ANTIINFLAMMATORY PEPTIDES (ANTIFLAMMINS) INHIBIT SYNTHESIS OF PLATELET-ACTIVATING FACTOR, NEUTROPHIL AGGREGATION AND CHEMOTAXIS, AND INTRADERMAL INFLAMMATORY REACTIONS

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Platelet-activating factor (PAF)[1] is a phospholipid (1-*O*-alkyl-2-*sn*-acetyl-glycero-3-phosphocholine) mediator of inflammation and endotoxic shock (1). Polymorphonuclear neutrophils (PMN), peritoneal macrophages, vascular endothelial cells, basophils, and platelets synthesize PAF rapidly after appropriate stimuli (1). For example, TNF or phagocytosis promote synthesis and release of PAF in PMN or macrophages within 10 min (2). These stimuli induce phospholipase A_2 (PLA_2) activity that cleaves membrane phospholipids into lyso-PAF and arachidonic acid, and acetyl-CoA:lyso-PAF acetyltransferase that produces PAF by acetylating lyso-PAF. Thrombin stimulates PAF synthesis in endothelial cells also within 10 min (3). Other proteinases, such as elastase, stimulate PAF synthesis rapidly in PMN, macrophages, and endothelial cells (4), and induce PLA_2 and acetyltransferase activity (5). Conversely, different proteinase inhibitors block PAF synthesis induced by TNF (2, 4). These findings led to the hypothesis that proteinases added to cells and cellular proteinases activated by TNF cleave proteins inhibitory for PLA_2, such as lipocortins (4).

Lipocortins belong to a family of related proteins that mediate the antiinflammatory activity of corticosteroids (6). Lipocortins inhibit PLA_2 activity in vitro by a mechanism still unclear (7–8). Furthermore, recombinant lipocortin I inhibits eicosanoid synthesis in vivo in perfused lungs (9). Cloning and sequencing of lipocortins cDNA has provided the amino acid sequence of these proteins (10–12). Another steroid-induced protein with PLA_2 inhibitory activity is uteroglobin, a rabbit secretory protein (13). Two identical subunits of 70 amino acids form uteroglobin (14); lipocortin I and II comprise four nonidentical repeats of 70 amino acids (see reference 15 for review). Miele et al. (15) have noticed a striking sequence similarity between amino

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[1] *Abbreviations used in this paper:* PAF, platelet-activating factor; PLA_2, phospholipase A_2; PMN, polymorphonuclear neutrophils; TT-BSA, Tris-buffered Tyrode's containing delipidated BSA.

acid residues 40–46 of uteroglobin and 247–253 of lipocortin I, repeat 3. Synthetic peptides designated "antiflammins" that correspond to such sequences show potent PLA_2 inhibitory activity in vitro and an antiinflammatory effect on carrageenan-induced rat foot pad edema in vivo (15).

In this paper we examine whether antiflammins inhibit synthesis of a phospholipid mediator of inflammation, such as PAF, in intact cells. These peptides inhibit PAF synthesis in PMN, macrophages, and endothelial cells stimulated by TNF, phagocytosis, or proteinases. The antiflammins inhibit also PMN aggregation and chemotaxis, and suppress the inflammatory reaction induced in rat skin by in situ formation of immune complexes or by intradermal injection of TNF and complement component C5a.

Materials and Methods

Materials. Human rTNF was a gift of the Suntory Institute for Biomedical Research, Osaka, Japan. Peptides MQMKKVLDS (antiflammin-1) and WKLFKKIEKV (a synthetic peptide corresponding to residues 2 to 11 of cecropin, a moth polypeptide) were a gift of Dr. Anil B. Mukherjee of the National Institutes of Health (Bethesda, MD). Peptide HDMNKVLDL (antiflammin-2) was purchased from Peptide Biotechnologies (Washington, DC). These peptides were stored under nitrogen in sealed glass vials and dissolved in Tris buffer, pH 8, containing 10 mM β-mercaptoethanol (ME) or 1 mM dithiothreitol to prepare 0.1 mM stock solutions that were kept at 2°C and diluted before each assay. Control incubations received the same amount of ME or dithiothreitol. Opsonized yeast spores (BYS-C3b) were prepared as described (4). The PAF receptor antagonist SRI 63072 was obtained from Sandoz (East Hanover, NJ).

Cell Preparation and PAF Assay. Human PMN were prepraed as described (4) and resuspended at 5×10^6/ml in Tris-buffered Tyrode's containing 0.25% delipidated BSA (TT-BSA) with calcium and magnesium. Rat peritoneal macrophages and human vascular endothelial cells were prepared and cultured as described (2). In standard assays, 2.5×10^6 PMN or 10^6 rat peritoneal macrophages were incubated at 37°C in 0.5 ml reactions with rTNF, 20 μl of BYS-C3b suspension (~10 spores/cell), and other additions indicated in the text. Endothelial cells were grown in multiwell plates (~5×10^5 cells/35 mm well) and treated in 1 ml of Iscove's medium containing 0.25% BSA. PAF released into the medium or associated with cells was isolated, characterized, and measured as described (2).

Enzymatic Assays. The preparation of cell homogenate and the assay conditions for acetyl-CoA:lyso-PAF acetyltransferase have been described in detail (16). This enzymatic activity is expressed in nanomoles of [^{3}H]acetate incorporated into PAF per minute of incubation and milligram of cell homogenate protein (16). PLA_2 activity was measured in PMN homogenates according to Blackwell et al. (17), with minor modifications. The reactions contained 0.1 ml of PMN sonicate (30–60 μg of protein) and 500 nM α'-palmitoyl, β-1-[^{14}C]oleyl, Lα-phosphatidylcholine (400 nCi) dispersed in 0.9 ml of 0.5 M Tris buffer, pH 8, and 50 mM $CaCl_2$. After 1 h at 37°C, 2 ml of methanol and 2 ml of chloroform were added. The samples were extracted and the chloroform phase was separated and dried. The product of hydrolysis, [^{14}C]oleic acid, was separated from unhydrolyzed substrate by TLC on silica gel using chloroform/methanol/acetic acid (70:10:1) as solvent. Unhydrolyzed phosphatide and oleic acid were eluted to measure percent hydrolysis. This enzymatic activity is expressed per microgram of PMN protein. The release of label from PMN preincubated with [^{14}C]arachidonic acid was assayed according to Hirata et al. (18). 10^7 PMN in 5 ml of modified Gey's solution were incubated with 1.25 μCi [^{14}C]arachidonic acid at 37°C for 45 min, washed twice, and resuspended in this solution; 50% of the label was incorporated by PMN. The release of label was measured by centrifuging the cells and counting the supernatant.

Preparation of Human C5a. Human C5a was prepared according to Vallota and Muller-Eberhard (19). Briefly, normal human serum was incubated with yeast spores after inhibition of the anaphylatoxin inactivator with ϵ-aminocaproic acid (19). The C5a was purified by three

sequential chromatographies on CM-cellulose, Sephadex G100, and CM-Sephadex C50; the biologic activity of C5a was assayed by testing its contractile property on guinea pig ileum (19). The minimal effective concentration was 2.5×10^{-10} M. C5a-des-Arg was prepared by digestion with carboxypeptidase B, as described (20).

Neutrophil Aggregation and Chemotaxis. PMN aggregation was measured following a modification (21) of the method of Craddock et al. (22). The PMN were resuspended at 1.5×10^6/ml in TT-BSA containing 1.5 mM Ca^{2+} and Mg^{2+}; 0.45 ml of this suspension were incubated in a silicone-coated cuvette of an aggregometer (Elvi 840, Milan, Italy). After 2 min at 37°C, 50 μl of C5a-des-Arg preparation were added. The resulting changes in light transmission were recorded as ΔT. Chemotaxis assays were carried out in Boyden chambers according to Venge (23). 1.5×10^6 PMN in 0.5 ml of TT-BSA were placed in the upper chamber that was separated from the bottom one by a Millipore filter of 3-μm pore size and 150-μm thickness. The bottom chamber contained TT-BSA with or without 0.2 μg/ml of C5a. After a 75-min incubation at 37°C, the upper chamber was emptied and washed with PBS containing 2 mM EDTA to remove the cells that had not entered into the filters. To quantitate chemotaxis, the filters were removed, washed in PBS, and stained with 0.2% crystal violet in 10% ethanol. The filters were thoroughly washed with water and the dye was eluted with 33% acetic acid to measure the A_{540}.

Arthus Reaction and Intradermal Injection of C5a or TNF. 20 mg of BSA and 25 mg of Evans blue in 0.5 ml of PBS were injected intravenously in female Lewis rats (130–150 g) per 100 g body weight. After 30 min, we injected intradermally 80 μg of purified rabbit anti-BSA antibody in 0.1 ml of PBS to induce an Arthus reaction (24). In control experiments, we injected 80 μg of nonimmune rabbit Ig. An injection of Evans blue alone was followed after 30 min by intradermal injection of either 50 μg C5a or 0.2 μg rTNF in 0.1 ml PBS. We injected PBS alone in control experiments. Antiflammin-2 or a control peptide were injected intradermally. The PAF receptor antagonist SRI 63072 was injected intraperitoneally (2.5 mg/100 g body weight). The rats were killed after 3 h and the area of blueing was first measured. Skin discs ~2.5 cm in diameter were then excised, fixed in 10% formalin, pH 7.2, embedded in paraffin, and processed for light microscopy examination. PMN present around venulae and arterioles in the deep layer of dermis were counted in an area of 690 × 46 μm in the center of the site of intradermal injections. The results are expressed as PMN per 0.032 mm^2.

Results

Antiflammins Inhibit PAF Synthesis. The peptide HDMNKVLDL (AF-2) corresponding to residues 246–254 of lipocortin I (12) inhibited PAF synthesis induced by rTNF in macrophages with an IC_{50} of ~100 nM (Fig. 1). The peptide MQMKKVLDS (AF-1) corresponding to region 39–47 of uteroglobin (13) was much less inhibitory for PAF synthesis than AF-2 (Fig. 1). The peptide WKLFKKIEKV was used as a control since it is similar in size to antiflammins but it is not inhibitory for PLA_2 (Mukherjee, A. B., personal communication). This peptide had no effect on PAF synthesis (data not shown).

To establish whether antiflammins are inhibitory in different cell types and for different species, we examined the effect of AF-2 on PAF synthesis also in human PMN and endothelial cells. At 100 nM concentration, this peptide inhibited PAF synthesis ~80% in rTNF-treated PMN and ~60% in PMN stimulated by phagocytosis or elastase (Table I). It should be pointed out that elastase did not inactivate AF-2, since its inhibitory activity did not decrease after a 30-min incubation with 1 μg/ml of this proteinase. PAF synthesis was also inhibited by 100 nM AF-2 in endothelial cells stimulated by thrombin (Table II) and in rat macrophages stimulated by phagocytosis (data not shown). Incubation with AF-2 had no effect on cell viability, since >95% of the endothelial cells and >90% of PMN stimulated by various

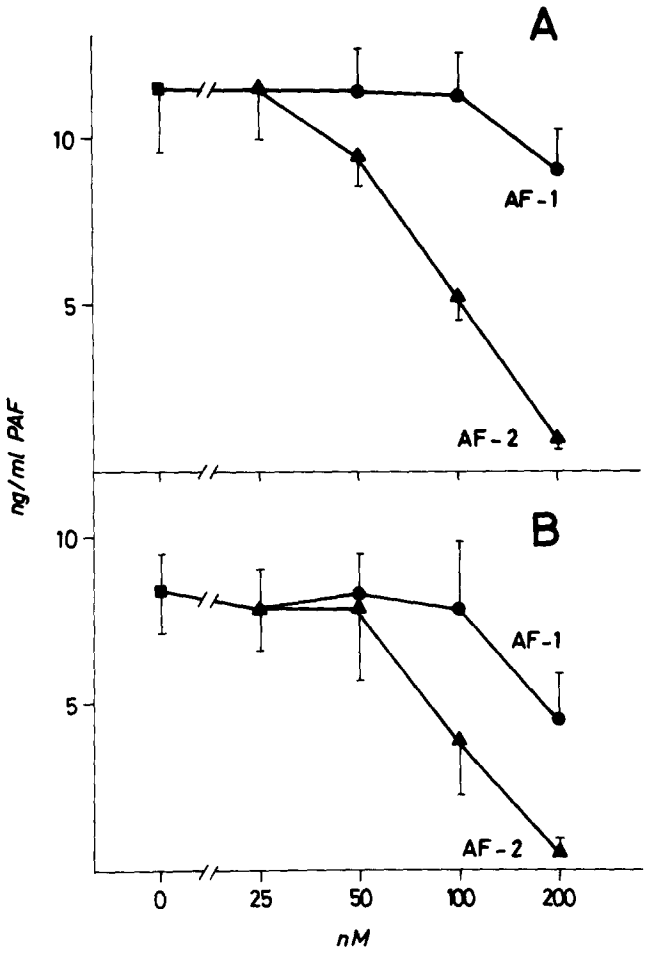

FIGURE 1. Antiflammins inhibit PAF synthesis induced by rTNF in rat peritoneal macrophages. These cells were preincubated for 30 min at 22°C with the concentration of AF-1 or AF-2 shown in the abscissa and then stimulated with 20 ng/ml of rTNF for 10 min at 37°C. (*A*) PAF released into the medium; (*B*) PAF cell-associated. The mean ± SD of three experiments is reported.

treatments excluded trypan blue. Therefore, AF-2 inhibited PAF synthesis in different cells stimulated by a variety of agents without apparent toxicity.

The inhibition of PAF synthesis by AF-2 was reversible when this peptide was removed by washing the cells. PMN and macrophages preincubated with 100 nM AF-2, washed and then stimulated by rTNF or phagocytosis, synthesized PAF in amounts comparable to control cells (Table III). These cells were preincubated without AF-2 and washed in the same way, since this treatment was found to reduce somewhat PAF synthesis in response to different stimuli. These results show that the continuous presence of AF-2 is required to inhibit PAF synthesis.

Inactivation of Antiflammins. In the experiments described above, we used 0.1 mM stock solutions of antiflammins dissolved in buffer containing 10 mM ME or 1 mM dithiothreitol. These reducing agents were added to protect methionine residues from oxidation and were present in incubations with different cells at concentrations up to 50 μM. Control experiments were carried out with PMN incubated in medium containing 0.1–1 mM ME, but no inhibition of PAF synthesis was detected (data

TABLE I

Antiflammin-2 Inhibits PAF Synthesis and Acetyl-CoA:lyso-PAF Acetyltransferase Activity Induced by Different Stimuli in Human Neutrophils

Inducer	Antiflammin-2	PAF		Acetyltransferase
		Released	Cell-bound	
	nM	*ng/ml*		*nmol/min/mg*
None	–	0.4 ± 0.2	0.4 ± 0.3	0.5 ± 0.1
rTNF	–	6.2 ± 1.3	4.5 ± 0.9	4.9 ± 1.5
rTNF	100	1.6 ± 0.7	1.1 ± 0.9	1.2 ± 0.2
rTNF	50	3.5 ± 0.9	2.8 ± 1.2	1.5 ± 0.2
BYS-C3b	–	10.4 ± 2.3	7.8 ± 2.5	6.4 ± 1.3
BYS-C3b	100	4.5 ± 0.8	2.1 ± 1.1	1.3 ± 0.2
BYS-C3b	50	6.3 ± 1.1	5.9 ± 1.3	1.8 ± 0.1
Elastase	–	7.4 ± 1.1	4.7 ± 1.2	3.8 ± 1.2
Elastase	100	2.5 ± 0.5	1.9 ± 1.3	0.9 ± 0.6

Antiflammin-2 was added for 30 min to PMN kept at 22°C; control PMN were preincubated without additions. PMN were stimulated with 10 ng/ml of rTNF or 1 μg/ml of elastase for 10 min, and with BYS-C3b for 20 min at 37°C. PAF released from 2 × 10^6 PMN and cell-associated was measured as described in Materials and Methods. Cell homogenates were prepared to assay the acetyl-CoA:lyso-PAF acetyltransferase activity (expressed in nmol lyso-PAF acetylated/min/mg of protein. The mean ± SD of PAF and acetyltransferase values obtained in three experiments are shown.

not shown). This finding indicated that ME alone had no effect on PAF synthesis. However, the antiflammins lost their inhibitory activity when the reducing agents were omitted from the incubations or stock solutions of peptides were frozen. We have no explanation for the loss of activity of antiflammins upon freezing, but we routinely kept antiflammin stock solutions at 2°C.

Experiments with PMN showed that AF-1 did not inhibit PAF synthesis after a 30 min preincubation at 22°C; such preincubation had little effect on the inhibitory activity of AF-2 (Fig. 2). In these experiments, PMN were stimulated by rTNF or phagocytosis after an increasing preincubation with antiflammins. Without preincubation, 500 nM AF-1 partially inhibited PAF synthesis; after 5 min preincubation, it was no longer inhibitory (Fig. 2). These results suggest that AF-1 is inacti-

TABLE II

Antiflammin-2 Inhibits PAF Synthesis and Acetyl-CoA:lyso-PAF Acetyltransferase Activity Induced by Thrombin in Human Endothelial Cells

Antiflammin-2	Cell-bound PAF	Acetyltransferase
nM	*ng/ml*	*nmol/min/mg*
Untreated control	0.4 ± 0.2	0.2 ± 0.1
0	5.8 ± 1.8	2.8 ± 0.9
100	3.7 ± 0.8	1.8 ± 0.3
500	0.3 ± 0.1	0.3 ± 0.2

Endothelial cells were treated with AF-2 as described in Table I and stimulated with 0.2 U/ml of thrombin for 30 min. The mean ± SD of PAF and acetyltransferase values determined in three experiments are shown.

TABLE III

Reversal of the Inhibitory Effect on PAF Synthesis by Washing Neutrophils or Macrophages after a Pretreatment with Antiflammin-2

Cells	Inducer	Antiflammin-2	PAF	
			Released	Cell-bound
		nM	*ng/ml*	
Neutrophils	rTNF	–	3.1 ± 1.2	1.5 ± 1.8
	rTNF	100	2.9 ± 1.1	1.3 ± 1.1
	BYS-C3b	–	8.2 ± 1.3	7.1 ± 1.9
	BYS.C3b	100	7.8 ± 1.2	6.9 ± 3.1
Macrophages	BYS-C3b	–	5.2 ± 1.3	3.1 ± 1.9
	BYS-C3b	100	4.2 ± 0.5	3.9 ± 1.8

The cells were preincubated with or without AF-2 for 30 min at 22°C, washed twice with TT-BSA, and treated with 10 ng/ml rTNF for 10 min or with BYS-C3b for 20 min. PAF released into the culture medium or cell-associated was measured as described in Table I. The mean ± SD values obtained in three experiments are shown.

vated by PMN. Therefore, the stability of different antiflammins may be important for their inhibitory activity. Furthermore, AF-2 inhibited PAF synthesis without a detectable lag.

Antiflammin-2 Inhibits Enzymatic Activities Induced for PAF Synthesis. According to Miele et al. (15), antiflammins at a 50 nM concentration inhibit ~90% of isolated porcine pancreatic PLA_2 activity in an in vitro assay. To confirm these data in a different system, we assayed the effect of antiflammins on PLA_2 activity in PMN homogenate. In this assay (see Materials and Methods), ~85% of the substrate was hydrolyzed in 1 h by homogenate of untreated PMN; 100 nM AF-2 inhibited this hydrolysis ~50%, whereas AF-1 was much less inhibitory (Table IV). It should be pointed out that this assay did not measure a specific PLA_2 activity but rather the combined activity of different PMN phospholipases that can hydrolyse the labeled substrate previously used by Blackwell et al. (17). Our results are in agreement with

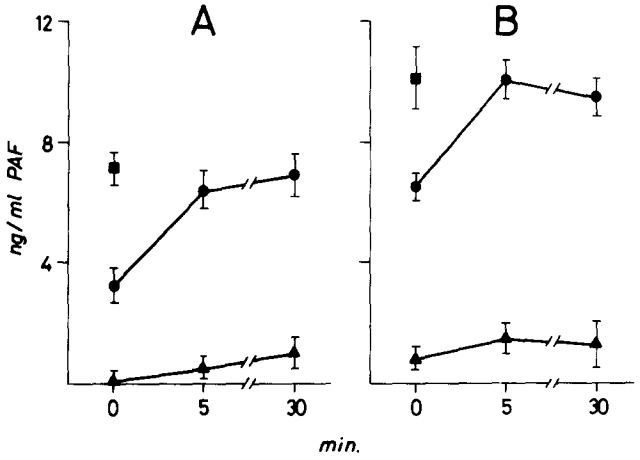

FIGURE 2. The effect of preincubation with human neutrophils on the inhibitory activity of antiflammins. PMN were preincubated with 500 nM AF-1 (●) or 200 nM AF-2 (▲) for the time shown in the abscissa and then stimulated with 10 ng/ml of rTNF (*A*) or by phagocytosis of yeast spores (*B*). PAF released into the medium is reported together with values for control cells incubated without antiflammins (■). Table I shows control values for released PAF of unstimulated cells. The mean ± SD of three experiments is reported.

TABLE IV

Antiflammins Inhibit Phospholipase A_2 Activity in Neutrophils Homogenate

Additions	nM	Hydrolysis	Inhibition
		%	%
None	–	1.59 ± 1.2	–
AF-1	100	1.34 ± 0.3	16
AF-1	1,000	0.31 ± 0.1	81
AF-2	10	1.71 ± 0.6	0
AF-2	100	0.75 ± 0.2	53
AF-2	200	0.41 ± 0.1	74

The PLA_2 activity was measured in sonicated human PMN according to Blackwell et al. (17). The [^{14}C]oleic acid hydrolyzed per microgram of protein is indicated as a percent of the substrate input. The mean ± SD values obtained in three experiments are shown.

those reported by Miele et al. (15) with isolated PLA_2, with the exception of the low inhibitory activity of AF-1. This peptide is apparently less active than AF-2 both in intact cells and in the crude cell-free system used in the present experiments.

To evaluate the effect of antiflammins on PLA_2 activity in intact cells, we labeled PMN with [^{14}C]arachidonic acid. The release of label in the supernatant was considered to reflect the cellular PLA_2 activity. TNF or phagocytosis stimulated PMN to release 2.3- and 5.4-fold more label than control cells, respectively; AF-2 inhibited such release with an IC_{50} of ~100 nM (Fig. 3 *A*). This IC_{50} is similar to that reported above for the inhibition of PAF synthesis by AF-2, suggesting that this peptide inhibits a phospholipase activity involved in both release of label from PMN and synthesis of PAF.

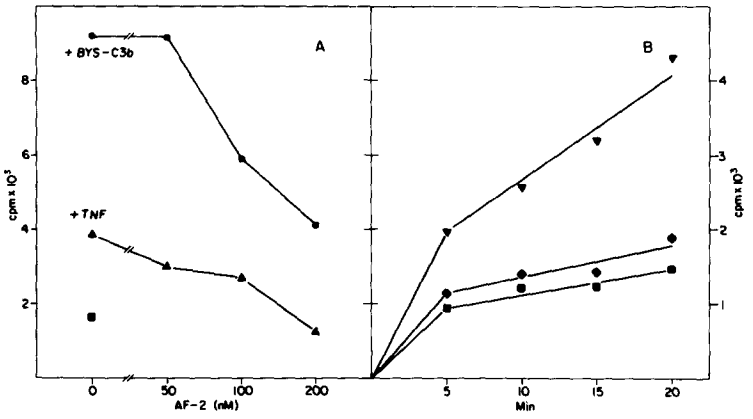

FIGURE 3. Antiflammin-2 inhibits the release of label from PMN preincubated with [^{14}C]arachidonic acid and stimulated by rTNF or phagocytosis. Human PMN were first labeled and then stimulated with 10 ng/ml of rTNF or with baker's yeast spores (BYS-C3b), as described in Materials and Methods. The AF-2 was added 5 min before stimulation. (*A*) The label released in the supernatant after 20 min by 2.5 × 10^6 PMN is shown for untreated cells (■) and for rTNF (▲) or BYS-C3b-treated cells (●). (*B*) The kinetics of label release from 1.25 × 10^6 PMN is shown for cells untreated (■) and stimulated by phagocytosis with (◆) or without (▼) 200 nM AF-2. The experiments were carried out in triplicate and the SD (not shown) was <10%.

The kinetics of label release was examined in PMN preincubated with [^{14}C]arachidonic acid and stimulated by phagocytosis (Fig. 3 *B*). Control cells released some label in the first 5 min of incubation and relatively little afterwards; PMN stimulated by phagocytosis released gradually up to 7% of the label incorporated during the preincubation in 20 min (the last point examined). This release was drastically inhibited by 200 nM AF-2 (Fig. 3 *B*).

The inhibition of PLA_2 activity by antiflammins may by itself account for their effect on PAF synthesis. However, the acetyl-CoA:lyso-PAF acetyltransferase is also induced in cells stimulated to produce PAF (1). It was therefore of interest to investigate the effect of antiflammins on this enzymatic activity. AF-2 did not significantly inhibit the acetyltransferase when added to homogenates prepared from PMN or macrophages stimulated by rTNF or phagocytosis. As an example, the homogenate of PMN stimulated by phagocytosis acetylated 6.3 nmol of lyso-PAF/min/mg of protein vs. 5.9 nmol in the presence of 100 nM AF-2. However, the activation of the acetyltransferase was inhibited when the cells were treated with AF-2 before stimulating PAF synthesis. Homogenates of TNF-treated rat macrophages showed a ~10-fold increase in acetyltransferase activity over control cells; addition of AF-2 to the culture medium inhibited this increase (Fig. 4). The acetyltransferase activity was 8–12-fold higher in homogenates of human PMN stimulated by TNF, phagocytosis, or elastase than in control untreated cells; 50 nM AF-2 inhibited this induction of acetyltransferase activity ~75% and 100 nM AF-2 inhibited >85% (Table I). We observed a similar inhibition in endothelial cells stimulated by thrombin (Table II). Therefore, AF-2 inhibited the activation of acetyltransferase at somewhat lower concentrations than those inhibitory for PAF synthesis.

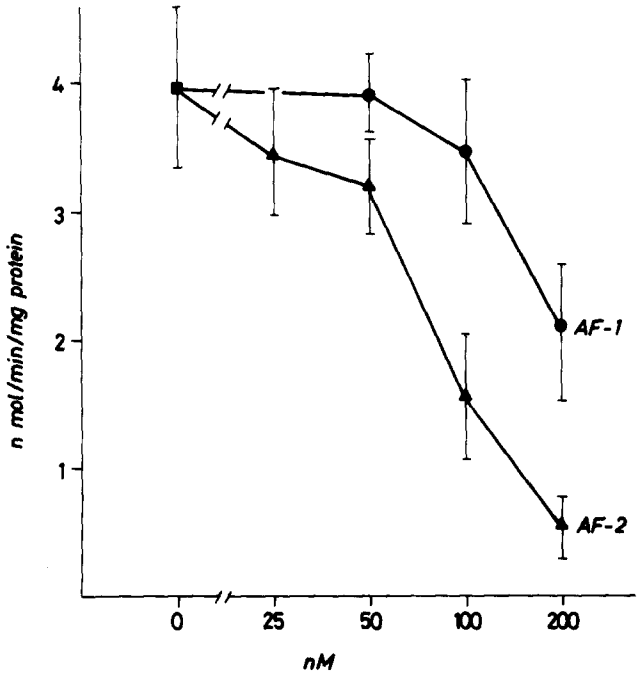

FIGURE 4. Antiflammins inhibit the acetyl-CoA:lyso-PAF acetyltransferase induced by rTNF in rat peritoneal macrophages. These cells were preincubated with AF-1 or AF-2 and then stimulated with rTNF, as indicated in Fig. 1. Cell homogenates were prepared and assayed for acetyltransferase activity. The mean ± SD of three experiments is shown.

TABLE V

Effect of lyso-PAF on the Inhibition of PAF Synthesis by Antiflammin-2 or p-Bromo-Diphenacylbromide in Neutrophils Stimulated by Phagocytosis

Additions	Antiflammin-2	PAF	
		Released	Cell-bound
	nM	*ng/ml*	
Untreated	–	0.4 ± 0.2	0.3 ± 0.2
Control	–	10.2 ± 2.5	9.7 ± 1.7
Control	100	5.1 ± 1.3	3.2 ± 1.2
Lyso-PAF	–	12.2 ± 1.9	11.2 ± 1.9
Lyso-PAF	100	4.2 ± 1.2	2.1 ± 1.7
PBDB	–	3.2 ± 1.5	1.7 ± 1.9
PBDB + lyso-PAF	–	12.3 ± 1.8	10.2 ± 1.9

A preincubation (10 min at 22°C) with AF-2 or 1 μM PBDB preceded the stimulation with BYS-C3b (20 min at 37°C). Control samples were treated with BYS-C3b only or with BYS-C3b and AF-2. Where indicated, we added 100 μM lyso-PAF during the incubation at 37°C. The antiflammin and PBDB were present throughout this incubation. The mean ± SD obtained in three experiments are shown.

This inhibition of acetyltransferase activation is surprising, since AF-2 is supposedly a specific PLA_2 inhibitor (15). Experiments carried out with PMN provided further evidence for this effect of AF-2 (Table V). Addition of the PLA_2 product lyso-PAF failed to promote PAF synthesis in PMN stimulated by phagocytosis but treated with AF-2. In contrast, lyso-PAF promoted PAF synthesis when the PLA_2 activity was inhibited by *p*-bromo-diphenacylbromide (PBDB). These results suggest that PBDB inhibits PLA_2 but not the acetyltransferase, whereas AF-2 inhibits both enzymatic activities. Such inhibition is not explained by a requirement for increased PLA_2 activity to induce the acetyltransferase, since in PMN treated with PBDB concentrations inhibitory for PLA_2 (2) the acetyltransferae is activated. For example, homogenates of control PMN acetylated 0.4 nmol of lyso-PAF/min/mg protein; homogenates of PMN treated with 1 μM PBDB and then stimulated by TNF or phagocytosis acetylated 3.2 and 6.1 nmol of lyso-PAF, respectively.

Antiflammins Inhibit Aggregation and Chemotaxis of Neutrophils. Stimuli that induce PAF release by PMN, such as C5a-des-Arg (20), promote aggregation of these cells (22, 25). To establish whether antiflammins inhibit this biological activity mediated by PAF, we treated human PMN with C5a-des-Arg for 2 min. We measured PMN aggregation by recording light transmission in a cuvette (21); 100 nM AF-2 inhibited completely PMN aggregation, but 100 nM AF-1 inhibited ~60% (Fig. 5 *A*). A control peptide did not inhibit PMN aggregation (Fig. 5 *A*). The PAF receptor antagonist SRI 63072 was ~70% inhibitory at 5 μM concentration (data not shown). These results show that antiflammins are potent inhibitors of a biological response mediated by PAF production. AF-1 may be more active in this assay because of the relatively short incubation time.

We examined next the effect of antiflammins on the stimulation of PMN chemotaxis by C5a. This assay was performed in Boyden chambers containing 0.2 μg/ml of C5a in the bottom compartment. The PMN migrating into the filter separating the two chambers were measured by staining with crystal violet (see Materials and Methods).

Both antiflammins were highly inhibitory for neutrophil chemotaxis (Fig. 5 *B*). In contrast, 5 μM SRI 63072 inhibited chemotaxis only ~25%. This finding indicates that antiflammins are inhibitory for the leukotactic activity of C5a that is presumably mediated by eicosanoids rather than by PAF, since it is marginally sensitive to a PAF receptor antagonist (26).

Antiflammin-2 Inhibits the Increase in Vascular Permeability and Leukocyte Infiltration Induced by an Arthus Reaction, C5a or TNF. A reverse passive Arthus reaction was induced in Lewis rats by intravenous injection of BSA and Evans blue followed after 30 min by intradermal injection of anti-BSA antibody (24). The rats were killed 3 h after the last injection and the area of Evans blue extravasation was first measured (Table VI). Circular skin areas were then excised and processed for light microscopy examination as described in Materials and Methods. We counted the PMN present around vessels at the center of the intradermal injection to quantitate leukocyte infiltration (see Materials and Methods). The Arthus reaction was characterized by severe inflammatory lesions in dermis around vessels, by edema and focal interstitial hemorrhage associated with increased vascular permeability, as judged by the extravasation of Evans blue (Table VI). An intradermal injection of 100 ng of AF-2 together with the anti-BSA antibody suppressed this increase in vascular permeability and the leukocyte infiltration. The PAF receptor antagonist SRI 63072 was less inhibitory than AF-2 (Table V) when injected intraperitoneally at 2.5 mg/100 g body weight (24).

In other experiments, we examined the effect of AF-2 on the increased vascular permeability and leukocyte infiltration induced by C5a or rTNF injected intradermally 30 min after Evans blue. Injection of C5a resulted in extravasation of this

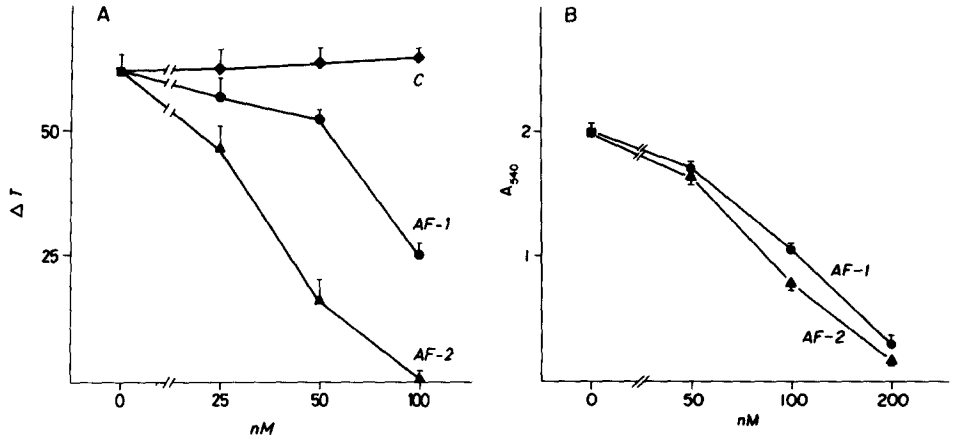

FIGURE 5. Antiflammins inhibit neutrophils aggregation induced by C5a-des-Arg (*A*) and chemotaxis induced by C5a (*B*). In the assay for aggregation, 0.45 ml of PMN suspension were incubated with 50 μl of C5a-des-Arg for 2 min, as described in Materials and Methods. The change in light transmission caused by PMN aggregation is reported as ΔT. PMN chemotaxis was measured in Boyden chambers as described in Materials and Methods and the A_{540} of cells that entered into the filters was measured after staining with crystal violet. The incubations contained no added peptide (■), AF-1 or AF-2, and the control peptide WKLFKKIEKV (◆) at the concentrations indicated in the abscissa. The mean ± SD of three experiments is reported.

TABLE VI

Effect of AF-2 and the PAF Antagonist SRI 63072 (SRI) on the Increase in Vascular Permeability and Leukocyte Infiltration Induced in Rats by an Arthus Reaction (BSA + anti-BSA), and by C5a or TNF

Treatment	Additions					
	–	AF-2	SRI	–	AF-2	SRI
	*mm**			*PMN/0.032 mm²*		
BSA + anti-BSA	17 ± 2	2 ± 1	7 ± 3	46 ± 12	3 ± 2	11 ± 4
C5a	15 ± 3	4 ± 1	11 ± 4	26 ± 8	3 ± 2	21 ± 3
TNF	7 ± 2	0	0	12 ± 4	3 ± 1	2 ± 1
Control	3 ± 1	ND	ND	3 ± 1	ND	ND

Treatments, additions, and examination of skin samples are described in Materials and Methods. The BSA was injected intravenously. The anti-BSA antibody, 50 μg of C5a, 0.2 μg of rTNF, and 0.1 μg of AF-2 were injected intradermally; 2.5 mg of SRI/100 g body weight were injected intraperitoneally. The mean ± SD values obtained in three experiments are shown.

* Diameter of blueing.

dye and leukocyte infiltration (Table VI). The AF-2 inhibited this activity of C5a, but the PAF receptor antagonist was scarcely inhibitory. Intradermal injection of rTNF increased vascular permeability and leukocyte infiltration much less than other treatments and leukocytes were mainly accumulated in the lumen of vessels as intravascular aggregates adherent to the endothelium. Both AF-2 and SRI 63072 inhibited this activity of rTNF (Table VI). These results show that AF-2 inhibits the increase in vascular permeability and leukocytes infiltration induced by all treatments tested. In contrast, the PAF receptor antagonist inhibits marginally the effect of C5a. This finding suggests that antiflammins inhibit a response to C5a that is not apparently mediated by PAF.

Discussion

The antiflammins are potent inhibitors of PAF synthesis induced in macrophages and PMN by rTNF or phagocytosis, and in endothelial cells by thrombin. These findings suggest that antiflammins inhibit enzymatic activities required for the synthesis of PAF, in agreement with the report by Miele et al. (15) that these peptides inhibit isolated PLA_2. Two features of the inhibitory activity of antiflammins were discovered by studying their effect on PAF synthesis in intact cells: (*a*) these peptides inhibit PAF synthesis without a significant lag; and (*b*) this inhibition is reversed by washing cells preincubated with antiflammins. This finding shows that antiflammins do not irreversibly inactivate enzymatic activities.

We measured the release of label from cells preincubated with [^{14}C]arachidonic acid to estimate the PLA_2 activity in PMN stimulated by rTNF or phagocytosis. Although arachidonic acid may be released from cellular phospholipids by the phospholipase C-diacylglycerol lipase pathway, it is well documented that in PMN most of the arachidonic acid is released by direct deacylation of phospholipids by PLA_2 (27). At the present time, this is the most convenient assay for PLA_2 activity in intact cells. In such an assay, antiflammins are inhibitory only in the presence of a reducing agent such as ME. This finding suggests that antiflammins are inactivated by oxida-

tion. However, AF-2 inhibits PAF synthesis even after a 30-min preincubation with PMN, whereas AF-1 is not inhibitory after a 5-min preincubation (Fig. 2). This finding shows that PMN inactivate AF-1 much faster than AF-2. The only differences in amino acid sequence between these antiflammins are the MQ→HD substitutions in residues 1–2, and the S→L substitution in residue 9. It is possible that the NH_2-terminal Met of AF-1 is oxidized by PMN secretory products. However, this Met residue is not essential for the biological activity of antiflammins, since it is substituted by His in AF-2 (15).

The first two residues of AF-1 can be replaced but not deleted without loss of activity, suggesting that the length of antiflammins is critical, possibly for conformational reasons (15). Oxidation of the NH_2-terminal Met may disrupt the conformation of AF-1 and account for the lower inhibitory activity of this peptide. However, we cannot exclude that AF-1 is less inhibitory than AF-2 for other reasons (e.g., cleavage by a proteinase with trypsin-like specificity of the Lys-Lys peptide bond that is not present in AF-2). Oxidation of the Met residue in position 3 may result in inactivation of all antiflammins, as suggested by the loss of activity after a 5-min incubation with 1 mM H_2O_2 (our unpublished observations). This Met residue may be less sensitive to oxidation than an NH_2-terminal Met since only AF-2 remains active in the presence of reducing agents.

Another novel finding is the inhibition of acetyl-CoA:lyso-PAF acetyltransferase activity in cells treated with antiflammins (Fig. 4). However, antiflammins do not inhibit this enzymatic activity in an in vitro assay with cell homogenate. This finding cannot be explained by AF-2 inactivation, since this peptide inhibits PLA_2 activity in PMN homogenate (Table IV). A major difference between the assays for PLA_2 and for acetyltransferase may possibly account for the lack of inhibition of the latter enzyme. PLA_2 can be activated in cell-free systems whereas the acetyltransferase can be activated only in intact cells. The activation of certain PLA_2 under specific conditions apparently involves dimerization of this enzyme (28–29). These findings cannot be presently generalized and certainly cannot be extrapolated to the action of PLA_2 on phospholipids in biological membranes. However, they provide an example of a specific activation mechanism for PLA_2. Our findings are consistent with the hypothesis that antiflammins impair PAF synthesis by inhibiting the activation of both PLA_2 and acetyltransferase. Such hypothesis explains why these peptides do not inhibit in cell-free systems the acetyltransferase already activated in intact cells.

Antiflammins inhibit neutrophil chemotaxis, and the increase in vascular permeability and leukocyte infiltration induced by C5a. These inflammatory responses are apparently not mediated by PAF since they are not significantly inhibited by the PAF antagonist SRI 63072. This indicates that antiflammins inhibit synthesis of other inflammatory mediators, such as leukotrienes, derived from arachidonic acid. Leukotriene B_4 is a potent chemotactic agent that mediates changes in vascular permeability and may be involved in the response to C5a (30). By inhibiting PLA_2, the antiflammins block synthesis of all eicosanoid mediators produced from arachidonic acid by specific enzymes. Therefore, antiflammins may display a wider antiinflammatory activity than drugs active on single enzymes, such as cyclooxygenase or lipooxygenase inhibitors.

The antiflammins are promising antiinflammatory agents. Our observations suggest that these peptides may produce a striking pharmacological effect at nM con-

centration. An intradermal injection of 100 ng of AF-2 suppresses the inflammatory response in an Arthus reaction (Table VI). This suppression is observed with a lower antiflammin dose than that used to inhibit the carrageenan-induced rat paw edema (15). Injection of ~500 μg AF-2 in the rat subplantar space inhibits 96% of the swelling caused by carrageenan, but lower amounts are much less inhibitory (15). These two inflammatory reactions are quite different and the effect of antiflammins cannot be meaningfully compared. Furthermore, in our experiments the antiflammins were injected together with 10 μM ME that may enhance their activity. In conclusion, AF-2 or another peptide of similar activity, but with the Met replaced by another amino acid residue to improve its stability, are good candidates as antiinflammatory agents in acute and chronic diseases.

Summary

Synthetic peptides corresponding to the region of highest similarity between human lipocortin I and rabbit uteroglobin inhibit phospholipase A_2 and show potent antiinflammatory activity on the carrageenan-induced rat footpad edema (15). The peptide HDMNKVLDL (antiflammin-2) inhibits the synthesis of platelet-activating factor (PAF) induced by TNF or phagocytosis in rat macrophages and human neutrophils, and by thrombin in vascular endothelial cells. The peptide MQMKKVLDS (antiflammin-1) is less inhibitory than antiflammin-2 for macrophages and not inhibitory for neutrophils after a 5-min preincubation. This finding suggests that antiflammin-1 is inactivated by neutrophils secretory products, possibly oxidizing agents. Synthesis of PAF is inhibited by antiflammin-2 without an appreciable lag, but this inhibition is reversed when neutrophils or macrophages are washed and incubated in fresh medium. Therefore, antiflammins must be continously present to inhibit PAF synthesis. Antiflammins block activation of the acetyltransferase required for PAF synthesis, suggesting that this enzyme is another target for the inhibitory activity of antiflammins. These peptides inhibit neutrophil aggregation and chemotaxis induced by complement component C5a. Antiflammin-2 suppresses the increase in vascular permeability and the leukocyte infiltration induced in rats by an Arthus reaction or by intradermal injection of rTNF and C5a.

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