Cancer Chemother Pharmacol (1993) 32: 109--115

Cancer Chemotherapy and Pharmacology

Identification of serum components that inhibit the tumoricidal activity of amphiphilic alpha helical peptides

Karen A. Peck-Miller, Richard P. Darveau, H. Perry Fell

Bristol-Myers Squibb Pharmaceutical Research Institute, 3005 First Avenue Seattle, WA 98121, USA

Received 25 August 1992/Accepted 10 November 1992

Abstract. Antimicrobial peptides that can form amphiphilic alpha helices were tested for their ability to lyse various human tumor cell lines in vitro. These peptides include C18G, whose sequence is a derivative of the carboxyl terminus of human platelet factor IV, and 399, an idealized amphiphilic alpha helix. Both peptides exhibited potent antitumor activity against all cell lines tested, unlike magainin 2, a naturally occurring antimicrobial peptide of similar structure, which was relatively inactive under the same conditions. Also, the lytic activity of C18G is specific for tumor cells versus human red blood cells. The effects of serum can be important when evaluating the potency of lytic peptides, since other tumoricidal peptides have been shown to be completely inactivated by low serum levels. Experiments with C18G and 399 revealed that their activity was indeed reduced in the presence of human serum, but that significant lytic activity remained even at relatively high serum concentrations. Various serum components were tested for their inhibitory activity. Whereas albumin and high-density lipoprotein had only slight inhibitory properties, low-density lipoprotein was found to be a potent inhibitor of peptide-mediated cell lysis. The peptide 399, which is more sensitive to serum inhibition than C18G, also binds more extensively to all serum components tested.

Introduction

Numerous protein or peptide toxins of varied structure have been identified and characterized from many sources, including bacteria, fungi, insects, amphibians, reptiles, and mammals (for reviews see [4, 23, 28]). Among the peptide toxins that have antimicrobial activity are magainins and cecropins, which are produced by the frog *Xenopus laevis* [45] and the giant silkworm moth, *H. cecropia* [21], respectively. Both magainins and cecropins are predicted to form amphiphilic alpha helices [5, 35]. Indeed, an alpha helical conformation has been demonstrated for several of these peptides [40]. It has been proposed that the antimicrobial activity of these peptides is likely to occur via the formation of peptide multimers which form channels in cell membranes [6, 39], thus disrupting the lipid bilayer and causing cell death. Westerhoff et al. [41] have also suggested that the ability of magainins to dissipate the electric potential across a cell membrane may be the cause of cell lysis. Whereas the antimicrobial activity of these and other amphiphilic alpha helical peptides has been well documented (for reviews, see [2, 3)], relatively little is known about their antitumor activity. However, magainin analogs (including magainin 2) [8, 27] and cecropin analogs [22] have been reported to lyse mammalian transformed cell lines in vitro.

The novel peptide C18G [10] has been shown to possess potent antibacterial activity both in vitro and in vivo. The sequence of C18G, which is derived from the carboxyl terminus of human platelet factor IV, also predicts the formation of an amphiphilic alpha helix. We therefore tested the potential antitumor activity of C18G, and compared it with both magainin 2 and an idealized amphiphilic alpha helical peptide [11] designated here as 399. Here we show that both C18G and 399 have significant lytic activity against tumor cells in vitro, and that they are much more potent than magainin 2 under the conditions tested. In addition, the lytic activity of C18G is specific for tumor cells versus red blood cells.

The in vitro antitumor activity of human defensins, the lytic peptides which are produced by neutrophils, is completely inactivated by low levels of serum [24]. Therefore, we have tested the effects of serum on C18G and 399, and found that relatively high levels of serum significantly reduced, but did not completely abolish, their lytic activity.

Abbreviations: IMDM, Iscove's modified Dulbecco's medium; FBS, fetal bovine serum; MD-PBS, modified Dulbecco's phosphate buffered saline; HDL, high density lipoprotein; LDL, low density lipoprotein; HSA, human serum albumin.

Correspondence to: K. A. Peck-Miller

These results indicate that serum inhibition of peptide-mediated tumor cell lysis is important to consider when measuring the potency of lytic peptides. Some aspects of serum inhibition are discussed. These include the identification of albumin and high- and low-density lipoprotein as components in serum that cause inhibition of peptide activity. We also show that these components can directly bind the peptides, and that the peptide that binds more extensively to various serum components is also the one which is more inhibited by serum.

Materials and methods

Proteins and reagents. The peptides 399 [(Leu-Lys-Lys-Leu-Leu-Lys-Leu)$_2$] [11], C18G (Ala-Leu-Tyr-Lys-Lys-Leu-Leu-Lys-Lys-Leu-Leu-Lys-Ser-Ala-Lys-Lys-Leu-Gly) [10], and magainin 2 (Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Asn-Ser) [45] were generously provided by Dr. J. Blake (from our institution), and were synthesized as described [9]. Peptide solutions were stored at 4° C as a 2 mg/ml stock in dH_20. ^{51}Cr (1 mCi = MBq) was purchased from NEN (Dupont, Wilmington, Del.).

Target cells. All tumor cell lines were from our own cell banks, except HCT 116 and Hs 578T, which were from the American Type Culture Collection, Rockville, MD (ATCC# CCL 247 and HTB 126, respectively). Cell lines were maintained in vitro in a humidified environment at 37° C in 6% CO_2 in Iscove's modified Dulbecco's medium (IMDM, purchased from GIBCO, Grand Island, NY) with 10% v/v heat-inactivated fetal bovine serum (FBS[1]), 60 μg/ml penicillin-G, and 100 μg/ml streptomycin sulfate. Red blood cells (RBCs) were purified from fresh blood of normal donors by Ficoll density gradient centrifugation. Packed RBCs (1 ml) were then washed twice in 50 ml IMDM plus 10% FBS and resuspended in medium before use in a cytotoxicity assay.

Cytotoxicity assays. Target cells (10^6 cells/300 μl IMDM plus 10% FBS) were mixed with ^{51}Cr (75 μCi/10^6 cells) and incubated for 2 h at 37° C. Cells were then washed twice and resuspended in media. Cytotoxicity assays of human tumor cells were performed in triplicate in Microtiter V-bottom plates purchased from Fisher Scientific (Santa Clara, Calif.) (#14-245-72). Per well, 2×10^4 labeled cells (67 μl) were added to 67 μl of peptide (diluted in media) and either 67 μl of pooled normal human serum or media for a total assay volume of 201 μl. Following a 2 h incubation at 37° C, the plates were centrifuged at 150 × g for 5 minutes. The radioactivity of 100 μl of supernatant per well was counted using a γ-counter. Percent cytotoxicity was calculated as described [18].

Because of the relatively inefficient labeling of RBCs with ^{51}Cr, all cytotoxicity assays of RBCs were performed using the same final concentrations of components as above, except that the total assay volume was 1.2 ml, and the radioactivity of 1 ml of supernatant was quantitated. Also, human serum used in these assays was from a single normal donor with the same blood type as the donor of RBCs.

Human serum and lipoproteins. Pooled normal human serum from five healthy donors was prepared and stored essentially as described [9]. Heat inactivated serum indicates human serum which was incubated at 56° C for 30 min before addition to cytotoxicity assays. Serum filtrate indicates the filtrate obtained when human serum is ultrafiltered through a Centrifree Micropartition System (purchased from Amicon, Beverly, Mass., #4104) which has a 30,000 MW cut-off and retains serum proteins and protein-bound ligands. Per filter, one ml of serum was loaded and centrifuged at 2000 *g* at 37° C for 40 min, and approximately 0.5 ml of serum filtrate was obtained. Human lipoprotein-deficient serum was purchased from Sigma, St. Louis, Mo. (#S5519). Since this serum had been dialyzed by the manufacturer against modified Dulbecco's phosphate buffered saline, pH 7.5 (MD-PBS per liter: 0.1 g $MgCl_2$, 0.05 g KCl, 0.05 g KH_2PO_4, 0.15 g Na_2HPO_4, and 2.0 g NaCl), normal pooled human serum which was compared to it was first dialyzed against the same buffer before use in a cytotoxicity assay. Human serum-free control assays contained 67 μl of MD-PBS instead of 67 μl of human serum.

Normal human high-density (HDL) and low-density (LDL) serum lipoprotein (solutions in 0.15 M NaCl plus 0.01% EDTA, pH 7.4, at 10 mg/ml and 5 mg/ml, respectively) and lyophilized human serum albumin (HSA) (#A3782) were purchased from Sigma. Per cytotoxicity assay, 2×10^4 labeled cells (67 μl in media) were added to 67 μl of peptide (diluted in media) and 67 μl of either HDL or LDL (4.5 mg/ml in NaCl/EDTA solution) or HSA (45 mg/ml in NaCl/EDTA solution), and treated as described above. Normal pooled human serum which was compared to HDL, LDL, and HSA was first dialyzed against NaCl/EDTA solution before use in a cytotoxicity assay. Human serum-free control assays contained 67 μl of NaCl/EDTA solution instead of 67 μl of human serum or purified serum components.

Peptide ultrafiltration. Peptide (20 mg/ml in dH_20) was added to media or normal pooled human serum to obtain a final concentration of 600 μg/ml peptide, and incubated at 37° C for 15 min. Alternatively, peptide was added to HSA, HDL, or LDL in 0.15 M NaCl plus 0.01% EDTA (pH 7.4), and treated in the same manner. The mixtures were then centrifuged through a Centrifree Micropartition System as described above. (The concentration of free peptide in the filtrate is equal to that in the pre-filtered mixtures [17, 32, 43].) The filtrates obtained were used in a cytotoxicity assay: 67 μl of filtrate was added to 2×10^4 target cells in 134 μl of media, and treated as outlined above. The concentration of free peptide which was present in the filtrates was determined by comparing the percent lysis elicited in a cytotoxicity assay to an appropriate standard

Table 1. In vitro peptide-mediated cytotoxicity of human solid and hematopoietic tumor cell lines and normal human red blood cells

Target cells		Percent peptide-mediated cell lysis[a]				
		Peptide 399	Peptide 399 + human serum	Peptide C18G	Peptide C18G + human serum	Magainin 2
CEM	Acute lymphocytic leukemia	98 ± 2	29 ± 15	81 ± 9	19 ± 6	3 ± 3
H3396	Breast carcinoma	94 ± 9	57 ± 14	91 ± 8	57 ± 8	9 ± 2
Hs 578T	Breast carcinoma	60 ± 8	29 ± 1	43 ± 1	8 ± 1	3 ± 2
HCT 116	Colon carcinoma	100 ± 5	15 ± 6	55 ± 4	10 ± 6	1 ± 0
H3347	Colon adenocarcinoma	92 ± 9	27 ± 9	49 ± 16	16 ± 7	5 ± 0
H2981	Lung adenocarcinoma	100 ± 13	38 ± 23	82 ± 3	14 ± 2	4 ± 1
H3740	Lung carcinoma	90 ± 8	11 ± 1	58 ± 11	10 ± 2	2 ± 1
H3759	Ovarian carcinoma	100 ± 0	17 ± 2	39 ± 5	17 ± 2	3 ± 1
Normal human red blood cells		73 ± 19	8 ± 1	1 ± 2	5 ± 6	3 ± 5

Target cells were labeled with ^{51}Cr and exposed to peptide (200 μg/ml) for 2 h at 37° C in the presence of IMDM plus 10% FBS, as described in Materials and methods. Where indicated, human serum was included in these assays (at a concentration of 33%)

[a] Mean ± SD of 2–4 experiments, each performed in triplicate

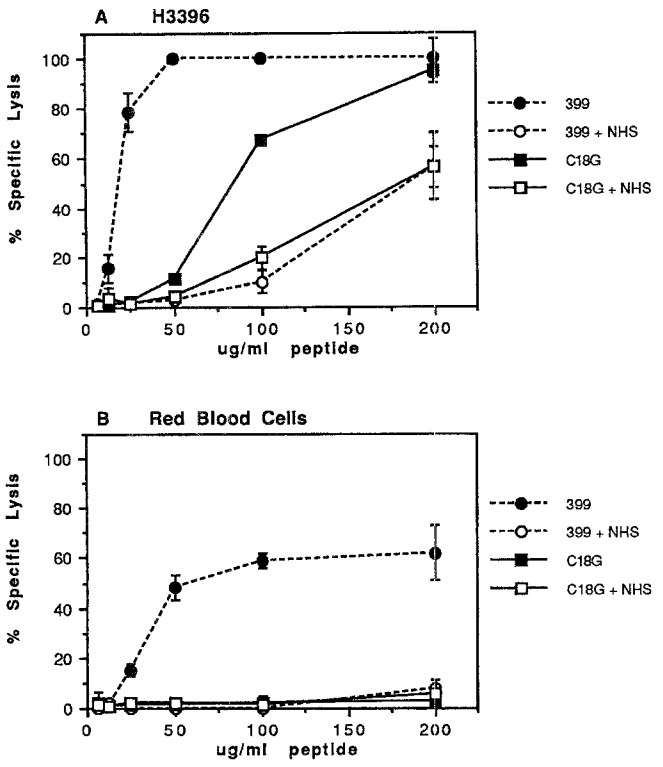

Fig. 1 A, B. Effects of serum on peptide-mediated cell lysis. **A** H3396, human breast adenocarcinoma cell line; **B** normal human red blood cells. Cytotoxicity assays were performed in IMDM plus 10% FBS (as described in Materials and methods), with the addition of 33% normal human serum (NHS), where indicated. Each *graph* shows the data from three experiments, each performed in triplicate. The *error bars* indicate the standard deviation from the mean

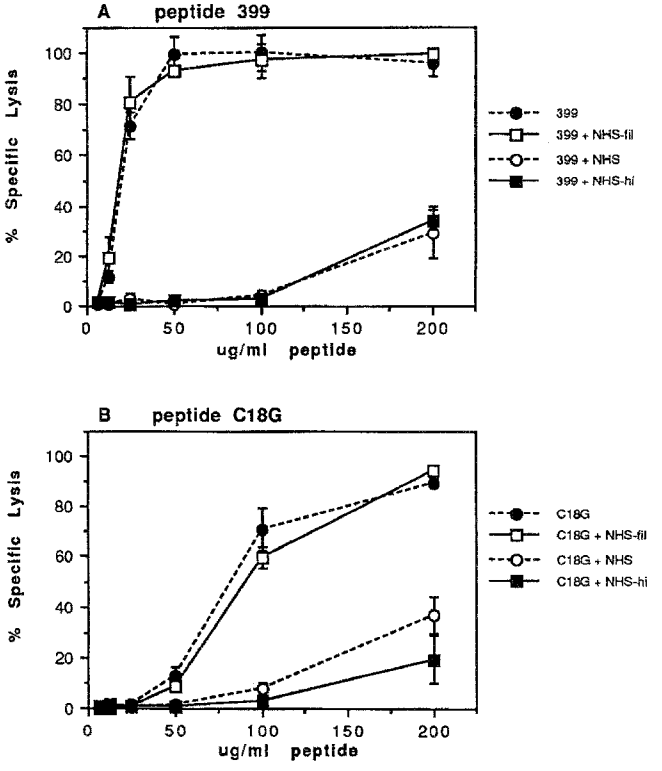

Fig. 2 A, B. Identification of serum components involved in inhibition of peptide-mediated cell lysis. The peptides 399 (**A**) and C18G (**B**) were used in cytotoxicity assays against the human breast adenocarcinoma cell line, H3396. All assays were performed in IMDM plus 10% FBS, with the addition of 33% normal human serum (NHS), 33% heat-inactivated NHS (NHS-hi), or 33% NHS filtrate (NHS-fil). See Materials and methods for details. Each *graph* shows the data from three experiments, each performed in triplicate. The *error bars* indicate the standard deviation from the mean

curve. To verify that free peptide is not retained on the filter, peptide which was diluted in dH_2O or 0.15 M NaCl plus 0.01% EDTA (pH 7.4) was ultrafiltered as described above. Peptide was found to be fully recovered in the filtrate, regardless of whether the initial concentration was 600 μg/ml or 30 μg/ml.

Results

Activity of lytic peptides against human transformed cell lines

The cytotoxic potential of the antibacterial peptide C18G [10] was tested on various solid and hematopoietic human tumor cell lines, as well as normal human red blood cells. Circular dichroism shows this peptide to be helical at pH 7.2. Two additional peptides, 399 and magainin 2, were also chosen for analysis. The peptide 399 is a synthetic sequence which is idealized for amphiphilic alpha helical character and possesses in vitro antibacterial activity [11]. Magainin 2 is a naturally occurring antimicrobial peptide originally isolated from the skin of *Xenopus laevis* [45]. Circular dichroism data indicate that 399 [12] and magainin 2 [40] also form alpha helices. Results from cytotoxicity assays of these peptides (described in Materials and Methods) are shown in Table 1.

Peptide C18G elicited a significant level of cell lysis against all transformed cell lines tested, while 399 was even more potent than C18G. Conversely, magainin 2 had little or no cytolytic activity against these cell lines under the conditions tested. Results from hemolysis assays (Table 1) indicate that 399 possesses significant hemolytic activity, whereas the less potent C18G is non-hemolytic [10], as is magainin 2 [45].

Serum inhibition of peptide cytotoxicity

Investigators working with defensins, the lytic peptides from human neutrophils, have reported that as little as 5% FBS almost completely inhibited defensin-mediated in vitro antitumor activity [24–26], whereas the synthetic peptides tested here have significant activity in the presence of 10% FBS (Table 1). In order to assess the effects of human serum in our in vitro assays, the antitumor activity of C18G and 399 was measured as described above, but with the addition of 33% human serum. The results presented in Table 1 indicate that the cytolytic activity of both peptides against all cell types tested was reduced in the presence of human serum, but that a significant amount of cell lysis was still apparent. Also, a titration of these peptides against

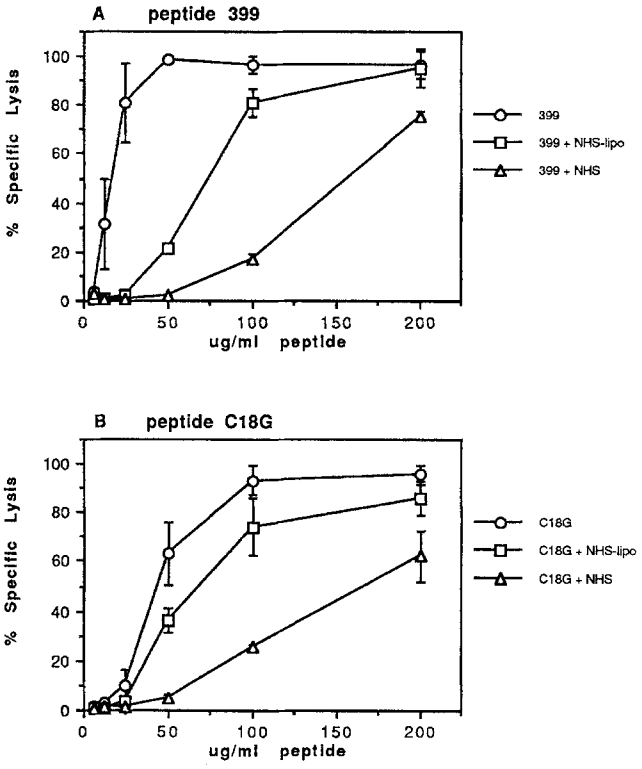

Fig. 3 A, B. Serum lipoproteins cause significant inhibition of peptide lytic activity. The peptides 399 (**A**) and C18G (**B**) were used in cytotoxicity assays against the human breast adenocarcinoma cell line, H3396. All assays were performed in IMDM plus 10% FBS, with the addition of 33% lipoprotein-deficient NHS (NHS-lipo) which was dialyzed against MD-PBS, 33% NHS which was dialyzed against MD-PBS (NHS), or 33% MD-PBS (*open circles*). See Materials and methods for details. Each *graph* shows the data from two experiments, each performed in triplicate. The *error bars* indicate the standard deviation from the mean

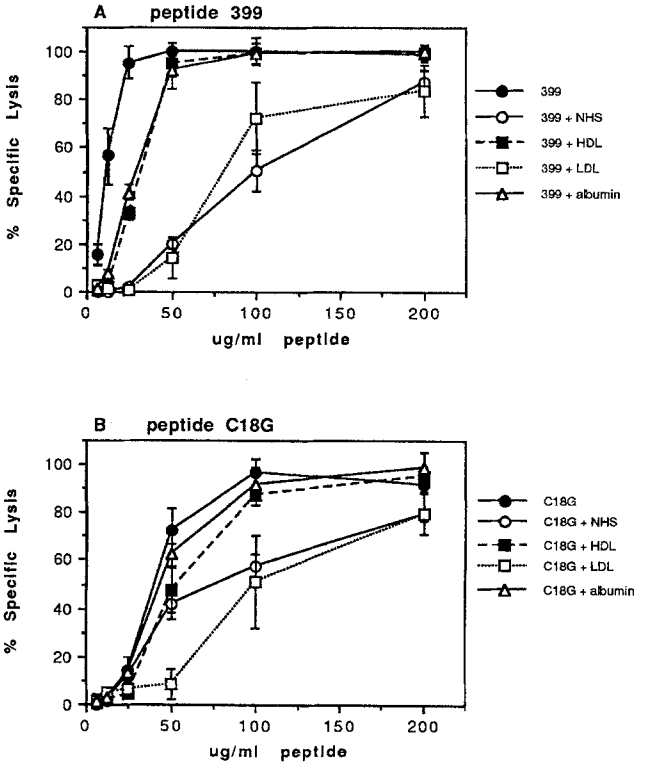

Fig. 4 A, B. Inhibition of peptide lytic activity by human serum albumin or human high and low density serum lipoproteins. The peptides 399 (**A**) and C18G (**B**) were used in cytotoxicity assays against the human breast adenocarcinoma cell line, H3396. All assays were performed in IMDM plus 10% FBS, with the addition of 33% NHS which was dialyzed against 0.15 M NaCl plus 0.01% EDTA (pH 7.4) (NHS), high-density (HDL) or low-density (LDL) lipoproteins in NaCl/EDTA solution (1.5 mg/ml final concentration), HSA (albumin) in NaCl/EDTA solution (15 mg/ml final concentration), or 33% NaCl/EDTA solution (*closed circles*). See Materials and methods for details. Each *graph* shows the data from two experiments, each performed in triplicate. The *error bars* indicate the standard deviation from the mean

the human breast carcinoma cell line H3396 (Fig. 1A) demonstrates that the reduction in activity of 399 is approximately five-fold greater than the reduction in activity of C18G. In a hemolysis assay (Fig. 1B), serum inhibition of lysis by 399 reduced activity to essentially zero, whereas the presence of human serum in assays with C18G did not alter the activity of this non-hemolytic peptide.

In an effort to identify the component(s) in human serum which cause a reduction in peptide lytic activity, human serum was either treated by heating at 56° C for thirty minutes to inactivate any heat-labile components, or was placed in 3,500 MW cut-off dialysis tubing and dialyzed against media, before use in a cytotoxicity assay. Both the heat inactivated serum (Fig. 2) and the dialyzed serum (data not shown) exhibit a level of inhibition of peptide lytic activity which is similar to the untreated serum. These results indicate that serum inhibition is not dependent on the active form of any heat-labile components (including complement), and that the components which mediate inhibition are larger than 3,500 mol wt. Also, normal human serum was ultrafiltered to remove all proteins (and protein-bound ligands) above 30,000 mol wt, before use in a cytotoxicity assay (see Materials and methods for details). The serum filtrate had no inhibitory activity compared with human serum-free assays (Fig. 2), indicating that serum inhibition is mainly dependent upon components larger than 30,000 mol wt [or any ligand(s) bound to them] which are removed from serum during the ultrafiltration step. Also, since peptides containing only D-amino acids are resistant to proteolytic cleavage [1, 40], an all-D-amino acid analog of C18G [10] was tested. This peptide possessed lytic activity and susceptibility to inhibition by serum which was identical to C18G, indicating that serum inhibition is not due to protease activity.

Serum lipoproteins are absent from the serum filtrate, and could theoretically inhibit peptide-mediated lysis by binding directly to peptides. In order to assess the possible inhibitory role of lipoproteins in human serum, cytotoxicity assays were performed in the presence of human lipoprotein-deficient serum versus normal human serum which had been dialyzed against the same buffer as the lipoprotein-deficient serum (see Materials and methods). Although the dialyzed normal human serum is less inhibitory than the untreated normal human serum (compare Figs. 2 and 3), the results presented in Fig. 3 clearly indicate that the absence of lipoproteins in serum alleviates serum inhi-

Table 2. Percent of free peptide in solution as determined by ultrafiltration

Peptide added to:	Percent free peptide[a]	
	Peptide 399	Peptide C18G
Human serum	0±0	16±3
IMDM plus 10% FBS	4±1	63±2
HSA (45 mg/ml in buffer[b])	22±4	46±1
HDL (4.5 mg/ml in buffer)	45±4	61±6
LDL (4.5 mg/ml in buffer)	12±1	33±5

Peptide was diluted directly into human serum (or other mixtures indicated) to obtain a final concentration of 600 μg/ml, then incubated at 37° C for 15 min and ultrafiltered. (The ratio of peptide to serum used here is equivalent to that in a standard cytotoxicity assay containing 33% human serum and 200 μg/ml peptide.) The amount of free peptide in the filtrate was subsequently determined (see Materials and methods for details). HSA, HDL, and LDL were tested at concentrations which are equivalent to those found in human serum.

[a] Mean ± SD of two experiments, each performed in triplicate

[b] Buffer contains 0.15 M NaCl plus 0.01% EDTA, pH 7.4

bition by approximately 80% for C18G, and by approximately 60% for 399. Therefore, serum lipoproteins play a major role in the inhibition of peptide activity in these assays.

Normal human high- and low-density serum lipoproteins were also added to cytotoxicity assays in an effort to directly identify which serum lipoproteins could be involved in inhibition of peptide lytic activity. Purified HDL or LDL was added to cytotoxicity assays at a final concentration of 1.5 mg/ml, which is equivalent to the concentration of these lipoproteins normally present in cytotoxicity assays containing 33% human serum. Results presented in Fig. 4 show that HDL does have a measurable inhibitory effect on 399 and C18G, but that LDL alone more closely approximates human serum in its inhibitory capacity.

Albumin is the most abundant protein in serum, and, because of its size (approximately 69,000 mol. wt), it is absent from the non-inhibitory serum filtrate. For this reason, the potential of human serum albumin (HSA) for inhibiting the lytic activity of 399 and C18G was also investigated. HSA was added to cytotoxicity assays at a final concentration of 15 mg/ml, which is equivalent to the amount of HSA that is present in cytotoxicity assays containing 33% human serum. Results presented in Fig. 4 indicate that HSA has only a slight inhibitory effect on 399, and no significant effect on C18G.

Interaction of serum components with peptide

Serum components could reduce the activity of lytic peptides by binding to peptide molecules, thereby lowering the concentration of free peptide. To determine the extent to which serum components bind directly to peptide molecules, C18G or 399 was preincubated with human serum, HSA, HDL, LDL, or media, then ultrafiltered to remove all proteins (and protein-bound ligands) above 30,000 mol. wt (see Materials and methods for details). Peptide which is bound to proteins (or protein-bound ligands) above 30,000 mol. wt, including albumin and serum lipoproteins, is retained on the filter, while only free peptide is recovered in the filtrate. The filtrate was then used in a cytotoxicity assay and the concentration of peptide in the filtrate was determined, as described in Materials and Methods.

Results from these experiments indicate that both C18G and 399 interact directly with human serum components (Table 2). This interaction is more extensive with 399, since no free peptide (i.e. no lytic activity) is detectable in filtrates of 399 plus human serum mixtures, while 16% of C18G is recovered in the filtrate under the same conditions. Also, even under conditions which support a high level of peptide-mediated cytotoxicity, i.e. IMDM plus 10% FBS, only 63% of C18G and 4% of 399 is recovered in the filtrate. Therefore, even if almost all of the lytic peptide molecules are bound to other components in an assay, a high percentage of cell lysis is still possible. Experiments with HSA, HDL, and LDL demonstrate that in every case, a larger percentage of C18G is free in solution compared with 399.

Discussion

The lytic activity of C18G and 399 was clearly demonstrated against eight different solid or hematopoietic human tumor cell lines (Table 1), showing that the activity of these peptides is not limited to a particular tumor cell type. Although C18G is non-hemolytic, the sensitivity of a normal human skin fibroblast cell line and human fresh peripheral blood lymphocytes to both C18G and 399 is similar to that of the tumor cell lines tested (data not shown). Nonetheless, it is important to note that numerous chemotherapeutic agents which elicit significant antitumor activity in vivo are known to possess nonspecific cytotoxicity against normal and transformed cell lines in vitro [reviewed in 13, 16, 27]. Also, nonspecific lysis of normal human nucleated cells versus transformed cell lines has been demonstrated previously for endogenous protein toxins, such as human defensins [24] and perforins [19].

The antimicrobial peptide magainin 2 was found to be almost completely inactive in our assays (Table 1). This result is in contrast to a previous report [8] in which magainin 2 was shown to be cytolytically active against a human tumor cell line (U937) at concentrations which are comparable to the levels used in the present study. The medium used in our cytotoxicity assays (IMDM) is more complex than that used previously [8], and also includes 10% FBS. It is likely that the difference in assay conditions (most notably the inclusion of serum), rather than the difference in target cells, accounts for the discrepancy observed in the activity of magainin 2.

We and others have found serum to be an important inhibitor of peptide-mediated cell lysis. Previous work has shown that as little as 5% FBS could completely inhibit the tumoricidal activity of human peptide defensins in vitro [24, 25]. Although the activity of C18G and 399 against various human tumor cell lines is greatly reduced in the presence of 33% human serum (Table 1), the human breast carcinoma cell line H3396 can still be lysed to approxi-

mately 50% under these conditions (Fig. 1 A). Conversely, the lytic activity of 399 against red blood cells is completely abolished (Fig. 1 B). Taken together, these results demonstrate that the inclusion of human serum in in vitro peptide-mediated tumor cell lysis assays is an important component necessary for accurately evaluating the potency and potential usefulness of lytic peptides for therapeutic use in vivo.

Because of the dramatic inhibitory effect observed when human serum is present in peptide-mediated antitumor assays, questions concerning which serum components reduce peptide lytic activity, and how this inhibition is mediated, were addressed. Serum inhibition is not dependent on small serum components or the native form of any heat-labile components (Fig. 2). The inhibitory component(s) were identified as protein(s) (or protein-bound ligands) of 30,000 mol. wt or higher (Fig. 2), however, human serum albumin, the most abundant protein in serum, mediates very little inhibition in these assays (Fig. 4). This is in contrast to the effect of albumin on defensin-mediated tumor cell lysis, in which the content of albumin (in this case bovine) in serum completely accounts for serum inhibition [25]. The difference in the effect of albumin on defensin activity versus the activity of C18G and 399 could be due to sequence specific and/or structural dissimilarities: defensins form beta sheets rather than alpha helices [20].

Other amphiphilic alpha-helical peptides have been shown to readily bind to artificial lipid monolayers and bilayers [4, 29, 37, 40]. It is possible that the hydrophobic quality of 399 and C18G allows these peptides to interact with the lipid bilayer of the cell membrane, and could allow them to bind to lipoproteins in serum, thereby leading to inhibition of peptide activity via a decrease in the concentration of free peptide. For this reason, lipoproteins were tested and were found to have significant inhibitory effects on peptide lytic activity (Fig. 3). Whereas purified HDL elicited only a low level of inhibition, purified LDL more closely approximated human serum in its inhibitory capacity (Fig. 4). The higher lipid content of LDL compared with HDL correlates with higher levels of inhibition, suggesting that the lipid portion of these lipoproteins may play a role in their ability to inhibit peptide lytic activity. In addition to lipoproteins and HSA, there may be other unidentified factor(s) in serum which mediate a reduction in peptide-mediated cell lysis. Other lytic proteins with similar secondary structural motifs, such as the ninth component of complement [31], perforin [38, 44], and *Staphylococcus aureus* δ-toxin [42], have also been shown to be partially inactivated by specific serum lipoproteins.

Examples of serum protein binding by various anticancer drugs and antibiotics have been well documented [7, 14, 15, 30, 33, 34, 36], and we have found that the peptides in these studies also bind serum proteins. Results shown in Table 2 demonstrate that the percent of 399 remaining free in solution is always less than that of C18G, regardless of which solution the peptides have been mixed with. Compared with C18G, 399 has more potent cytolytic activity against erythrocytes and tumor cells, but is also more inhibited by the presence of serum. In addition, 399 potentially forms a more idealized amphiphilic alpha helix than C18G or magainin 2 (in descending order). It is likely that the idealized structure of 399 relative to the other two peptides renders it more successful at interacting with the hydrophobic environment of the cell membrane, thus leading to a higher level of cell lysis. This quality of 399 could also lead to a more extensive and/or tighter association with serum components relative to C18G, which could be a factor in the greater serum inhibition observed with 399. Thus, direct binding of serum components to peptide molecules could contribute to inhibition of peptide lytic activity.

However, the results in Table 2 also suggest that the binding of peptide to other molecules in solution is not irreversible. For example, 399 elicits a significant level of cell lysis in the presence of human serum (Fig. 1 A) even though there is no detectable free peptide in human serum (Table 2), while the amount of free peptide under relatively non-inhibitory conditions (IMDM plus 10% FBS) is only marginally higher (approximately 4%). Although residual free 399 could be present in filtrates of peptide plus human serum, there is not enough to cause any measurable lytic activity. Therefore, in standard cytotoxicity assays containing 399 and human serum (Fig. 1 A), the residual free 399 which could be present is not enough to account for the significant cell lysis observed. Also, HSA and HDL extensively bind both peptides (Table 2), but do not significantly inhibit peptide activity (Fig. 4). Either bound peptide is active in cell lysis, or, more likely, the cell surface successfully competes with serum components (especially HSA and HDL) for binding to peptide. LDL in serum may bind peptide more extensively and less reversibly than HSA or HDL, thereby accounting for the higher level of inhibition of peptide activity observed with LDL. Other possibilities for the mediation of serum inhibition in our assays include binding of serum components to target cells, thereby blocking areas on the cell surface such that peptide molecules must compete for these sites in order to cause cell lysis.

The data presented here, and by other investigators [8, 22, 27], clearly demonstrate that peptides which theoretically form amphiphilic alpha helices can possess both antibacterial and antitumor properties. On the other hand, the inhibitory effects of human serum on peptide-mediated tumor cell killing are dramatic, and should be considered when determining the potency of various lytic peptides and in evaluating them for use in in vivo studies. It will be important to identify peptides that do not linearly succumb to serum inhibition as potency increases. In addition, direct binding of peptide to specific serum components which do not inhibit lytic activity could actually increase the half-life of the peptide in vivo. It may also be possible to favorably affect the pharmacokinetic properties of conventional drugs by conjugating them to peptides which interact with serum components.

References

1. Bessalle R, Kapitkovsky A, Gorea A, Shalit I, Fridkin M (1990) All-D-magainin: chirality, antimicrobial activity and proteolytic resistance. FEBS Lett 274: 151

2. Bevins CL, Zasloff M (1990) Peptides from frog skin. Annu Rev Biochem 59: 395
3. Boman HG, Hultmark D (1987) Cell-free immunity in insects. Annu Rev Microbiol 41: 103
4. Boman HG (1991) Antibacterial peptides: key components needed in immunity. Cell 65: 205
5. Chen H-C, Brown JH, Morell JL, Huang CM (1988) Synthetic magainin analogues with improved antimicrobial activity. FEBS Lett 236: 462
6. Christensen B, Fink J, Merrifield RB, Mauzerall D (1988) Channel-forming properties of cecropins and related model compounds incorporated into planar lipid membranes. Proc Natl Acad Sci USA 85: 5072
7. Craig WA, Suh B (1986) Protein binding and the antimicrobial effects: methods for the determination of protein binding: In: Lorian (ed) Anti- biotics in laboratory medicine, 2nd edn., Williams and Wilkins, Baltimore, p 477
8. Cruciani RA, Barker JL, Zasloff M, Chen H-C, Colamonici O (1991) Antibiotic magainins exert cytolytic activity against transformed cell lines through channel formation. Proc Natl Acad Sci USA 88: 3792
9. Darveau RP, Cunningham MD, Seachord CL, Cassiano-Clough L, Cosand WL, Blake J, Watkins CS (1991) β-Lactam antibiotics potentiate magainin 2 antimicrobial activity in vitro and in vivo. Antimicrob Agents Chemother 35: 1153
10. Darveau RP, Blake J, Seachord CL, Cosand WL, Cunningham MD, Cassiano-Clough L, Maloney G (1992) Peptides related to the carboxyl terminus of human platelet factor IV with antibacterial activity. J Clin Invest 90: 447
11. DeGrado WF (1983) Solution phase synthesis of cecropin A 1–22 and potent analogues thereof using segments prepared on an ozime solid phase support: In: Hruby V, Rich DH (eds) Peptides: structure and function. Pierce Chemical Co., Rockford, Ill, p 195
12. DeGrado WF, Lear JD (1985) Induction of peptide conformation at apolar/water interfaces. 1. A study with model peptides of defined hydrophobic periodicity. J Am Chem Soc 107: 7684
13. Foley GE, Epstein SS (1964) Cell culture and cancer chemotherapy: In: Goldin A, Hawking F (eds) Advances in chemotherapy, vol. 1. Academic Press, New York, p 175
14. Gera S, Musch E, Osterheld HKO, Loos U (1989) Relevance of the hydrolysis and protein binding of melphalan to the treatment of multiple myeloma. Cancer Chemother Pharmacol 23: 76
15. Gewirtz DA, Holt S (1985) Protein binding as a component of drug interaction in cellular pharmacokinetic studies. Biochem Pharmacol 34: 747
16. Goldin A, Carter S (1982) Screening and evaluation of antitumor agents. In: Holand JF, Frei E III (eds) Cancer medicine, 2nd edn., Lea and Febiger, Philadelphia, Pa, p 633
17. Hammond GL, Nisker JA, Jones LA, Siiteri PK (1980) Estimation of the percentage of free steroid in undiluted serum by centrifugal ultrafiltration-dialysis. J Biol Chem 255: 5023
18. Hellström I, Brankovan V, Hellström KE (1985) Strong antitumor activities of IgG3 antibodies to a human melanoma-associated ganglioside. Proc Natl Acad Sci USA 82: 1499
19. Henkart PA, Millard PJ, Reynolds CW, Henkart MP (1984) Cytolytic activity of purified cytoplasmic granules from cytotoxic rat large granular lymphocyte tumors. J Exp Med 160: 75
20. Hill CP, Yee J, Selsted ME, Eisenberg D (1991) Crystal structure of defensin HNP 3, an amphiphilic dimer: mechanisms of membrane permeabilization. Science 251: 1481
21. Hultmark D, Steiner H, Rasmuson T, Boman HG (1980) Insect immunity. Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of *Hyalophora cecropia*. Eur J Biochem 106: 7
22. Jaynes JM, Julian GR, Jeffers GW, White KL, Enright FM (1989) In vitro cytocidal effect of lytic peptides on several transformed mammalian cell lines. Peptide Res 2: 157
23. Kini RM, Evans HJ (1989) A common cytolytic region in myotoxins, hemolysins, cardiotoxins and antibacterial peptides. J Peptide Protein Res 34: 277
24. Lichtenstein A, Ganz T, Selsted ME, Lehrer RI (1986) In vitro tumor cell cytolysis mediated by peptide defensins of human and rabbit granulocytes. Blood 68: 1407
25. Lichtenstein AK, Ganz T, Nguyen T-M, Selsted ME, Lehrer RI (1988) Mechanism of target cytolysis by peptide defensins. Target cell metabolic activities, possibly involving endocytosis, are crucial for expression of cytotoxicity. J Immunol 140: 2686
26. Lichtenstein A (1991) Mechanism of mammalian cell lysis mediated by peptide defensins. Evidence for an initial alteration of the plasma membrane. J Clin Invest 88: 93
27. Ohsaki Y, Gazdar AF, Chen H-C, Johnson BE (1992) Antitumor activity of magainin analogues against human lung cancer cell lines. Cancer Res 52: 3534
28. Ojcius DM, Young JD-E (1991) Cytolytic pore-forming proteins and peptides: is there a common structural motif? TIBS 16: 225
29. Roise D, Horvath SJ, Tomich JM, Richards JH, Schatz GA (1986) A chemically synthesized pre-sequence of an imported mitochondrial protein can form an amphiphilic helix and perturb natural and artificial phospholipid bilayers. EMBO J 5: 1327
30. Rolinson GN (1980) The significance of protein binding of antibiotics in antibacterial chemotherapy. J Antimicrob Chemother 6: 311
31. Rosenfeld SI, Packman CH, Leddy JP (1983) Inhibition of the lytic action of cell bound terminal complement components by human high density lipoproteins and apoproteins. J Clin Invest 71: 795
32. Sophianopoulos JA, Durham SJ, Sophianopoulos AJ, Ragsdale HL, Cropper WP Jr (1978) Ultrafiltration is theoretically equivalent to equilibrium dialysis but much simpler to carry out. Arch Biochem Biophys 187: 132
33. Steele WH, Haughton DJ, Barber HE (1982) Binding of vinblastine to recrystallized human α_1-acid glycoprotein. Cancer Chemother Pharmacol 10: 40
34. Steele WH, King DJ, Barber HE, Hawksworth GM, Dawson AA, Petrie JC (1983) The protein binding of vinblastine in the serum of normal subjects and patients with Hodgkin's disease. Eur J Clin Pharmacol 24: 683
35. Steiner H (1982) Secondary structure of the cecropins: antibacterial peptides from the moth *Hyalophora cecropia*. FEBS Lett 137: 283
36. Svensson CK, Woodruff MN, Baxter JG, Lalka D (1986) Free drug concentration monitoring in clinical practice. Rational and current status. Clin Pharmacokinet 11: 450
37. Tamm LK, Tomich JM, Saier MH Jr (1989) Membrane incorporation and induction of secondary structure of synthetic peptides corresponding to the N-terminal signal sequences of the glucitol and mannitol permeases of *Escherichia coli*. J Biol Chem 264: 2587
38. Tschopp J, Masson D, Schäfer S (1986) Inhibition of the lytic activity of perforin by lipoproteins. J Immunol 137: 1950
39. Urrutia R, Cruciani RA, Barker JL, Kachar B (1989) Spontaneous polymerization of the antibiotic peptide magainin 2. FEBS Lett 247: 17
40. Wade D, Boman A, Wåhlin B, Drain CM, Andreu D, Boman HG, Merrifield RB (1990) All-D amino acid-containing channel-forming antibiotic peptides. Proc Natl Acad Sci USA 87: 4761
41. Westerhoff HV, Juretic D, Hendler RW, Zasloff M (1989) Magainins and the disruption of membrane-linked free-energy transduction. Proc Natl Acad Sci USA 86: 6597
42. Whitelaw DD, Birkbeck TH (1978) Inhibition of Staphylococcal delta-haemolysin by human serum lipoproteins. FEMS Microbiol Lett 3: 335
43. Whitlam JB, Brown KF (1981) Ultrafiltration in serum protein binding determinations. J Pharm Sci 70: 146
44. Young J D-E, Damiano A, DiNome MA, Leong LG, Cohn ZA (1987) Dissociation of membrane binding and lytic activities of the lymphocyte pore-forming protein (perforin). J Exp Med 165: 1371
45. Zasloff M (1987) Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc Natl Acad Sci USA 84: 5449