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Interaction of Melittin With a Human Lymphoblastoid Cell Line, HMy2

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Abstract We have examined the cytolytic effects of the membrane-active peptide, melittin, on a human lymphoblastoid cell line (HMy2) in the context of the use of melittin as the toxic component of an immunotoxin. The toxicity of melittin for HMy2 cells was linear over the concentration range 0.875–3.5 μM. Increased incubation times failed to result in significant cell death at concentrations of melittin below 0.875 μM. Kinetic analysis revealed that the cytolytic activity of melittin was independent of time of exposure beyond 90 min. Flow cytometric analysis of HMy2 cells incubated with FITC-labeled melittin demonstrated that the cells could incorporate up to 2.5×10^5 FITC-melittin molecules per cell with no reduction in viability. Extrapolation of this data indicates that 10^6 melittin molecules per cell are required for maximum cytotoxicity to HMy2 cells. Further analysis of HMy2 cells that incorporated melittin, but that remained viable, revealed that these cells were able to reduce the number of melittin molecules per cell over time. The data indicate a potential threshold value for the number of melittin molecules that may be required to be delivered to the cell surface in the form of an immunotoxin if effective selective cell death is to be achieved. J. Cell. Biochem. 68:164–173, 1998.

Key words: melittin; flow cytometry; cytotoxicity; immunotoxin; HMy2

Melittin is a 26-amino acid peptide that constitutes the major protein component of the venom of the European honey bee, *Apis mellifera* [Habermann, 1972]. The structure of melittin is that of a bent α-helical rod with the C-terminal 6 amino acids forming a charged tail, which is thought to target the molecule to the cell membrane [Terwilliger and Eisenberg, 1982; Vogel and Jahnig, 1986]. Insertion of melittin into the cell membrane and formation of tetrameric or higher oligomers results in the disruption of the membrane and lysis of the cell [Sessa et al., 1969; Vogel and Jahnig, 1986; Rex, 1996]. The direct cytolytic action of melittin is demonstrated by the release of hemoglobin from erythrocytes and histamine from mast cells [Habermann, 1972]. While the action of melittin has been thought to involve the formation of membrane pores [Vogel and Jahnig, 1986; Laine et al., 1988; Tosteson and Tosteson, 1991], it has been recently suggested that the effect of melittin on lipid membranes is a more general one of membrane perturbation rather than pore formation [Benachir and Lafleur, 1995; Pawlak et al., 1994]. We have previously shown that human lymphoblastoid cells (HMy2) incubated with melittin (7-260 μM) form membrane vesicles and that binding and cytotoxicity at 35 μM melittin is very rapid with 100% death of HMy2 cells occurring within 2 min [Weston et al., 1994]. A rapid permeabilisation of human promyelocytic cells (HL60) was also demonstrated by Midoux et al. [1995] who showed that less than 5-min incubation of melittin with HL60 cells resulted in cell lysis.

The cytotoxicity of melittin to erythrocytes is well documented. De Grado et al. [1982] showed that the binding of melittin to erythrocytes was rapid but that the subsequent release of hemoglobin followed a biphasic pattern: a rapid early phase of a few minutes duration followed by a slower phase lasting several hours. Tosteson et al. [1985] also demonstrated a rapid binding of melittin and lysis of red cells such that after 1-h exposure lysis was complete. They also reported that a minimum of 2 x 10^5 melittin molecules per cell were required to induce detectable lysis of erythrocytes and 2×10^6 melit-

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tin molecules per erythrocyte were required for 50% cytotoxicity.

Because of its direct effect on the cell membrane, melittin has been proposed as a possible toxic component of a novel class of immunotoxins that act at the cell surface rather than requiring internalisation to be effective. We have previously shown that a recombinant immunotoxin utilising melittin as the toxic component specifically bound to and killed target cells [Dunn et al., 1996]. In this case each immunotoxin molecule incorporated a single melittin molecule. In order to further assess its potential as the toxic component of an immunotoxin we investigated the action of melittin and the minimum requirements for cytotoxicity to a mammalian lymphoblastoid cell line (HMy2).

In this study, melittin (up to 7 μM) was shown to cause lysis of 90% of HMy2 cells within 30-min incubation. Maximum lysis occurred after 90-min incubation. FITC-labelled melittin was used to show that an estimated 10^6 melittin molecules per cell were required for death of 100% of HMy2 cells. Moreover, cells that incorporated melittin, but that remained viable, were able to reduce the number of melittin molecules per cell over time.

METHODS

Melittin and FITC-Melittin

Melittin and FITC-melittin (synthesised with one molecule of fluorescein isothiocyanate at the N-terminus of each melittin molecule) were obtained from Auspep, Victoria, Australia. Melittin was dissolved in dH_2O and diluted in RPMI to the desired concentration. FITC-melittin was dissolved in a minimum volume of dimethyl sulfoxide and reconstituted in RPMI to the desired concentration.

Cells

The human lymphoblastoid cell line (LICRLON-HMy2) was maintained in in vitro culture in RPMI supplemented with 10% fetal bovine serum (FBS), HEPES (4.76 g/L), sodium bicarbonate (8.5 mg/L), L-glutamine (2 μM), and penicillin (100 IU/ml) at 37°C in an atmosphere of 5% CO_2.

Flow Cytometric Assessment of Cytotoxicity and Incorporation of FITC-Melittin

HMy2 cells (10^5) were cultured with melittin at various concentrations at 37°C for 30, 90, 180 min, and 25h. At each time point viability was assessed by addition of ethidium bromide (10 μg/ml final concentration) to the culture. The cells were then immediately analysed by flow cytometry using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Cells fluorescing due to incorporated ethidium bromide were considered nonviable. In order to verify that the incorporation of ethidium bromide equated to cell death, HMy2 cells were incubated with melittin for 90 min. Triplicate aliquots of 10^5 cells were then added to ethidium bromide (10 μg/ml final concentration) and analysed by flow cytometry. Quadruplicate aliquots (10^5)were cultured in vitro for 48 h at 37°C after the melittin treatment. Tritiated (^{3}H-) thymidine was added for the final 24 h of culture. The cells were then harvested using a PHD cell harvester and the uptake of ^{3}H-thymidine determined as a measure of cell proliferation.

In the experiments in which the incorporation of FITC-melittin was determined, HMy2 cells were incubated with FITC-melittin at various concentrations at 37°C for 90 min. In one set of experiments, HMy2 cells were incubated with FITC-melittin for 90 min, 6, 24, and 48 h. Incorporation of FITC-melittin was determined by flow cytometry using a FACScan flow cytometer. Simultaneous analysis of cell viability by ethidium bromide incorporation as described above was also undertaken. Each experiment was accompanied by analysis of reference fluorescent microbeads at the same flow cytometer instrument settings (see below). All analyses were performed using the CellQuest program (Becton Dickinson). The values for mean fluorescence intensity (MFI) of cells incorporating FITC-melittin were obtained for each population of viable cells. The range of fluorescence intensities for all samples was similar with the lower 10% of cells having a MFI of 46.2% of the average and the upper 10% of cells having a MFI of 207% of the average. The values for MFI for each population of cells incorporating FITC-melittin were converted to Molecules of Equivalent Soluble Fluorochrome (MESF) after reference to fluorescent microbead standards.

Fluorescent Microbeads

Fluorescent microbeads (Quantum Fluorescence Kit 26p) were obtained from Australian Laboratory Services, Rockdale, NSW. The beads are a series of reference microbead standards whose intensities have been calibrated in Mol-

ecules of Equivalent Soluble Fluorochrome (MESF units). There were five discrete populations of beads per test. The average MESF values were 0, 7378, 34798, 105364, and 307461 MESF per bead. Analysis of each population of beads based on MESF fluorescence yielded corresponding values for mean fluorescence intensity (MFI) and a standard curve relating MESF to MFI could be constructed. From this curve the MESF of a population of cells incorporating FITC-melittin could be determined from the MFI of the same cells according to the formula:

$$\text{MESF} = (5815 \times \text{MFI}) - 5966 \quad (R^2 = 1.000).$$

The highest MESF value analysed was 3.07×10^5 per bead, which equated to a MFI of approximately 54 arbitrary units. In some experiments the MFI of cells treated with FITC-melittin exceeded this number. In order to verify the assumption of linearity of the above relationship for MESF values greater than 3×10^5, analysis of fluorescent microbeads with a range of MESF values of $0–2 \times 10^6$ was undertaken using the same instrument settings as those yielding the relationship above. The standard curves for both sets of microbeads were almost identical, thus justifying the extrapolation (data not shown).

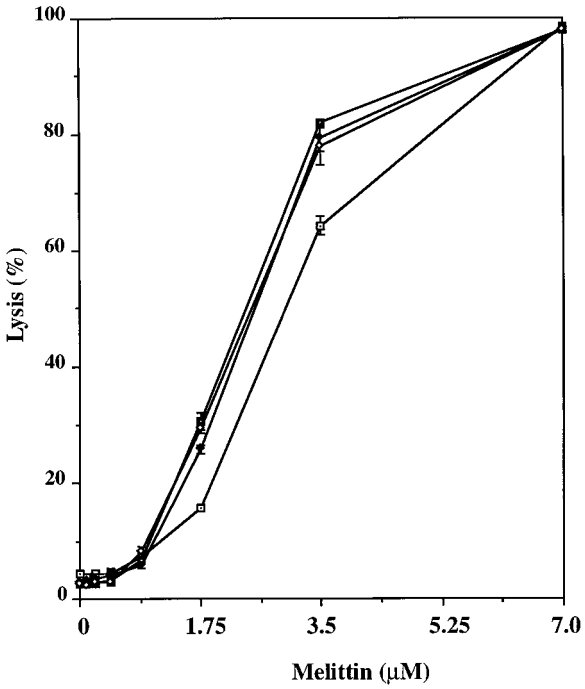

Fig. 1. Time course of melittin cytotoxicity. HMy2 cells (10^5) were incubated with melittin (0–7 μM) for 30 min, 90 min, 3 h, and 25 h at 37°C. At the time points specified, the cells were analysed for viability by a flow cytometric assay based on the exclusion of ethidium bromide by viable cells. —⊡— 30 min; , —◆— 90 min; , —◘— 3 h; , —◇— 25 h.

RESULTS

Time Course of Melittin Cytotoxicity

HMy2 cells were incubated with melittin (0–7 μM) and cell viability was assessed after various times over a 25-h period (Fig. 1). The majority of cell death occurring at each concentration was completed within 30 min. Incubation times of 90 min or longer resulted in similar plots of cytotoxicity versus melittin concentration. In order to verify that uptake of ethidium bromide correlated to cell death, cells were simultaneously assessed for uptake of ethidium bromide and ^{3}H-thymidine incorporation (Fig. 2). The plots of uptake of ethidium bromide and uptake of ^{3}H-thymidine versus melittin concentration were similar and justified the method of ethidium bromide uptake as an indicator of cell death.

Incorporation of FITC-Melittin Into HMy2 Cells

A standard curve relating MFI to the number of FITC molecules (MESF) was generated using fluorescent-labeled microbead standards. The MFI of cells incorporating FITC-melittin was then related to the number of FITC molecules present, as indicated under Methods. Since each FITC-melittin molecule incorporated a single FITC moiety the value for MESF equated to the number of melittin molecules associated with the cell. HMy2 cells incubated with FITC-melittin were analysed by flow cytometry. On the basis of light-scatter profiles, it was possible to distinguish viable from nonviable cells (R2 and R1, respectively, in Fig. 3) and to determine the incorporation of FITC-melittin into each of the cell populations. Analysis of cells incubated with 3.5 μM FITC-melittin revealed 40% of the population in R1 and 50% in R2 (Fig. 3A) on the basis of light-scatter properties. The cells exhibiting reduced forward light-scatter characteristics (R1) were deemed nonviable on the basis of their failure to exclude ethidium bromide (solid histogram, Fig. 3B). Cells in R2 were those that remained viable despite the FITC-melittin treatment as indicated by their exclusion of ethidium bromide (solid histogram, Fig. 3C). The nonviable cells (R1) incorporated significantly more FITC-melittin (Fig. 3D) than did the cells that remained viable (R2; Fig. 3E).

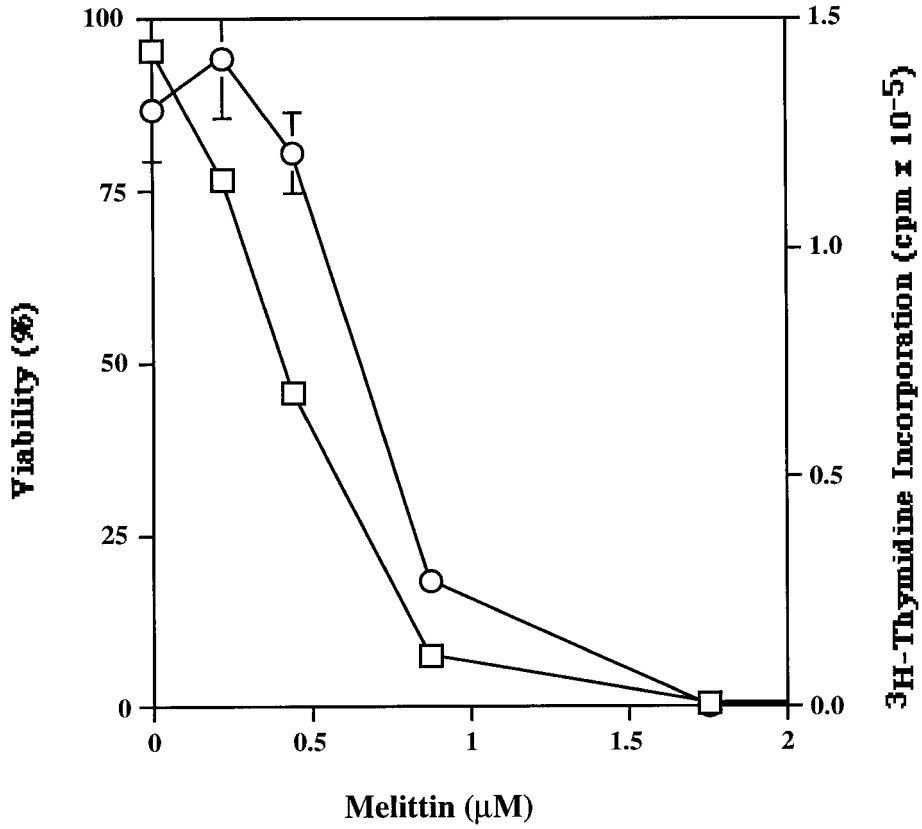

Fig. 2. Comparison of two indicators of cell viability: ethidium bromide uptake and ^{3}H-thymidine incorporation. HMy2 cells were incubated with melittin for 90 min. Triplicate aliquots of 10^5 cells were then added to ethidium bromide (10 μg/ml final concentration) and analysed by flow cytometry. The results are expressed as percentage cells incorporating ethidium bromide ±1 SD (—□—). Quadruplicate aliquots (10^5) of melittin-treated cells were cultured in vitro in 96-well plates for 48 h at 37°C. Tritiated (^{3}H-) thymidine was added for the final 24 h of culture. These cells were then harvested using a PHD cell harvester and the uptake of ^{3}H-thymidine determined as a measure of cell proliferation (—○—). The results are expressed as counts per minute (mean ±1 SD).

The extent to which nonviable cells incorporated FITC-melittin was determined by the concentration of FITC-melittin in the culture (Fig. 4). As the concentration increased there was a concomitant increase in uptake of FITC-melittin. In contrast, cells that remained viable exhibited a much lower level of incorporation of FITC-melittin, although this incorporation was also concentration dependent.

The preceding experiment suggested that nonviable cells continued to take up FITC-melittin beyond the threshold level at which death occurred. Thus, it was impossible to analyse these cells to determine the number of FITC-melittin molecules required for cell death. Hence, viable cells were analysed in order to assess the number of FITC-melittin molecules that could be incorporated with the cells remaining viable. An estimate was then made of the minimum requirement for cell death. Analysis of the incorporation of FITC-melittin into viable cells showed that up to 2.5 x 10^5 melittin molecules could be inserted into the cells with viability remaining essentially unchanged (Fig. 5). However, with increasing concentrations of FITC-melittin and the concomitant increase in the number of FITC-melittin molecules per cell there was a decrease in viability to the point where few viable cells could be detected. Extrapolation of the data of MESF suggests that 100% cell death would occur with approximately 10^6 FITC-melittin molecules per cell. We note that 7μM FITC-melittin caused approximately 70% cell death (Fig. 5), while the same concentration of melittin was cytotoxic to 100% HMy2 cells (Fig. 1). The solution of FITC-melittin contained a small amount of insoluble material and while the concentration shown is

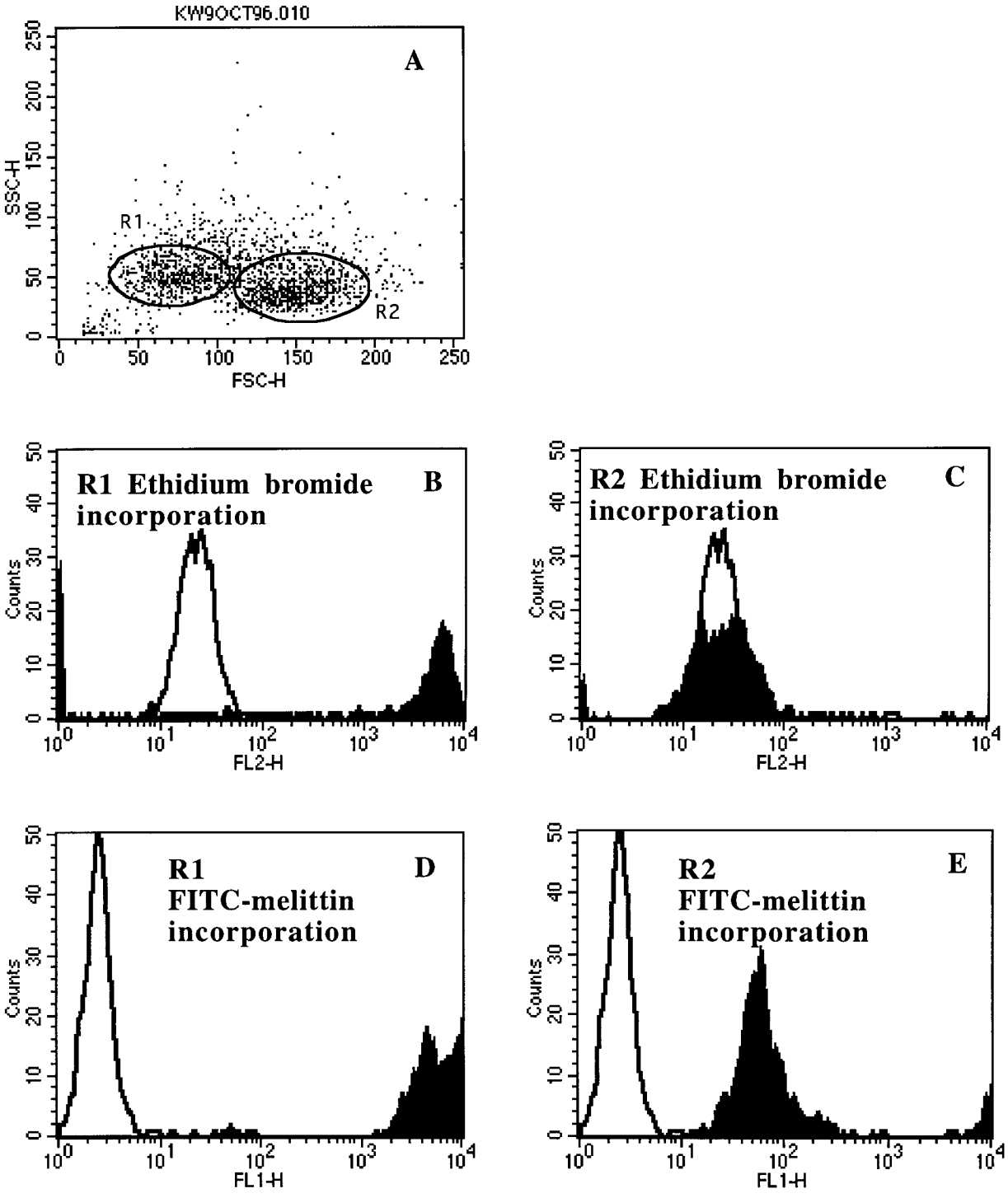

Fig. 3. Ethidium bromide and FITC-melittin incorporation into viable and nonviable cells. HMy2 cells (10^5) were incubated with FITC-melittin (3.5 µM) for 90 min at 37°C. Viability was determined based on the exclusion of ethidium bromide (B,C) and FITC-melittin incorporation assessed (D,E). **A:** Light-scatter profile of HMy2 cells incubated with FITC-melittin (3.5 µM); y-axis: 90° light scatter; x-axis: forward light scatter. **B:** Incorporation of ethidium bromide into cells in R1 (solid histogram). **C:** Incorporation of ethidium bromide into cells in R2 (solid histogram). **D:** Incorporation of FITC-melittin into cells in R1 (solid histogram). **E:** Incorporation of FITC-melittin into cells in R2 (solid histogram). Each outline histogram represents the fluorescence of cells incubated in the absence of FITC-melittin.

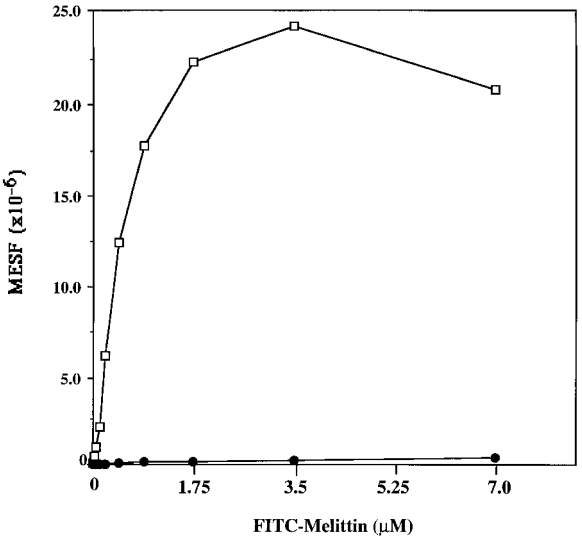

Fig. 4. Uptake of FITC-melittin by viable and nonviable cells. HMy2 cells (10^5) were incubated with FITC-melittin (0–7 μM) for 90 min at 37°C and analysed by flow cytometry. Based on their light-scatter profiles, viable and nonviable cell populations were analysed separately for incorporation of FITC-melittin. —●—, viable cells; —□—, nonviable cells.

based on weight per volume of FITC-melittin, the effective concentration is likely to be slightly lower. This would account for the slight discrepancy in cytotoxicity of FITC-melittin compared to melittin.

Fate of FITC-Melittin Incorporated into Viable Cells

HMy2 cells were incubated with FITC-melittin (0–7 μM) for 90 min, 6, 24, and 48 h. At these time points, cells were removed and assessed for incorporation of FITC-melittin and viability. In one set of experiments (Fig. 6B), 75% of the culture supernatant was removed after 6 h and replaced with culture medium lacking FITC-melittin. Viable cells were distinguished on the basis of their light-scatter characteristics (as in Fig. 3) and the results presented in Figure 6 show the incorporation of FITC-melittin into viable cells only. There was a concentration-dependent increase in the number of FITC-melittin molecules per cell up to 24-h incubation (Fig. 6A). Thereafter, the fluorescence was reduced so that by 48 h, the number of FITC-melittin molecules per cell was similar to the 6-h value. This result was similar over a range of FITC-melittin concentrations. The effect of reducing the melittin concentration in the culture was investigated (Fig. 6B). With removal of 75% of the unbound FITC-melittin from the culture after 6 h, there was no further increase in uptake of FITC-melittin and a more rapid reduction in the number of FITC-melittin molecules in the cells than occurred in cultures in which FITC-melittin was not removed (Fig. 6A).

DISCUSSION

It has been previously demonstrated that melittin causes lysis of human erythrocytes by perturbation of the cell membrane and subsequent leakage of cellular contents [Habermann, 1972; Tosteson et al., 1985]. We have also shown that melittin is able to promote the formation of vesicles in the membranes of a human lymphoblastoid cell line (HMy2) [Weston et al., 1994]. In the present study we have investigated the cytotoxicity of melittin and the fate of melittin incorporated into HMy2 cells that are not killed.

After 30 min, the melittin-induced death of HMy2 cells was approximately 90% complete at a concentration of 3.5 μM. Comparison of cells incubated with melittin for 90 min and longer showed no difference in plots of cytotoxicity versus melittin concentration. Moreover, increased incubation times failed to result in significant cell death. It has been proposed that melittin requires the formation of oligomers in order for lysis to occur [Vogel and Jahnig, 1986; Tosteson and Tosteson, 1991; Rex, 1996]. It may be expected that low concentrations of melittin may require a longer incubation period for lysis to occur as a result of melittin molecules in the membrane taking longer to randomly interact and associate. The results presented here suggest that this is not the case, indicating that the multimerisation of melittin required for lysis occurs very rapidly and that long-term (>90 min) movement and association of melittin within the cell membrane has no influence on subsequent lysis of the cells.

FITC-melittin was used to determine the number of melittin molecules required for cell lysis. When the population of viable cells was analysed it was revealed that HMy2 cells could incorporate up to 2.5×10^5 FITC-melittin molecules per cell and remain viable (Fig. 5). Lysis of 50% of the cells occurred when approximately 7×10^5 FITC-melittin molecules per cell were incorporated. Extrapolation of the data in Figure 5 indicates that 100% cell death would

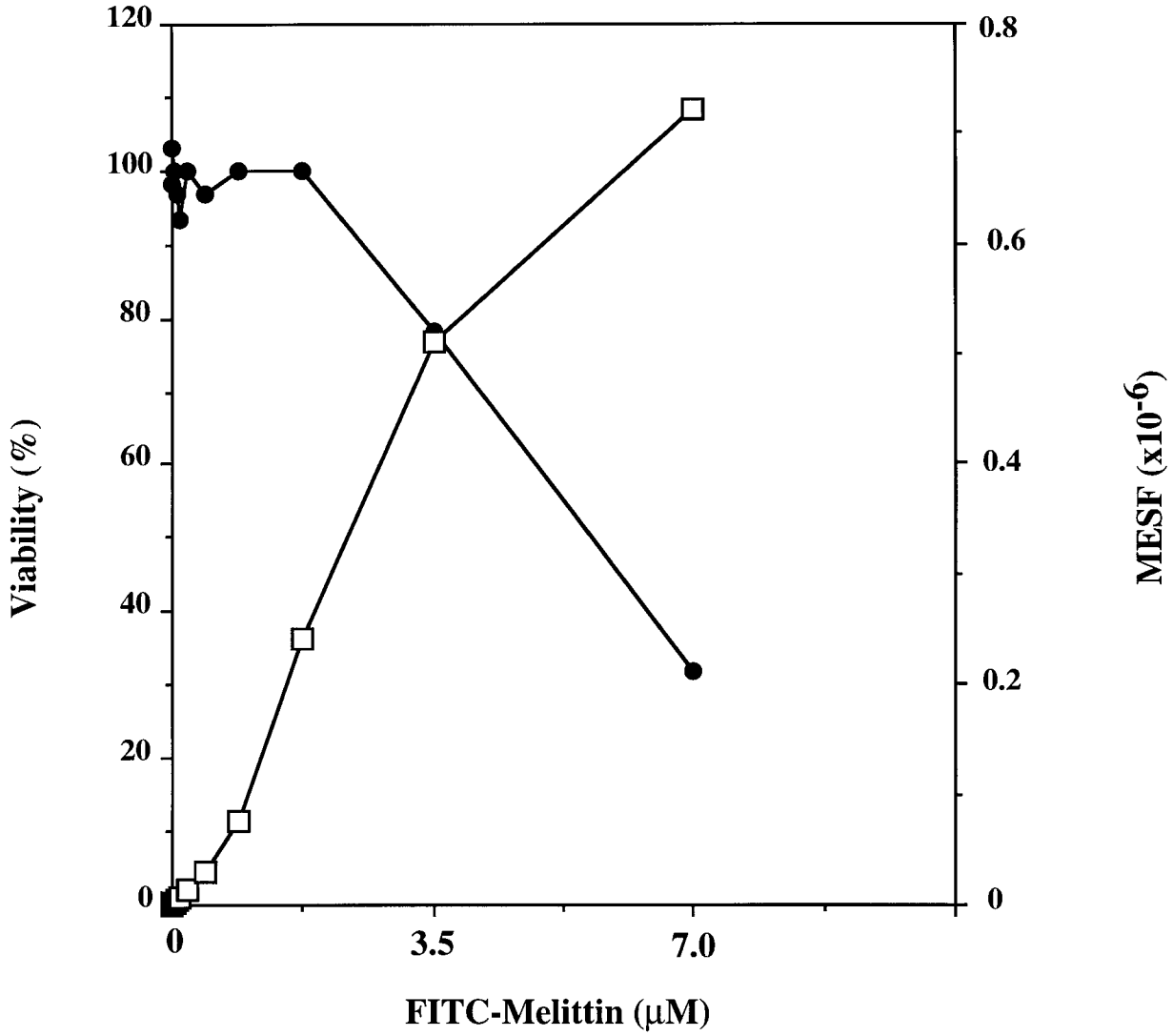

Fig. 5. Relationship between FITC-melittin incorporation and cytotoxicity. HMy2 cells (10^5) were incubated with FITC-melittin (0–7 μM) for 90 min at 37°C and analysed by flow cytometry. Based on their light-scatter profiles, populations of viable cells were analysed for incorporation of FITC-melittin. —●—, % viable; —□—, MESF.

result when approximately 10^6 FITC-melittin molecules were incorporated per cell. This result is similar to that obtained from binding data for erythrocyte ghosts [Tosteson et al., 1985]. It might be expected that higher numbers of melittin molecules are required for lysis of HMy2 cells compared to erythrocytes since the HMy2 cells have a larger surface area. However the value determined for erythrocytes may not have taken into account the continued uptake of melittin by dead cells. Alternatively, the result may be influenced by the lipid composition of the two cell types [Batenberg et al., 1988; Ohki et al., 1994].

Finally, the fate of melittin incorporated into viable cells was addressed. After 6- and 24-h incubation, the uptake of melittin increased (Fig. 6A) despite the finding (Fig. 1) that only 90-min incubation was required for cell death. Moreover, the number of FITC-melittin molecules incorporated after 24 h exceeded 10^6 per cell and yet viability was unaffected. This apparent paradox can be explained if FITC-melittin initially incorporated into the cell membrane was internalised. Following internalisation, more FITC-melittin could then be incorporated into the membrane. The combined effect of both intracellular and membrane FITC-melittin

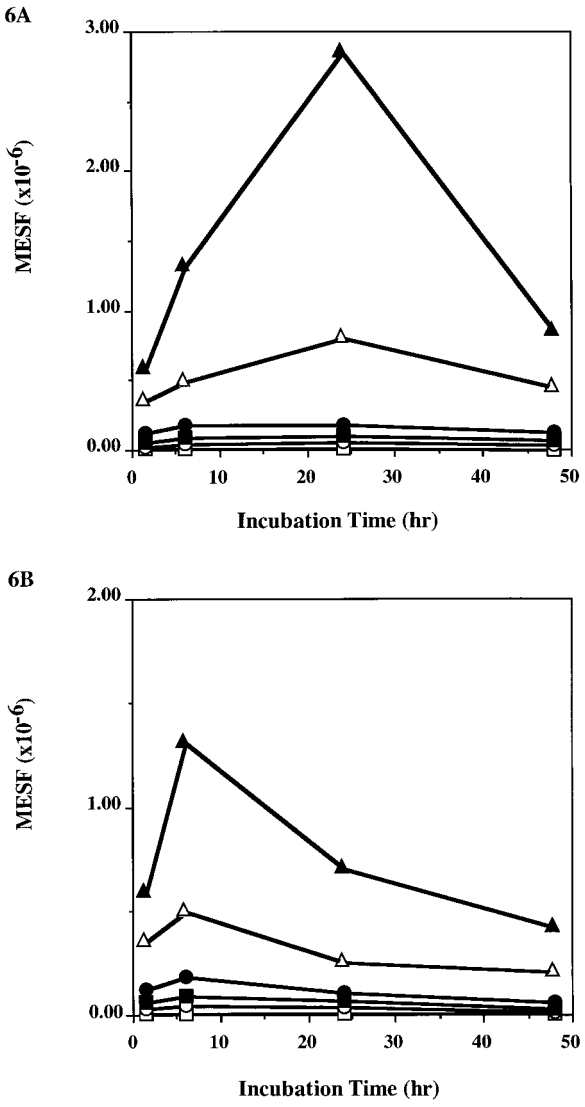

Fig. 6. Fate of FITC-melittin incorporated into viable cells. HMy2 cells (10^5) were incubated with FITC-melittin (0–7 μM) for 90 min, 6 h, 24 h, and 48 h at 37°C. Incorporation of FITC-melittin into viable cells was assessed by flow cytometry. **A:** FITC-melittin concentration was kept constant during the course of the experiment. **B:** 75% of the culture supernatant was removed at 6 h and replaced with culture medium lacking FITC-melittin. —□—, 0 μM; —○—, 0.4 μM; —■—, 0.9 μM; —●—, 1.8 μM; —△—, 3.5 μM; —▲—, 7 μM FITC-melittin.

would result in an increase in MESF. By 48 h incubation with FITC-melittin (Fig. 6A) the MESF decreased indicating that HMy2 cells that had incorporated melittin and remained viable were able to reduce the number of melittin molecules inside the cell. Whether the FITC-melittin molecules were degraded or exocytosed could not be determined. However, it would be important to determine the fate of internalised melittin to assess the effect of melittin-containing immunotoxins taken up nonspecifically by bystander cells.

The concentration dependence of incorporation of FITC-melittin into viable cells was demonstrated by the removal of 75% of the unbound melittin from the culture after 6 h, followed by incubation of the cells for a further 48 h (Fig. 6B). The total FITC-melittin incorporated into the cells (Fig. 6B) was lower than that for cells where the initial concentration of FITC-melittin was maintained (Fig. 6A). HMy2 cells that had initially bound and internalised FITC-melittin bound fewer additional molecules in the cultures depleted of FITC-melittin (Fig. 6B) than in cultures with the original concentration of FITC-melittin (Fig. 6A). The loss of FITC-melittin from viable cells was again demonstrated by a reduction in the number of FITC-melittin molecules per cell over time.

The ability of HMy2 cells to take up melittin continuously and yet remain viable suggests different mechanisms are involved for uptake of melittin and uptake of ethidium bromide. Ethidium bromide does not readily cross functionally intact extracellular cell membranes. However, once the cell has been permeabilised or nuclei isolated, the molecule is quickly incorporated [Watson, 1991]. In addition, ethidium bromide interacts with both anion and cation transporters [Tekle et al., 1994]. Thus, when cells are viable, they are able to actively export any ethidium bromide that may have entered the cells. In contrast, melittin has been demonstrated to bind rapidly to membranes and induce rapid changes to the lipid membrane. Aggregation of sufficient numbers of melittin molecules perturbs the cell membrane. Formation of a channel in this way may allow melittin access to the interior of the cell, where it may be degraded or stored in vesicles for later export. Alternatively, endocytosis of affected membrane regions may result in internalisation of melittin in lipid vesicles, thus allowing continued binding of extracellular melittin. If the interaction of melittin with the cell membrane is not too extensive, it is possible that the cell can continue to repair the membrane at a rate similar to the rate at which melittin binds, thus allowing continued uptake of melittin while maintaining viability. However, if melittin uptake causes extensive membrane perturbation

and results in the loss of membrane integrity, the resultant permeabilisation and death of the cell may facilitate entry of ethidium bromide or additional melittin molecules into the cell since no active transport mechanism or repair mechanism is available to prevent this occurring. Thus the ability of melittin to enter viable cells is likely to occur in a controlled manner in situations where the membrane integrity is maintained. This would still prevent entry of ethidium bromide or allow transport mechanisms to exclude it from the viable cells. Interestingly, it has been demonstrated that mammalian cells that have encountered a sublethal dose of melittin become resistant to the effects of melittin [Reiter et al., 1995]. This may also account for the ability of cells continuously exposed to melittin to be unaffected as seen in Figures 5 and 6. This particular result is interesting in the light of our earlier immunotoxin results where an extended incubation period with the antibody-melittin conjugate was required for lysis to occur [Dunn et al., 1996]. In this case, melittin was linked at the gene level to a single-chain Fv fragment of a murine monoclonal antibody, K121, specific for a tumour associated cell surface antigen, KMA (kappa myeloma antigen) [Boux et al., 1983]. KMA is not internalised upon binding K121 and thus a surface active toxin (melittin) was necessary for this immunotoxin to be effective. In contrast to native melittin, which rapidly binds and kills cells, the immunotoxin, although rapidly bound by the cells, was only significantly toxic after 18 h incubation. This may have resulted from the inability of KMA to internalise bound immunotoxin and thus reliance on the gradual association of immunotoxin molecules in the membrane to form the multimers required for membrane perturbation and consequent lysis. Since this long-term association does not appear to be involved in the lysis of cells by native melittin the results indicate that the kinetics of action of native melittin and melittin in the form of an immunotoxin are different. Whether the same result would occur for immunotoxin molecules that target other antigens remains to be determined.

In summary, approximately 2.5×10^5 melittin molecules could be incorporated into HMy2 cells with viability remaining unchanged. Moreover it was estimated that 10^6 molecules per cell were required for maximum cytotoxicity. The results indicate that a threshold level of incorporation of melittin will be required when this toxin is administered in the form of an immunotoxin for the purposes of inducing selective cell death. Moreover, the amount of melittin that was incorporated into viable cells was reduced over time, demonstrating the ability of the viable HMy2 cells to degrade or exocytose melittin over time. The precise fate of internalised melittin remains to be elucidated.

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