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Flow Cytometric Analysis of Cell Killing by the Jumper Ant Venom Peptide Pilosulin 1

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Pilosulin 1 is a synthetic 56–amino acid residue polypeptide that corresponds to the largest allergenic polypeptide found in the venom of the jumper ant *Myrmecia pilosula*. Initial experiments showed that pilosulin 1 lysed erythrocytes and killed proliferating B cells. Herein, we describe how flow cytometry was used to investigate the cytotoxicity of the peptide for human white blood cells. Cells were labeled with fluorochrome-conjugated antibodies, incubated with the peptide and 7-aminoactinomycin D (7-AAD), and then analyzed. The effects of varying the peptide concentration, serum concentration, incubation time, and incubation temperature were measured, and the cytotoxicity of pilosulin 1 was compared with that of the bee venom peptide melittin. The antibodies and the 7-AAD enabled the identification of cell subpopulations and dead cells, respectively. It was possible, using the appropriate mix of antibodies and four-color analysis, to monitor the killing of three or more cell subpopulations simultaneously. We found that 1) pilosulin 1 killed cells within minutes, with kinetics similar to those of melittin; 2) pilosulin 1 was a slightly more potent cytotoxic agent than melittin; 3) both pilosulin 1 and melittin were more potent against mononuclear leukocytes than against granulocytes; and 4) serum inhibited killing by either peptide. Cytometry 32:268–273, 1998. © 1998 Wiley-Liss, Inc.

Key terms: flow cytometry; cytotoxic peptides; pilosulin 1; melittin; 7-aminoactinomycin D

The sting of the jumper ant *Myrmecia pilosula* contains the polypeptides Myr p 1 and Myr p 2 (5,7,21), which cause severe allergic reactions in sensitized individuals. Pilosulin 1 is a synthetic peptide (MW = 6045, 56 amino acid residues: GLGSVFGRLARILGRVIPKVAKKLGPK-VAKVLPKVMKEAIPMAVEMAKSQEEQQPQ$_{56}$) corresponding to the largest polypeptide of the Myr p 1 group. Experiments with pilosulin 1 have revealed that 1) pilosulin 1 lyses human erythrocytes; 2) pilosulin 1 kills proliferating Epstein-Barr virus (EBV)-transformed B cells; 3) pilosulin 1 has the potential to adopt an α-helical secondary structure (which is a common feature of many cytolytic polypeptides [1,9,12]); and 4) the 22 N-terminal amino acid residues are critical for the cytotoxic activity of pilosulin 1 (24).

Melittin (26 amino acid residues, from bee venom) is the most extensively studied of the cytolytic peptides. Residues 1–20 are able to form an amphiphilic α-helix and are important for binding to the cell membrane; the carboxy-terminal hexapeptide contains four basic residues and seems to be vital for cytolysis (3,19). In recent years, the cytotoxic effects of melittin on human cell lines have been studied using flow cytometry (8,14,15,23). The criterion for cell death was the uptake (and consequent fluorescence) of either ethidium bromide or propidium iodide.

This report describes investigations into the cytotoxicity of pilosulin 1 and melittin when incubated with a heterogeneous population of nonproliferating human white blood cells, rather than a proliferating cell line. The flow cytometric technique used 7-aminoactinomycin D (7-AAD), which fluoresces at a longer wavelength than either ethidium bromide or propidium iodide (the 7-AAD emission maximum is approximately 660 nm), to distinguish live cells from dead cells (17). The use of 7-AAD allowed the cells to be labeled by up to three fluorochrome-conjugated antibodies in one tube, making it possible to assay the effect of a cytotoxic agent on a number of cell subpopulations simultaneously. (Theoretically, with the use of three antibodies, the killing of eight subpopulations could be monitored.)

MATERIALS AND METHODS

Peptides

Pilosulin 1 was synthesized as described previously (6,10). Honey bee venom melittin was obtained from Sigma Chemical Co. (St. Louis, MO).

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Antibodies

The following antibodies were used: CD3-FITC, NKH1-RD1 (CD56), MY7-RD1 (CD13), Mo2-RD1 (CD14), and B4-ECD (CD19) (Coulter, Miami, FL). FITC, RD1, and ECD are equivalent to fluorescein isothiocyanate, phycoerythrin, and phycoerythrin–Texas Red, respectively.

Cells

Peripheral blood mononuclear cells (PBMCs) were prepared from normal heparinized blood by diluting the blood with an equal volume of modified Hanks' Balanced Salt Solution (HBSS; Sigma) supplemented with 10 mM sodium bicarbonate, 60 U/ml penicillin G (Sigma), 100 μg/ml streptomycin sulfate (ICN Biomedicals Inc., Aurora, OH), and 2 U/ml heparin (Calbiochem-Novachem Corp., La Jolla, CA) and performing density gradient centrifugation over Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden). The PBMCs were washed with HBSS and suspended in RPMI 1640 (GIBCO BRL, Life Technologies Inc., Grand Island, NY) supplemented with 24 mM sodium bicarbonate, 20 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES; ICN), 3 mM L-glutamine (Sigma), 60 U/ml penicillin G, and 100 μg/ml streptomycin sulfate. Total white blood cells (WBCs) were prepared by centrifuging heparinized blood at 600*g* for 5 min and resuspending the cell pellet in freshly made ammonium chloride solution (0.83% ammonium chloride, 0.1% potassium bicarbonate) for 2–5 min at room temperature. The cells then were washed with RPMI 1640 and suspended in the same medium.

Serum Fractionation

Fresh serum was collected from the clotted blood of four healthy individuals. Aliquots of each serum were heat treated (56°C, 30 min) to deactivate complement. Serum from one individual was fractionated by agar electrophoresis. Eighty milliliters of 1.5% Agar Purum (Behring Diagnostics Australia, Sydney, New South Wales, Australia) in 1.2% barbital buffer (pH 8.6) was poured into an 11 × 14-cm tray (Horizon 11.14; GIBCO BRL), fitted with a 14-tooth comb (1 mm thick), to give a 5-mm-deep gel; 20 μl of serum was added to each well and electrophoresed (H5 Apparatus, GIBCO BRL) for 2 h at 140 V. Horizontal slices were removed from the top and bottom of the gel for staining with Paragon Blue stain (Beckman Instruments, Fullerton, CA) to locate the positions of the serum fractions, and then the rest of the gel was sliced vertically in the appropriate places. The gel slices were eluted with, and the eluates dialyzed against, phosphate-buffered saline (PBS). The fractions were concentrated with an Amicon 8010 cell (Amicon, Beverly, MA) fitted with a YM10 membrane, and the purity of each fraction was assessed by electrophoresis (Beckman Paragon system) and nephelometric assays for albumin, α_1-antitrypsin, ceruloplasmin, transferrin, and immunoglobulin G (IgG) (Beckman Array 360). Fraction 1 was 98% albumin; fraction 2 was 58% albumin, 42% α_1-globulin and α_2-globulin; fraction 3 was 90% α_2-globulin and β-globulin; fraction 4 was 90% β-globulin and γ-globulin.

Cell Labeling

WBCs were labeled with CD3-FITC and CD13-RD1 to identify T cells and myeloid cells (granulocytes and monocytes), respectively. PBMCs were labeled with CD3-FITC, CD19-ECD, and either CD56-RD1 or CD14-RD1 to identify T cells, B cells, and either NK cells (CD3-negative, CD56-positive) or monocytes (CD14-positive). Cells (200–500 μl, 10^6 cells/ml) were incubated with antibodies (at the concentrations recommended by the manufacturer) for 30 min on ice, then washed once with cold RPMI 1640 and resuspended to the initial volume.

Incubation of Cells With Peptides

Antibody-labeled (or, occasionally, -unlabeled) cells were incubated with peptide and 7-AAD (4 μg/ml; Sigma) in the dark. Different incubation times and temperatures were tested, as was the effect of serum. Usually, the cells were incubated in serum-free medium (supplemented RPMI 1640) for 5 min at room temperature, then placed on ice until analysis by flow cytometry (within 30 min). When investigating the effects of incubation time and temperature, each parent tube was maintained at 0°C, room temperature, or 37°C, and samples were removed from it at different times for immediate analysis on the flow cytometer.

Flow Cytometry

Cells were analyzed with the Coulter ELITE, and the air-cooled argon laser was operated at 15 mW. FITC fluorescence was deflected by a 550DL filter and collected through a 525BP filter. RD1 fluorescence was similarly collected via 600DL and 575BP filters, ECD fluorescence via 650DL and 610BP filters, and 7-AAD fluorescence through a 675BP filter. Fluorescence signals were amplified logarithmically, and data were stored as list mode. The viability of cells before and after incubation with peptide was determined by the following data analysis procedure. First, a broad light-scatter gate was used to include the cells of interest, live and dead, but excluding small debris (e.g., Fig. 1, histograms F and J, gate A for lymphocytes). Cell subpopulations within that light-scatter gate could then be studied if the cells had been labeled with antibody. Cells labeled with three antibodies, as in Figure 1, for example, were subjected to three-dimensional (3D) color analysis by the flow cytometer's PRISM electronics (11,16), thereby defining (in this example) T, B, and NK cells. Each subpopulation was then further analyzed separately: 1) the antibody fluorescence criteria defining that subpopulation (live and dead) were used as a gate, and the light scatter of those cells was displayed (Fig. 1, histogram K); 2) two gates were drawn on the latter histogram, one around the cells with the distinctive scatter of healthy cells (Fig. 1, histogram K, gate B) and the other around those cells displaying reduced forward scatter (gate C); 3) the 7-AAD fluorescence of cells satisfying the combined criteria of the antibody fluorescence gate and light-scatter gate B was compared with that of cells expressing the same antibody fluorescence but in light-scatter gate C (Fig. 1, histograms

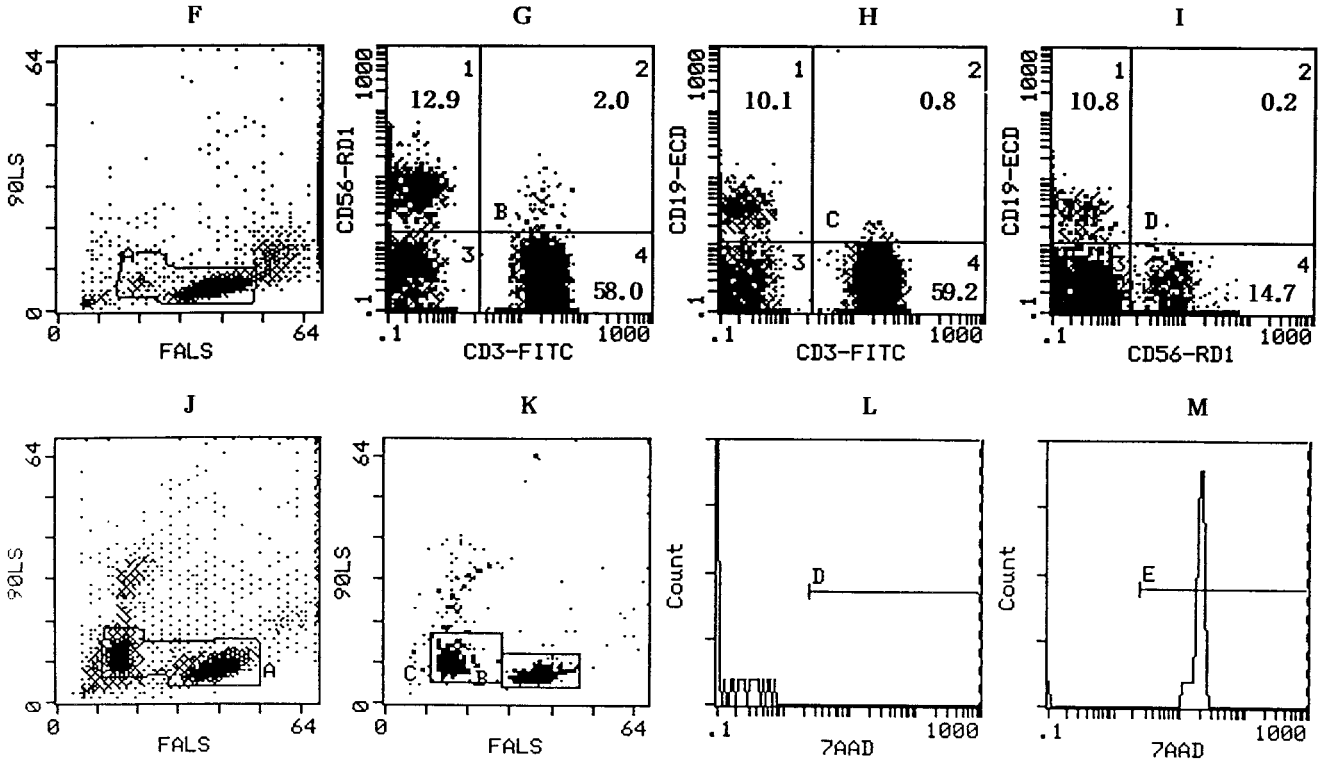

FIG. 1. Killing of peripheral blood mononuclear cells (PBMCs) by pilosulin 1. Cells (from Donor 1) were labeled with CD3-FITC, NKH1-RD1 (CD56), and B4-ECD (CD19), then incubated with 1 μM peptide and 7-aminoactinomycin D (7-AAD) for 5 min at room temperature. Histograms **F–I** and **J–M** are from cells before and after peptide incubation, respectively. Histograms G–I illustrate the antibody fluorescence of the lymphocytes within light-scatter gate A (histogram F; FALS, forward light scatter; 90LS, 90° light scatter). Comparison of histograms J and F clearly shows that the peptide has caused a marked reduction in the forward scatter of approximately one-half the lymphocytes. Three-dimensional color analysis was performed (by PRISM) on the antibody fluorescence of the lymphocytes with normal and reduced light scatter (i.e., the cells in histograms F and J, gate A), thus defining the T, B, and NK cell subpopulations. The light scatter of cells after peptide incubation—which satisfied the criteria CD19-positive, CD3-negative, CD56-negative (i.e., B cells)—is shown in histogram K. Histogram K, gates B and C, define B cells with normal and abnormal light scatter, respectively. Histogram L shows the 7-AAD fluorescence of cells that satisfied the combined criteria of the B cell antibody fluorescence gate and light-scatter gate B. Similarly, histogram M shows the 7-AAD fluorescence of the B cells with reduced light scatter (gate C). Thus, histograms L and M demonstrate that B cells with normal light scatter were 7-AAD-negative (i.e., viable) and that those with reduced light scatter were 7-AAD-positive (i.e., dead).

L and M, respectively). Thus, live and dead cells within each subpopulation were distinguished by both 7-AAD fluorescence and light scatter: Live cells were defined as those that were 7-AAD-negative and inside gate B; dead cells were defined as those that were 7-AAD-positive and in either gate B or gate C.

RESULTS

Analysis of Killing of Subpopulations Within a Heterogeneous Cell Population

Figure 1 demonstrates the result of incubating PBMCs with 1 μM pilosulin 1 for 5 min at room temperature in serum-free medium. First, comparing the light scatter of the cells before (Fig. 1, histogram F) and after (Fig. 1, histogram J) incubation, it is clear that pilosulin 1 caused a marked decrease in the forward light scatter and a slight increase in the 90° light scatter of approximately one-half the lymphocytes and more than one-half the monocytes. (The monocytes in Fig. 1, histogram F, are near the top right corner of light-scatter gate A; in Fig. 1, histogram J, many of them appear above the left of gate A.) Second, because the lymphocytes in this experiment were labeled with antibodies, each subpopulation could be identified by the fluorescence of the cell-bound antibodies and then separately assessed for its viability, before and after incubation with peptide, as described in Materials and Methods. Thus, for example, histograms K, L, and M illustrate the light scatter and 7-AAD fluorescence of the B cells (CD19-positive, CD3-negative, CD56-negative) after pilosulin 1 and show that 55% of the B cells had abnormally low forward scatter and were 7-AAD-positive; that is, they had been killed by the peptide.

Figure 2 illustrates the killing of mononuclear cells and granulocytes over a range of pilosulin 1 concentrations. This result is typical of experiments with PBMCs and WBCs from six individuals. Cells from different individuals varied in their lability to the peptide (e.g., the peptide concentration required to kill 50% of the T cells varied as much as fivefold), but for each individual, the granulocytes were more resistant to the cytotoxic effect of the pilosulin 1 than the mononuclear cells. The most dramatic peptide-induced change in the light scatter of the granulocytes was

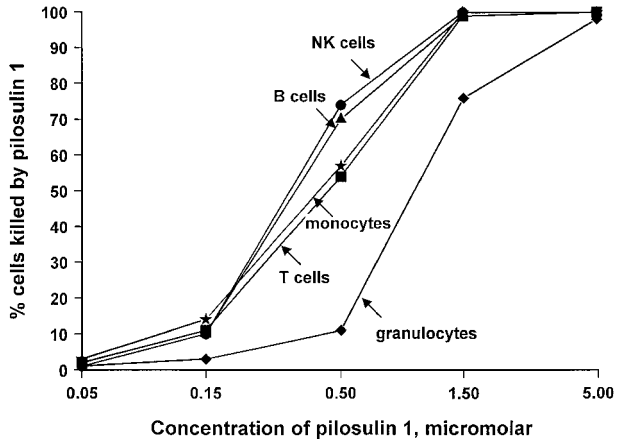

FIG. 2. Dose–response curves for pilosulin 1 killing of mononuclear cells and granulocytes. Antibody-labeled PBMCs and white blood cells (WBCs) from the same individual (Donor 2) were incubated with 7-AAD and different concentrations of pilosulin 1 for 5 min at room temperature. Three tubes were prepared for each concentration of peptide: 1) PBMCs labeled for T, B, and NK cells; 2) PBMCs labeled for T cells, B cells, and monocytes; and 3) WBCs labeled for T cells and myeloid cells (granulocytes and monocytes). Killing of the different cell subpopulations was determined according to the procedure described in Materials and Methods and in Figure 1. Granulocytes were defined as CD13-positive with higher 90° light scatter than monocytes.

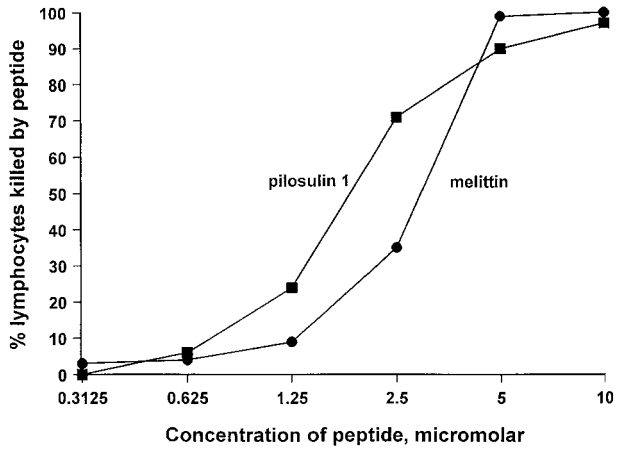

FIG. 3. Dose–response curves for lymphocyte killing by pilosulin 1 and melittin. PBMCs (from Donor 3) were incubated with 7-AAD and various concentrations of either pilosulin 1 or melittin for 5 min at room temperature. The PBMCs were not labeled with antibody, so the viability of the entire lymphocyte population was analyzed (by light scatter and 7-AAD uptake).

the decrease in forward scatter, as was the case with mononuclear cells.

Cytotoxicity of Pilosulin 1 Versus Melittin

Figure 3 shows the result of an experiment with unlabeled PBMCs incubated for 5 min at room temperature with different concentrations of either pilosulin 1 or melittin. It is evident that pilosulin 1 was a slightly more potent cytotoxic agent for the lymphocytes than melittin. Experiments with WBCs from other individuals confirmed this. They also demonstrated that melittin caused similar changes in cellular light scatter to pilosulin 1, and was more potent against mononuclear cells than granulocytes (data not shown).

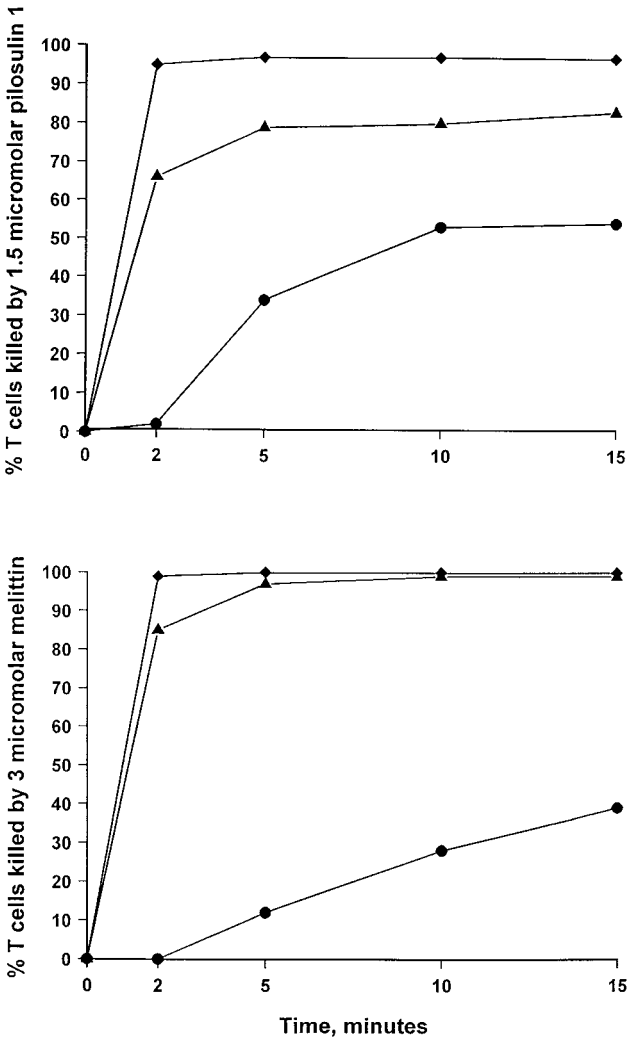

FIG. 4. Kinetics of pilosulin 1 (**top**) and melittin (**bottom**) killing at different temperatures. Antibody-labeled WBCs (from Donor 4) were incubated with 7-AAD and either 1.5 μM pilosulin 1 or 3 μM melittin. Three tubes were prepared for each peptide and incubated at 0°C (●), room temperature (▲), or 37°C (◆). Samples were removed from each tube at time 0 and at 2, 5, 10, and 15 min for immediate analysis on the flow cytometer.

Kinetics of Killing by Pilosulin 1 and Melittin at Different Temperatures

Figure 4 shows the results of an experiment with labeled WBCs incubated with either 1.5 μM pilosulin 1 or 3 μM melittin, for 15 min at various temperatures. It is derived from T cell data and illustrates that 1) the two peptides killed with similar kinetics; 2) the maximum cell kill was achieved by 5 min at room temperature and by 2 min at 37°C; and 3) increasing the temperature increased the maximum cell kill. The granulocyte data were similar, although the cell kill was lower.

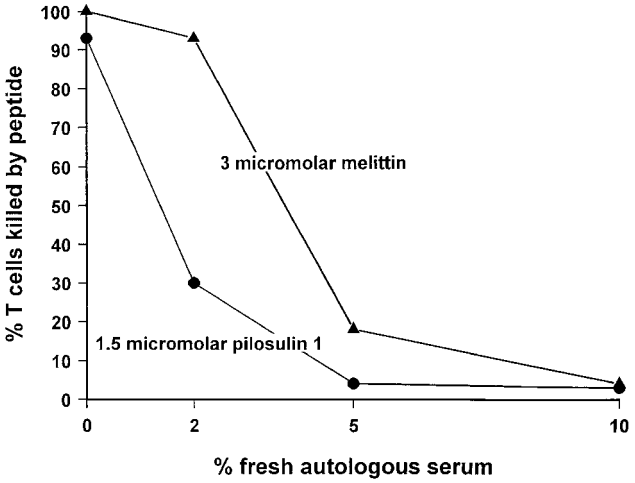

FIG. 5. Serum inhibition of T cell killing by pilosulin 1 or melittin. Antibody-labeled PBMCs (from Donor 4) were incubated with 7-AAD and either 1.5 μM pilosulin 1 or 3 μM melittin for 10 min at 37°C with different concentrations of fresh autologous serum.

Serum Inhibition of Peptide Cytotoxicity

Figure 5 is from an experiment where PBMCs labeled for T cells were incubated with pilosulin 1 or melittin for 10 min at 37°C in the presence of several concentrations of fresh autologous serum. Serum clearly inhibited killing by either peptide. This result is typical of experiments with cells and either fresh or heat-treated autologous sera from four individuals. Fetal calf serum was also inhibitory. An experiment with autologous serum fractions was then performed. First, PBMCs were incubated with pilosulin 1, without serum, to determine the concentration of peptide that killed 80% of the lymphocytes; subsequent incubations showed that serum fractions 1, 2, 3, and 4 (see Materials and Methods, Serum Fractionation) at a concentration of 200 μg protein/ml inhibited 23, 62, 63, and 45%, respectively, of the killing. There was a significant correlation (Spearman rank correlation, $P < 0.001$) between the concentration of α_1-globulin and α_2 -globulin and the inhibition of killing.

DISCUSSION

The results described previously (24) prove that pilosulin 1 is one of many polypeptides isolated from a variety of living organisms that have affinity for biological membranes and are cytotoxic. These results show that pilosulin 1 killed nonproliferating human leukocytes with kinetics similar to those of melittin and, indeed, was slightly more potent than melittin. Our flow cytometric technique allowed us to monitor the killing of different cell subpopulations simultaneously and revealed that both peptides were more potent against mononuclear leukocytes than against granulocytes. Peptide cytotoxicity was inhibited by serum proteins, in particular (with respect to concentration expressed as weight/volume) by the α-globulins.

To the best of our knowledge, our flow cytometric technique is novel because it uses 7-AAD to monitor cell death in conjunction with three-color immunophenotyping, using a single laser. A previous report used 7-AAD to assess cell viability in a four-color assay, but it was a dual-laser technique (18).

The change in the light scatter of cells killed by pilosulin 1 or melittin that we observed is different from that reported by Weston et al. (23). They found a large increase in 90° scatter, but they used different cells (Hmy2 lymphoblastoid) and, probably more importantly, higher concentrations of melittin (up to 266 μM). It is quite conceivable that these peptides might have a different molecular form in solution (1) and a more drastic effect on cell membranes (4) at higher concentrations than at lower concentrations. That would explain why Weston et al. (23) found that with 35 μM melittin (100 μg/ml), cell kill reached its maximum within 20 s. With the concentrations of pilosulin 1 and melittin that we used (<10 μM) the biggest change in the peptide-induced cellular light scatter was a striking decrease in forward scatter, which is indicative of a loss of cell membrane integrity and a consequent decrease in refractive index (20).

The ability of amphiphilic polypeptides to penetrate and disrupt the lipid bilayer of a cell membrane depends on the ratio of hydrophobic to hydrophilic residues in the polypeptide chain, the relative positions of those residues, and the number and distribution of charged groups (2,9). It is therefore of interest that the basic amino acid residues lysine (K) and arginine (R) are found at regular intervals along the polypeptide chain of pilosulin 1, amidst a larger number of hydrophobic residues (A, F, I, L, M, P, V), and that the molecule is potentially α-helical (24). The different susceptibilities of PBMCs and granulocytes to lysis are probably due to differences in membrane lipid structure (13).

The inhibition of pilosulin 1 and melittin cytotoxicity by serum proteins is interesting. Relative to protein concentration, the α-globulins were the most inhibitory proteins in serum, although albumin contributed substantially to the inhibition because of its higher concentration. Electrostatic charge may play a role in the inhibition, because the peptides are basic, whereas albumin and the α-globulins are acidic. However, electrostatic charge does not explain why the α-globulins were more inhibitory than albumin. Another possible explanation is that the peptides bind to the phospholipids of the lipoproteins that migrate on electrophoresis (at pH 8.6) in the α-globulin region. The question that arises is, What is the biological relevance of the serum protein inhibition? It has been calculated that the concentration of melittin in the worker bee venom sac is at least 20 mM (22). Possibly, the spread of cell damage caused by the release of such highly concentrated melittin directly into the skin from a bee sting may be limited by the neutralizing effect of proteins from the lymph and from the blood. However, even bound to protein or lipoprotein, peptides such as melittin and pilosulin 1 may still be capable of triggering the release of granules from mast cells.

The activity of pilosulin 1 is not merely of academic interest. Such molecules are small and highly cytotoxic, and they do not have to be internalized by a cell to kill it.

This activity has recently led to a novel and potentially clinically important use of melittin as part of a recombinant immunotoxin (8). The immunotoxin retained the specificity of the original antibody and its cytotoxic activity, when tested against the specific cellular target in vitro, was significantly greater on a molar basis than that of native melittin, although the kinetics of killing were very different.

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