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The random co-polymer glatiramer acetate rapidly kills primary human leukocytes through sialic-acid-dependent cell membrane damage

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ABSTRACT

The formulation glatiramer acetate (GA) is widely used in therapy of multiple sclerosis. GA consists of random copolymers of four amino acids, in ratios that produce a predominantly positive charge and an amphipathic character. With the extraordinary complexity of the drug, several pharmacological modes-of-action were suggested, but so far none, which rationalizes the cationicity and amphipathicity as part of the mode-of-action. Here, we report that GA rapidly kills primary human T lymphocytes and, less actively, monocytes. LL-37 is a cleavage product of human cathelicidin with important roles in innate immunity. It shares the positive charge and amphipathic character of GA, and, as shown here, also the ability to kill human leukocyte. The cytotoxicity of both compounds depends on sialic acid in the cell membrane. The killing was associated with the generation of CD45+ debris, derived from cell membrane deformation. Nanoparticle tracking analysis confirmed the formation of such debris, even at low GA concentrations. Electric cell-substrate impedance sensing measurements also recorded stable alterations in T lymphocytes following such treatment. LL-37 forms oligomers through weak hydrophobic contacts, which is critical for the lytic properties. In our study, SAXS showed that GA also forms this type of contacts. Taken together, our study offers new insight on the immunomodulatory mode-of-action of positively charged co-polymers. The comparison of LL-37 and GA highlights a consistent requirement of certain oligomeric and chemical properties to support cytotoxic effects of cationic polymers targeting human leukocytes.

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1. Introduction

Polymer-based formulations are currently among the most widely sold drugs [1]. One of these is Copaxone™ with the pharmaceutical active ingredient glatiramer acetate (GA), approved for treatment of relapsing-remitting multiple sclerosis (MS). Both experimental and clinical studies suggest that GA has a broad impact on the immune system. Unlike other immunomodulatory MS drugs, GA has few side effects and does not increase the incidence of infections in treated patients. Among several proposals, the pharmacological mode-of-action appears to involve attenuation of the autoimmune response in MS by skewing of the T lymphocyte response from a Th1 to Th2 profile. More recently, cellular compartments and molecular mechanisms of the innate immune response have also been considered as targets for GA [2–5].

GA is prepared from random co-polymerization of *N*-carboxy-α-amino acid anhydrides using well-established polymerization techniques. The copolymers are composed of four amino acids, *i.e.*, L-glutamate, L-lysine, L-alanine, and L-tyrosine in molar ratios of 1.4 (Glu):3.4 (Ala):4.2 (Lys):1 (Tyr) [2]. The M_r varies from 5000 to 9000, corresponding to ~45–80 residues with an experimentally mean M_r of 8030 and a narrow standard deviation of only 170 [6]. Considering these properties, treatment with GA theoretically could generate $>10^{30}$ different amino acid sequences [2,7]. At physiological pH, the amphipathic co-polymers would carry an average net charge of ~+3 as judged from the molar ratios of glutamate and lysine. Synchrotron radiation circular dichroism (CD) suggests that the copolymers are largely disordered in aqueous solution, while membrane-like environments induce

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a high level of α-helical structure [8]. Recently, it was appreciated that GA belongs to the realm of first-generation nanomedicines from the observation that co-polymers appear to form oligomers in solution [9,10]. However, structural and functional characteristics of GA are poorly understood, in particular with regard to what forces drive such oligomerization and the significance of this observation in terms of GA pharmacology.

GA treatment involves daily, subcutaneous injections of 18 mg GA or, more recently, 36 mg GA three times a week. Following subcutaneous injection, a substantial fraction of the GA dose is hydrolyzed [11], while the remaining GA copolymers interact with peripheral blood lymphocytes locally in the skin [7]. Koenig et al. [12] reported that GA copolymers binds onto human cell lines, apparently through electrostatic contacts with heparan sulfate (HS) as well as proteins carrying negatively charged carbohydrates expressed in the cell membrane. It was already known that lysine residues are critical for GA prevention in experimental autoimmune encephalomyelitis (EAE) [13], an animal model for multiple sclerosis. The simpler poly L-lysine can also prevent EAE in guinea pigs [14]. Hence, the positively charged residues of GA may play an important role in its pharmacological mode-of-action, but insight on mechanisms relating the requirement of positive charge to its immunomodulatory effects are lacking.

The cationicity and amphipathicity of GA are strikingly similar to the positively charged antimicrobial peptide (AMP) LL-37, which is the only human cathelicidin among a family of proteins widely found in mammals [15]. AMPs were originally described on the basis of their striking ability to destroy the integrity of microbial membranes. Similar to GA [8], LL-37 also takes a mainly helical secondary structure in lipid membranes or membrane-like environments [16]. It is now clear that functions of AMPs expand into several immunomodulatory properties [15, 17–19]. In psoriatic lesions, concentrations of LL-37 may reach ~6 mg/ml [20]. Intriguingly, this is close to the concentration of GA co-polymers injected into the subcutaneous tissue of MS patients. LL-37 has long been known to kill leukocyte cell lines [21]. While the general chemical nature of GA and its similarity with LL-37 does not, *per se*, dictate similar functions, no previous report has experimentally compared GA with such immunomodulatory peptides of the innate immune system.

Here, we report that GA kills primary human PBMCs, notably T lymphocytes, but also, albeit to a lesser extent, monocytes, at therapeutically relevant dosages. This occurred in the presence of albumin, physiological salt concentration, and divalent cations. Decoration with sialic acid was critical for such lysis. Both in qualitative and quantitative terms, LL-37 had similar effects. Imaging flow cytometry of cells treated with fluorescently-tagged GA found the compound to bind T lymphocytes extensively with indications of concomitant cell membrane deformation. LL-37 fragmented leukocytes into pieces easily quantitated by the forward scatter in flow cytometry. By contrast, the fragments generated by GA were smaller, in a size range of 180–500 nm as investigated by nanoparticle tracking analysis (NTA). GA was also capable of lysing liposomes with a zwitterionic composition similar to mammalian cell membranes. GA or LL-37 reduced the ability of T lymphocytes to form contact with the substrate, agreeing well with the notion that these compounds influences properties of the cell membranes. Taken together, our report now points to GA as a potential limiter of T lymphocyte functions. Surprisingly, the comparison with LL-37 reveals new aspects of the immunomodulatory effects of both compounds, notably with GA potentially mimicking functions of the evolutionary highly conserved LL-37.

2. Materials & methods

2.1. Blood collection

PBMC were isolated from buffy coats from The Blood Bank, Dept. of Clinical Immunology, Aarhus University Hospital made available through ethically approved procedures.

2.2. T lymphocyte and monocyte viability following GA and LL-37 exposure

Buffy coats were depleted of erythrocytes by Ficoll-Paque PLUS gradient centrifugation (GE Health Care Bioscience AB, Sweden). Immediately afterwards, T lymphocytes and monocytes, respectively, were isolated using Dynabeads® Untouched™ Cell Isolation kits according to manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). T lymphocytes and monocytes were either left untreated or stimulated for 30 min with GA (PubChem CID: 3,081,884; Copaxone®, Teva Pharmaceutical Industries Ltd., Petah Tikva, Israel; Batches X07861 & P63010) or LL-37 (PubChem CID: 16198951), made by Innovagen, Lund, Sweden as described [22], at concentrations of 2, 10, 25, or 50 μg/ml in temperate serum-free Gibco® AIM-V medium (Thermo Fisher Scientific) supplemented with 1% (w/v) GlutaMax (Thermo Fisher Scientific) and 10 mM HEPES (Thermo Fisher Scientific). All indicated concentrations were calculated from dilutions of the Copaxone stock at 20 mg/ml GA. Cells were centrifuged at 230 × *g* for 7 min at room temperature. Cells were resuspended in 500 μl PBS. Staining with mAb was conducted at room temperature for 20 min by addition of 10 μl of anti CD45-FITC (DAKO A/S, Copenhagen, Denmark), 3 μl of CD3-PE (DAKO), 10 μl of CD19-FITC (DAKO), and 10 μl of anti CD14-APC (BD Biosciences, Franklin Lakes, NJ). To quantify viability, cells were stained with 10 μl of 7-amino-actinomycin D (7AAD, BD Biosciences). All samples were stained for 20 min at room temperature in complete darkness. Then, cells were washed twice with 2 ml PBS supplemented with 0.5% (w/v) BSA and 0.09% (w/v) NaN_3 and centrifuged at 230 × *g* for 7 min and finally resuspended in 200 μl supplemented PBS. To quantify the absolute count of cells in samples, fluorescent counting beads, with laser excitation at 488 nm and light emission at 530–700 nm, were applied according to manufacturer's protocol (Cytocount™, DAKO). In these experiments, all flow cytometry data were collected on a LSRFortessa (BD Biosciences) instrument and analyzed using FlowJo V.10.0.8 software (FlowJo, Inc., Ashland, OR).

2.3. Quantification of cell death induced by GA and LL-37 in physiological media

Prior to analysis, PBMCs from three separate donors were stored at −134 °C in round-bottom cryo tubes (Nunc®, Thermo Scientific) in RPMI 1640 with L-glutamine, pH 7.2, 20% (v/v) heat-inactivated fetal calf serum (FCS; Gibco®, Thermo Fisher Scientific), and 10% (v/v) DMSO. Cells were thawed quickly at 37 °C, transferred to 15 ml tubes with 9 ml PBS, pH 7.4, and 20% (v/v) FCS, and centrifuged for 10 min at 200 × *g* at 20 °C with slow braking. Supernatants were discarded and cells resuspended in HEPES-buffered saline (HBS) (pH 7.4, NaCl 0.15 M, KCl 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1.8 mM; HEPES 10 mM) or HBS (pH 7.4, NaCl 0.15 M, KCl 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1.8 mM; HEPES 10 mM) supplemented with 40 mg/ml of human serum albumin, close to the concentration in human plasma [23], to a final concentration of 1×10^6 cells/ml. PBMCs were either left untreated or stimulated for 60 min with GA or LL-37 at concentrations of 2, 10, 25, or 50 μg/ml. Then, cells were washed once with PBS, stained with 7AAD and prepared for flow cytometry following procedures as previously described.

2.4. Leukocyte viability following neuraminidase treatment and GA and LL-37 exposure

Freshly isolated PMNCs were prepared from buffy coats as above. Immediately after isolation, the MNCs were either left untreated or incubated with 50 mU/ml neuraminidase obtained from *Vibrio cholerae* (50 mU/ml; Cat. no: 72197-1ML, Sigma-Aldrich, St. Louis, MO) at 37 °C for 30 min. Subsequently, cells were left untreated or treated with 50 μg/ml GA (Teva) or LL-37 (Innovagen) for 30 min at 37 °C as described above. Staining with mAb was conducted in complete darkness at room temperature for 20 min by addition of 5 μl CD45-FITC + CD14-RPE (FR700, DAKO), 3 μl of CD3-PE (R0810, DAKO), 10 μl of CD14-PE

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