic transformation of the DBA/2 lymphoid cell resulted in a loss of expression (structure or synthesis) of these sites on L1210 leukemia cells. This loss may explain the increased sensitivity of the leukemia cells to lysis by melittin. These same sites may be involved in more physiological events, such as complement-mediated, antibody-dependent cell lysis, since $GalNH_2$ and $GluNH_2$ are potent inhibitors of lysis by complement (17). This speculation seems reasonable in light of the evidence that lysis by the sequential events of the complement pathway and melittin involve an ability of polypeptides to penetrate and disrupt the plasma membrane. Perhaps these sites are involved in "antigenic modulation," allowing leukemia cells to escape complement-mediated lysis (18). In contrast, the rapid penetration by melittin leads to an increase of sensitivity of the leukemia cell to lysis by this molecule.

Collectively, these results support the notion that normal lymphoblastic cells (BM) are rich in amino-group/carbohydrate binding sites, the expression of which decrease in mature splenic cells, but appear to be lost after neoplastic transformation. We have recently exploited the cytolytic properties of melittin in the therapy of L1210 leukemia-bearing mice (19).

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