Serum amyloid A-derived peptides, present in human rheumatic synovial fluids, induce the secretion of interferon-γ by human CD_4^+ T-lymphocytes

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Abstract Serum amyloid A (SAA) is a major acute-phase protein whose biochemical functions remain largely obscure. Human rheumatic synovial fluids were screened by high performance liquid chromatography mass spectrometry for SAA-derived peptides, specifically the sequence AGLPEKY (SAA_{98-104}) which was previously shown to modulate various leukocyte functions. Two such fluids were found to contain a truncated version of SAA_{98-104}. Synthetic SAA_{98-104} and several of its analogs were shown capable of binding isolated human CD_4^+ T-lymphocytes and stimulating them to produce interferon-γ. Given the high acute-phase serum level of SAA and its massive proteolysis by inflammatory related enzymes, SAA-derived peptides may be involved in host defense mechanisms.

Key words: Serum amyloid A; Inflammation; Interferon-γ; T-lymphocyte

1. Introduction

Human serum amyloid A (SAA) is a major acute-phase protein (APP), whose blood concentration may soar dramatically, as high as 1000-fold above the normal level during inflammatory events. However, the precise biochemical and immunological roles of this APP have not been fully established. SAA, a single chain 104 amino acid protein, exists in the body in complex with the third fraction of high density lipoprotein, and is primarily synthesized by hepatocytes [1]. As inflammation subsides, SAA is quickly cleared from the plasma and very low basal levels are re-established [2–4]. Although the catabolic pathway of SAA has not been completely defined, studies with isolated human neutrophils and monocytes provided evidence for SAA degradation by cell-associated elastase-like proteases [5–8]. Several other serine proteases found in serum, including kallikrein and plasmin, also have been shown to degrade SAA [9,10]. However, the particular sites cleaved by the various proteases were not identified in these studies. Recently, the effects of neutrophil elastase and cathepsin B on SAA degradation were examined [11]. Both enzymes degraded SAA at the carboxy-terminus and generated fragments comprised of the first 58 or 76 residues of SAA.

In previous studies, we have demonstrated that SAA is proteolysed by the lysosomal enzymes of human neutrophils to generate peptides which may be intimately involved in the acute-phase response [12]. Markedly enhanced levels of SAA and neutrophils are found in various inflammatory diseases in which synovial fluid accumulates within joints [13]. Therefore, we screened human inflammatory synovial fluids for specific SAA-derived peptides, previously shown in our laboratory to induce interferon-γ (IFNγ) secretion by human T-lymphocytes (Oren Rosen, 1995, Ph.D. thesis, Weizmann Institute of Science, unpublished data).

2. Materials and methods

Unless otherwise stated, all chemicals and reagents were obtained from Sigma Chemicals (St. Louis, MO, USA). Phosphate-buffered saline (PBS), HEPES, DMEM medium and RPMI 1640 medium were obtained from the Biological Services of the Weizmann Institute of Science. Tissue culture reagents were purchased from Beit-Haemek Biological Industries. Human recombinant SAA (rSAA) was kindly donated by Peprotec Ltd. (Ness-Ziona, Israel). Human recombinant IFNγ was purchased from Cytolab Ltd. (Ness-Ziona, Israel). Recombinant human SAA was purified from bacterial phospholipids as previously described [14]. From 1 g of crude SAA, 28 mg of pure SAA was obtained.

2.1. Synovial fluid processing

Synovial fluids (approximately 50 ml each) were collected following knee perfusions, centrifuged for 15 min at 3000 rpm and stored at −20°C. The cell free solutions were thawed and diluted with three volumes of ethanol and allowed to stand overnight at 4°C. The white precipitates, composed primarily of proteoglycans, were then centrifuged for 15 min at 5000 rpm (4°C), the supernatants were evaporated to a minimum volume, suspended in 10% trichloroacetic acid (TCA) and allowed to stand overnight at 4°C to pellet the high molecular weight proteins. The solutions were centrifuged for 15 min at 8000 rpm (4°C) and the supernatants extracted with three volumes of cold ether. The processed fluids were then purified by high performance liquid chromatography (HPLC) (Spectra-Physics SP-8800) using a prepacked Lichrospher-100 RP-18 column (4×25 mm, 5 μm bead size) employing a binary gradient formed from 0.1% trifluoroacetic acid (TFA) in water (solution A) and 0.1% TFA in acetonitrile (solution B). The typical elution gradient used was: $t = 0$ min B = 5%, $t = 5$ min B = 5%, $t = 65$ min B = 70% (flow rate 0.8 ml/min). Solvents and columns were obtained from Merck (Darmstadt, Germany).

2.2. Mass spectrometry (MS)

Electrospray (ES) mass spectra were recorded on an API-III triple quadrupole mass spectrometer (Perkin Elmer Sciex, Thornhill, Ont., Canada) equipped with an ES source using atmospheric pressure ion-

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Abbreviations: APP, acute-phase protein; IFNγ, interferon-γ; PHA, phytohemoagglutinin; SAA, human serum amyloid A

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ization. For MS detection, the positive ion mode was used and the orifice voltage was set to 80 V. For multiple reaction monitoring (MRM) using MS/MS mode, argon was used as a collision gas. For HPLC-MS analysis, samples were chromatographically separated on Nucleosil C-18 2×100 column (Grom, Herrenberg, Germany) using an Applied Biosystems 140A HPLC (Weiterstadt, Germany) connected to the ES source. A linear gradient 10% B to 100% B in 45 min was used (solvent A: 0.1% TFA in water; solvent B: 0.1% TFA in acetonitrile).

2.3. Peptide synthesis

Peptides were prepared by Fmoc strategy of solid phase peptide synthesis using an Abimed AMS-422 peptide synthesizer, purified to above 98% by preparative HPLC, and analyzed using an HP-1090 amino acid analyzer as previously described [15]. Without exception, amino acid analysis of all peptides yielded excellent correspondence to calculated ratios (±10%).

2.4. Iodination of SAA_{98-104}

SAA_{98-104} was labeled with ^{125}I by employing the chloramine T procedure. $Na^{125}I$ (Amersham, UK; 1 mCi, specific activity 644 MBq/μg iodine) was added to a solution of SAA_{98-104} (10 μg) and chloramine T (1.5 μg) in 0.5 M sodium phosphate buffer, pH = 7.5 (11.5 μl). The iodination reaction was stopped after 3 min at room temperature (RT) by addition of sodium metabisulfite (3.5 μg) in sodium phosphate buffer (35 μl). Free radioactive iodine was separated from the labeled protein by adding a solution of KI (20%; 10 μl) followed by loading the reaction mixture on a Sephadex G10 column (0.5×15 cm; Pharmacia, Sweden). The column was eluted with calcium and magnesium free PBS containing 0.1% bovine serum albumin (BSA). The specific activity was 1.5 μCi/μg SAA_{98-104}.

2.5. Peripheral human CD_4^+ T-lymphocytes

Human CD_4^+ T-cells were purified from the peripheral blood leukocytes of healthy donors as previously described [14]. Over 90% of the resulting cells were CD_4^+ T-lymphocytes, as determined by FACScan analysis.

2.6. IFNγ secretion and detection

T-cells were suspended in RPMI medium (RPMI 1640 supplemented with 2% BSA, 1 mM Ca^{2+}, 1 mM Mg^{2+}, 1% sodium pyruvate, 1% glucose and 1% HEPES buffer) and seeded in 24-well plates (Nunc, Denmark) (1.5×10^6 cells/well). The cells were incubated with SAA-derived peptides at various concentrations (1–500 μM) for 18 h, 37°C, in a humidified 10% CO_2 atmosphere. As a positive control of IFNγ secretion, phytohemoagglutinin (PHA) stimulation at a concentration of 1 μg/ml was used. Subsequently, the media contents in wells were collected and centrifuged, and the supernatants were assayed for the cytokine protein level using two-sites sandwich enzyme-linked immunosorbent assay: first antibody attachment to the 96 microtiter wells (Nunc, Denmark) was performed by incubating (18 h, 4°C) purified rat anti-human IFNγ monoclonal antibody (Pharmingen, CA, USA) (2 μg/ml) in a final volume of 50 μl/well. The coating buffer contained 0.1 N $NaHCO_3$, pH 8.2. The plates were then washed twice with 0.05% Tween 20/PBS and blocked with 3% BSA/PBS solution for 1 h at RT. Following extensive washes, supernatants obtained from incubation of T-cells with SAA and SAA peptides were added to the wells (100 μl/well) and left for 2 h at RT. For calibration of secretion, a double-diluted series of recombinant IFNγ was used. Following incubation and washing, biotinylated anti-IFNγ (2 μg/ml, 100 μl/well) was added and left for 2 h at RT. Alkaline phosphatase-conjugated streptavidin (Jackson Immunoresearch Laboratories, PA, USA) (1:1000 dilution, 100 μl/well) was added for 30 min at RT. Following extensive washing and substrate addition (PNPP), the absorbance was measured at 405 nm with a microplate reader (Dynatech, CA, USA).

2.7. Binding and displacement experiments

For binding assays, two series having identical concentrations of ^{125}I-SAA_{98-104} in PBS containing 0.1% BSA were prepared by sequential double dilutions. One, designed for evaluation of non-specific binding, contained 10–0.1 μM SAA_{98-104} and 100–1 nM ^{125}I-SAA_{98-104}, respectively. The other designed for the total binding curve contained only 100–1 nM ^{125}I-SAA_{98-104}. Human T-cells adhered to polystyrene surfaces (96-well microtiter plates; 2×10^5 cells/100 μl/well) were incubated at 4°C for 40 min with appropriate ligands. Following incubation, reaction was stopped by aspirating unbound ligands followed by several washes (three times) with ice cold PBS. Cells were lysed with 0.1 N NaOH solution (150 μl/well) containing 1% Tween 20, the mixture was then transferred to γ-counter tubes and radioactivity was measured (Kontron MD-480). In displacement experiments with ^{125}I-SAA_{98-104}, a concentrated series of either unlabeled SAA_{98-104} or other competing ligands (peptides related to SAA_{98-104}) were prepared in binding mixture (i.e. 40 nM ^{125}I-SAA_{98-104}, 0.1% BSA in PBS). The initial concentration of cold ligands was 10 μM and by sequential double dilutions reached a concentration of 10 nM. Incubation with cells and lysis were performed as described above.

3. Results and discussion

The carboxy-terminal sequence of SAA (AGLPEKY, SAA_{98-104}) was found in a synovial fluid of an arthritic patient. Preliminary results have demonstrated that the peptide stimulates IFNγ production by mononuclear cells as well as inhibiting superoxide production and chemotaxis of activated neutrophils (Oren Rosen, Ph.D. thesis, Weizmann Institute; unpublished data).

To establish the physiological existence of peptides related to SAA_{98-104}, eight additional synovial fluids were processed. These fluids were obtained from patients afflicted with rheumatoid arthritis and processed by ethanol/TCA precipitation (see Section 2). The peptide-containing lyophilized fractions were purified by reversed phase HPLC. Optimal chromatographic conditions were achieved by injecting 25% of each sample (approximately 10–20 mg dry weight containing 0.5–1 mg peptide) on an analytical column and resulted in complex chromatograms containing a large number of unidentified peaks (not shown). Peptide content was estimated by amino acid analysis of combined eluted peaks.

Fractions were collected 3 min prior and after the elution time of the synthetic peptide $AGLPEKY_{98-104}$ chromatographed under identical conditions (i.e. 26–32 min). From the eight synovial fluids evaluated, five samples which contained significant UV absorbing peaks in the elution region of 26–32 min were characterized by HPLC-MS. The SAA-derived peptide $A_{98}GLPEK_{103}$ (614 amu) was unambiguously identified by ES-MS in two different synovial fluids (Table 1). Synthetic AGLPEK peptide was also prepared and shown to match the retention times and molecular weight values obtained from the physiological samples. In a further experiment, MRM was performed which considers only ion intensities from characteristic fragments, directed towards increasing sensitivity. The characteristic fragment 373.2 amu assigned to AGLP in the MRM run was found in both synovial fluids and in the synthetic peptide. $A_{98}GLPEK_{103}$ differs from $A_{98}GLPEKY_{104}$ by a single C-terminus tyrosine residue.

Table 1
Mass spectrometric data of HPLC-purified fractions from two different synovial fluids

Sample	Observed *m/z*	Sequence	Calculated mass
Fluid # 5	614.1 ($M+H^+$)	AGLPEK	614.3 ($M+H^+$)
	636.3 ($M+Na^+$)	AGLPEK	636.3 ($M+Na^+$)
	485.1	fragment: AGLPE	485.5
	485.1	fragment: LPEK	485.5
Fluid # 8	614.2 ($M+H^+$)	AGLPEK	614.3 ($M+H^+$)
	286.8	fragment: GLP	286.3
	228.6	fragment: LP	228.8

Ionization conditions for the two samples are not identical, resulting in different fragmentation patterns.

Table 2
IFNγ secretion activity of human T-lymphocytes, stimulated by SAA-derived peptides, analogs, rSAA and PHA (positive control), measured 20 min following substrate addition (see Section 2)

Substance	Sequence	Concentration	IFNγ secretion (OD units 405 nm)
rSAA	104 amino acids	100 μM	2.0 ± 0.2
Medium	–	–	0.2 ± 0.1
PHA+medium	–	1 mg/ml	1.9 ± 0.2
Peptide **1**	AGLPEKY	100 μM	1.5 ± 0.2
Peptide **2**	AGLPEK	100 μM	0.3 ± 0.1
Peptide **3**	AGLPE	100 μM	0.2 ± 0.1
Peptide **4**	GLPEKY	100 μM	0.2 ± 0.1
Peptide **5**	LPEKY	100 μM	0.2 ± 0.1
Peptide **6**	AGLPEK**A**	100 μM	0.3 ± 0.1
Peptide **7**	AGLPE**A**Y	100 μM	0.2 ± 0.1
Peptide **8**	AGLP**A**KY	100 μM	0.6 ± 0.1
Peptide **9**	AGL**A**EKY	100 μM	0.2 ± 0.1
Peptide **10**	AG**A**PEKY	100 μM	1.1 ± 0.2
Peptide **11**	AGLPEK**F**	100 μM	1.5 ± 0.2
Peptide **12**	AGLPE**R**Y	100 μM	0.9 ± 0.2
Peptide **13**	AGLP**D**KY	100 μM	0.8 ± 0.2
Peptide **14**	AGL-**Sar**-EKY	100 μM	0.2 ± 0.1
Peptide **15**	**YKEPLGA**	100 μM	0.2 ± 0.1

Experiments were performed using leukocytes obtained from three different blood donations. Bold letters denote amino acid substitution. Sar is sarcosine (*n*-methyl-glycine). Peptide **15** is the reverse sequence of SAA_{98-104}.

Cleavage of SAA by a trypsin-like enzyme may perhaps account for formation of a shorter sequence since Arg_{97} and Lys_{103} precede the Ala_{98} and Tyr_{104} residues, respectively.

In order to study bio-activity and structure–function relationships of SAA_{98-104} and several analogs, they were synthesized, purified, analyzed and evaluated for their ability to modulate the IFNγ secretory activity of human CD_4^+ T-lymphocytes, at concentrations comparable with the acute-phase levels of intact SAA (Table 2). SAA-derived peptide **1** (AGLPEKY), similar to the intact rSAA, increased substantially the secretion of IFNγ by human T-lymphocytes. From the various synthetic peptide analogs tested (peptides **2**–**15**), peptide length seems to be crucial for its biological activity. Eliminating C- or N-terminus residues of peptide **1** (peptides **2**–**5**) abolished the IFNγ secretory activity. Systematic single alanine substitutions of residues 100–104 (peptides **6**–**10**) demonstrates the functional importance of the amino acids P_{101}-E-K-Y_{104}. In contrast, the Leu_{100} residue may be replaced by alanine without effecting substantially the IFNγ secretory activity (peptide **10**). Noteworthy, the aromatic ring of Tyr_{104} may be substituted by phenylalanine without loss of stimulatory activity (peptide **11**). In a similar fashion, the positive charge at the Lys_{103} residue and the negative charge at the Glu_{102} residue may be replaced by similarly charged Arg and Asp residues, respectively, with only a 2-fold reduction in activity (peptides **12**–**13**). When Pro_{101} was replaced by sarcosine (a non-bridged analog of proline), the enhancing capability was almost totally abolished (peptide **14**), stressing the unique structural importance of proline in this peptide. The retro-peptide (peptide **15**), used as control compound, proved to have no influence on T-cell IFNγ secretion.

Peptide **1** and its active peptide analogs were found capable of modulating IFNγ secretion levels by T-cell in a dose-dependent manner (Fig. 1). As a rule, higher activities were apparent at peptide concentrations of 100–500 μM.

SAA was previously shown to associate avidly with specific extra-cellular matrix (ECM) moieties [14]. Upon complexation

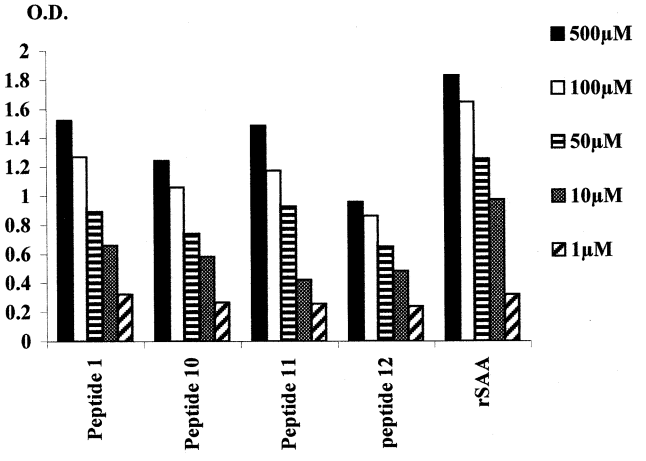

Fig. 1. IFNγ secretion by human T-lymphocytes: a dose-dependent effect. Results are mean values from three experiments with S.D. values below 10%. Medium control, PHA values and experimental conditions are identical to those described in Table 2.

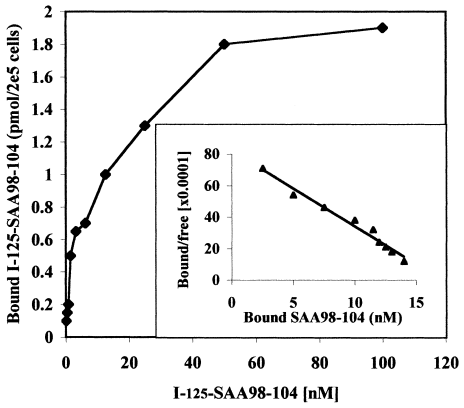

Fig. 2. Specific binding of ^{125}I-SAA_{98-104} to human T-lymphocytes. The mean values from two experiments are shown (S.D. values are below 10%). Insert: Scatchard analysis of the ^{125}I-SAA_{98-104} binding to human T-lymphocytes. Considering a homogeneous population of binding sites, a dissociation constant of 12 nM was found.

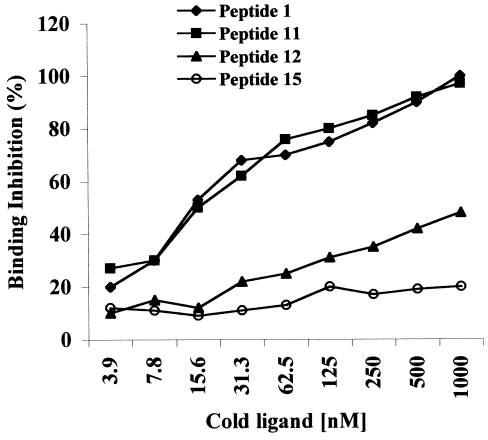

Fig. 3. Inhibition of ^{125}I-SAA_{98-104} binding to human T-lymphocytes by peptide analogs of SAA_{98-104}. The retro-peptide (peptide **15** was used as a negative control). Results are mean values from two experiments (S.D. values are below 10%).

with SAA, immobilized ECM, laminin and vitronectin induced markedly the adhesion of human CD_4^+ T-cells in a β1-integrin-mediated manner. Another study described the binding characteristics of SAA to human neutrophils [12]. Specifically, the C-terminus fragment SAA_{77-104} was shown to inhibit the binding of intact SAA to neutrophils.

To study the mechanism of activity, SAA_{98-104} (peptide **1**) was tested for its ability to bind specifically to human T-lymphocytes. The binding of peptide **1** was saturable and specific (Fig. 2). The plotted data provided curves which were expressed as Scatchard plots upon transforming experimental bound and free values according to the Langmuir binding model. The dissociation constant (K_d) determined was 12 nM, considering an averaged homogeneous population (Fig. 2, insert). Non-specific binding, i.e. the fraction of ^{125}I-SAA_{98-104} detected in the presence of 100-fold excess of unlabeled SAA_{98-104}, represented 18 ± 5% (S.D.) of the total cell-associated radioactivity in the absence of competing ligand.

Competition assays were also performed with synthetic peptides related to SAA_{98-104}. Only peptide **11** completely inhibited the binding of ^{125}I-SAA_{98-104} to human T-lymphocytes. Partial inhibition (50%) was obtained with peptide **12** (Fig. 3). These results correlate well with the stimulatory values determined for peptides **12** and **13** and with the lack of activity observed in peptide **15**.

The issue of the physiological function(s) of SAA is largely unresolved. In view of our present and previous findings [12,14], it is most plausible that SAA, either intact or through its proteolytic fragments, is associated with immune cell function and activation. The levels of SAA in various pathological conditions, such as acute inflammation, may reach concentrations of 1–2 mg/ml (~100–200 μM). Considering the fast proteolysis of SAA by different enzymes, most notably those derived from polymorphonuclear leukocytes, levels of related peptide derivatives may reach similar values. Moreover, local concentrations of SAA-derived peptides may even be much higher at inflicted tissues where leukocytes and SAA co-accumulate and specifically associate with each other. At these levels, SAA-derived peptides (such as SAA_{98-104}) may possess a substantial in vivo modulatory effect on the T-cell secretion of IFNγ and thus be directly involved in host defense immune response.

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References

[1] Mackiewicz, A., Kushner, I. and Baumann, H. (1993) Acute Phase Proteins, Molecular Biology, Biochemistry and Clinical Application, CRC Press.
[2] Hoffman, J.S. and Benditt, E.P. (1983) J. Clin. Invest. 71, 926–934.
[3] Tape, C. and Kisilevsky, R. (1990) Biochim. Biophys. Acta 1043, 295–300.
[4] Husby, G., Marhaug, G., Dowton, B., Sletten, K. and Sipe, J.D. (1994) Int. J. Exp. Clin. Invest. 1, 119–137.
[5] Lavie, G., Zucker-Franklin, D. and Franklin, E.C. (1978) J. Exp. Med. 148, 1020–1031.
[6] Lavie, G., Zucker-Franklin, D. and Franklin, E.C. (1980) J. Immunol. 125, 175–180.
[7] Silverman, S.L., Cathcart, E.S., Skinner, M. and Cohen, A.S. (1982) Immunology 46, 737–744.
[8] Shephard, E.G., deBeer, F.C., deBeer, M.C., Jeenah, M.S., Coetzee, G.A. and van der Westhuyzen, D.R. (1987) Biochem. J. 248, 919–926.
[9] Skogen, B. and Natvig, J.B. (1981) Scand. J. Immunol. 14, 389–396.
[10] Bausserman, L.L. and Herbert, P.N. (1984) Biochemistry 23, 2241–2245.
[11] Yamada, T., Liepnieks, J.J., Kluve-Beckerman, B. and Benson, M.D. (1995) Scand. J. Immunol. 41, 94–97.
[12] Preciado-Patt, L., Levartowsky, D., Pras, M., Hershkovitz, R., Lider, O. and Fridkin, M. (1994) Eur. J. Biochem. 223, 35–42.
[13] Sukenik, S., Gross, J., Buskila, D. and Weitzman, S. (1988) J. Rheumatol. 15, 942–945.
[14] Preciado-Patt, L., Hershkovitz, R., Fridkin, M. and Lider, O. (1996) J. Immunol. 156, 1189–1195.
[15] Yavin, E.J. and Fridkin, M. (1998) J. Pept. Res. 51, 282–289.