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Effect of tuftsin and its retro-inverso analogue on the release of interferon (IFN-γ) and tumor necrosis factor (TNF-α) by human leucocytes

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1. Summary

The aim of this work was to demonstrate whether natural tuftsin or a retro-inverso (r.i.) analogue may induce interferon (IFN) and tumor necrosis factor (TNF) in peripheral-blood-mononuclear-cells (PBMC). For this purpose tuftsin or its analogue were added at different molar concentrations to PBMC and the supernatants were tested for IFN and TNF activity. Both cytokines were released after 12 hours incubation with r.i. tuftsin at an optimum concentration of 10^{-10} M. Under the same conditions no activity was observed in the presence of natural tuftsin. In comparison to natural tuftsin the stimulatory activity of this tuftsin analogue is likely to be due to its high stability.

2. Introduction

Tuftsin, the tetrapeptide threonyl-lysyl-prolyl-arginine (H-Thr-Lys-Pro-Arg-OH) is a natural factor released in the plasma following the enzymatic cleavage of the Fc fragment of the immunoglobulin G [1,2].

It is known that tuftsin exerts a wide range of biological functions that confer upon it a key regulatory role in the activation of peripheral-blood-mononuclear-cells (PBMC). These effects consist of the enhancement of phagocytosis [3], motility, chemotaxis of macrophages [3,4] as well as their increased bactericidal and tumoricidal activity [5,6]. Tuftsin is also able to increase natural cell-mediated cytotoxicity [7], and, when conjugated with antigens, to enhance the antigen presenting capacity of macrophages to T cells, triggering their immune response [8]. Recent reports have shown that the immunogenic effect of tuftsin conjugates is mediated by the synthesis of mRNA encoding for interleukin-1 (IL-1) in macrophages [9]. The production of cytokines such as IL-1 and tumor necrosis factor (TNF) has also been reported in mouse peritoneal macrophages and in human monocytes stimulated by glyco-tuftsin derivatives [10]. Moreover, TNF is induced by tuftsin on HL60 in vitro [11] and, after intraperitoneal injection, in mice [12].

It is not known whether natural tuftsin or, even more interestingly, a synthetic analogue obtained by retro-inversion of the Thr-Lys bond (r.i. tuftsin), which has been shown to be a more potent stimulator of several immune functions as compared with natural tuftsin [13], may induce interferon (IFN) and TNF production by PBMC. We thought it worthwhile to investigate this problem. Since cytokines have potent and pleiotropic functions, the release of these substances may explain most of the described biological activities elicited

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by tuftsin or its retro-inverso analogue. Moreover, from a practical point of view, it may be useful to ascertain whether the r.i. tuftsin is more stable and active than natural tuftsin.

3. Materials and Methods

3.1. PBMC preparation

Human peripheral venous blood (100–200 ml) was withdrawn from healthy donors, who were hepatitis B surface antigen and HIV negative. Donors ranged in age from 25–40 years. Blood samples were diluted 1:1 with phosphate buffered saline (PBS) supplemented with heparin (10 IU/ml) and layered over Ficoll-Hypaque for PBMC separation according to Boyum's method [14]. The PBMC fraction was resuspended in RPMI 1640 medium (DUTCH modified, Flow Laboratories, Irvine, Scotland) supplemented with 10% heat-inactivated (56°C, 60 min) fetal calf serum (Seromed, Biochrom KG, Germany), penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM) at a final concentration of 1.5×10^6 viable cells/ml. The cell viability was assayed by trypan blue exclusion technique and light microscope observation.

3.2. Treatment of samples

Either natural or r.i. tuftsin (kindly provided by Sclavo SpA, Siena, Italy) at the following final concentrations, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-10}, 10^{-12}, 10^{-14}, 10^{-16}, were added to the cell suspensions, incubated in 48-well culture plates (Costar, Badhoevedorp, the Netherlands) (0.5 ml per well) at 37°C in a humidified atmosphere (95% air-5% CO_2). At different times: 0, 12, 24, 48, 72 hours, samples were centrifuged at 1000 × *g* and the supernatants were frozen at −80°C until IFN and TNFα determination. Controls underwent the same procedure except that PBS was added instead of either tuftsin or its r.i. analogue.

In order to verify cell responsiveness, some of the PBMC samples were challenged with 5 μg/ml phytohaemagglutinin (PHA).

When experiments were carried out with polymyxin B sulphate (Serva Feinbiochemica, Heidelberg, Germany) concentrations of up to 2000 μg/ml were used.

3.3. IFN determination and characterization

Supernatants were titrated for IFN activity with an antiviral assay [15] using human amniotic cells (Wish) and vesicular stomatitis virus (VSV, Indiana strain) as challenge virus. Titrations were always performed using the international reference preparations for human IFN-α (Ga23-902-530), human IFN-β (G023-902-527) and human IFN-γ (Gg23-901-530). Titers were expressed as International Units (IU) per ml. Characterization of the antiviral activity as IFN was carried out according to classical procedures, namely acidification of samples at pH 2.0, followed by neutralization at pH 7.0 prior to the assay, heating at 56°C for 1 h, dialysis for 1 day at 2°C, ultracentrifugation at 105000 × *g* and proteolytic (trypsin: 0.25% for 30 min at 37°C) treatment. Neutralization of IFN was carried out as follows: samples containing about 100 IU/ml as well as international standards were incubated for 60 min at 37°C in the presence of individual and pooled anti-IFN-α, -IFN-β and -IFN-γ antisera obtained from NIAID, NIH, Bethesda, MD and the residual antiviral activity was then assayed. For each experiment the amount of anti-human IFN-α, -β and -γ used were sufficient to completely neutralize the corresponding IFNs.

3.4. TNF-α determination

Concentrations of TNF-α were measured simultaneously by a sandwich enzyme immunoassay (BIOKINE TNF test kit, T Cell Sciences, Cambridge, MA) and by a bioassay using L929 and actinomycin D [16]. Both tests are highly specific for TNF-α activity. Having calculated a conversion factor by using a TNF-α standard solution measured by bioassay it has been possible to report all data as pg/ml.

4. Results

In order to examine whether natural tuftsin or its r.i. analogue were able to induce the secretion of cytokines, suspensions of PBMC were added with different molar concentrations (10^{-4}–10^{-16})

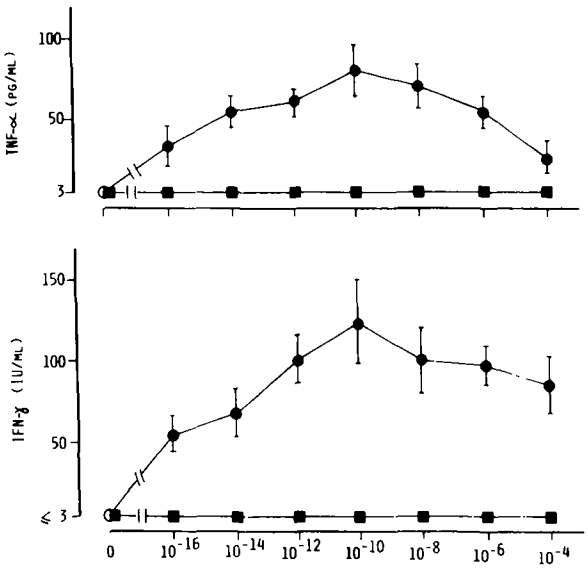

Fig. 1. IFN-γ and TNF-α activity in the supernatant of Ficoll-purified mononuclear cells either untreated (○) or treated with natural tuftsin (■) and r.i. tuftsin (●) at different molar concentrations (abscissa).

of the potential inducers and incubated for 12 hours. Fig. 1 shows that discrete amounts of either IFN and TNF activity were released in the supernatant of the cells treated with r.i. tuftsin while no activity was observed after treatment with natural tuftsin. For both cytokines, the optimal concentration of r.i. tuftsin was 10^{-10} M but even a femtomolar concentration showed some activity. It then became useful to know the kinetic of release and when the peak of activity occurred. To this end PBMC suspensions, stimulated with r.i. tuftsin at 10^{-10} M, were incubated for different times and the supernatants tested for TNF and IFN activity.

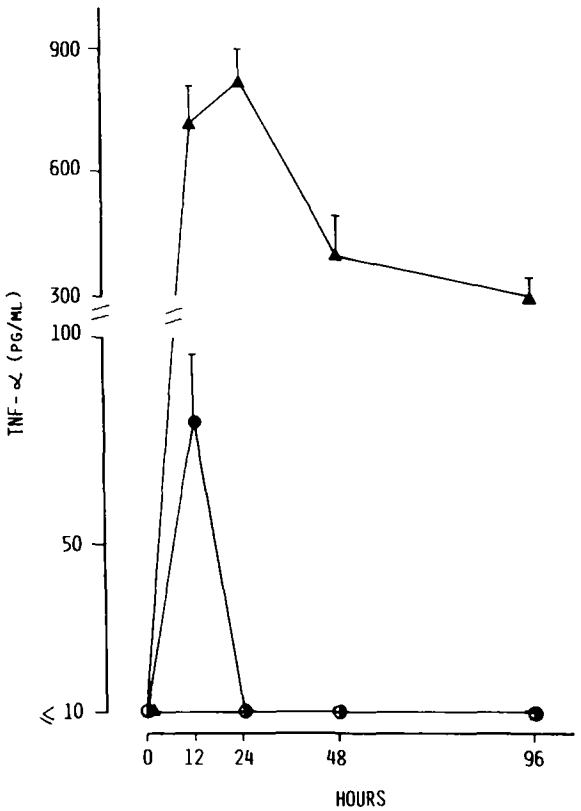

Fig. 2. Kinetic of TNF-α production on Ficoll-purified mononuclear cells either untreated (○) or treated with either r.i. tuftsin (10^{-10}) (●) or PHA (5 μg/ml) (▲).

Figs. 2 and 3 show the kinetic of release of TNF-α and IFN-γ, both with a maximal production in the first 12 hours. After that, TNF-α activity rapidly fell reaching the basal level within 24 hours, while IFN-γ activity, although drastically decreased, still persisted up to 96 hours of incubation. Controls (untreated samples) did not show

TABLE 1

Effect of polymyxin B on the release of TNF and IFN activity by Ficoll-purified mononuclear cells untreated or treated with r.i. tuftsin (10^{-10} M) with or without polymyxin B at different concentrations when incubated for 12 hours.
Values are reported as mean of two samples.

	Control	r.i. tuftsin alone	r.i. tuftsin + polymyxin B 20 (μg/ml)	r.i. tuftsin + polymyxin B 200 (μg/ml)	r.i. tuftsin + polymyxin B 2000 (μg/ml)
TNF-α	< 2	90	90	75	75
IFN-γ	< 2	40	35	25	25

TABLE 2

Characterization of the antiviral activity as an interferon in the supernatant of Ficoll-purified mononuclear cells treated with r.i. tuftsin (10^{-10} M) and incubated for 12 hours

Treatment	IFN (IU/ml)	Degree of inactivation (percentage)
None	110	0
pH 2.0 for 1 day at 2°C	< 3	100
Heating at 56°C for 1 h	< 3	100
Ultracentrifugation	110	0
Trypsin digestion	< 3	100
Anti-Hu-IFN-α	110	0
Anti-Hu-IFN-β	110	0
Anti-Hu-IFN-γ	< 3	100

any activity, while very high cytokine titres were observed after PHA addition (Figs. 2 and 3), confirming that this mitogen is a far more potent inducer than r.i. tuftsin.

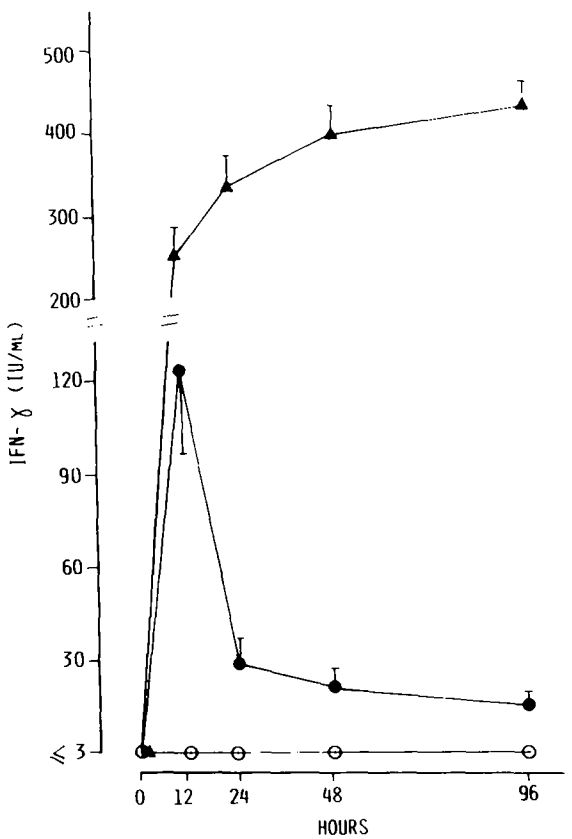

Fig. 3. Kinetic of IFN-γ production on Ficoll-purified mononuclear cells either untreated (○) or treated with either r.i. tuftsin (10^{-10}) (●) or PHA (5 μg/ml) (▲).

The next step aimed to clarify whether the cytokine release was due to the eventual presence of endotoxins present in r.i. tuftsin chemical preparation. Table 1 shows that preincubation of r.i. tuftsin with polymyxin B sulphate did not have any effect on cytokine release except at very high polymyxin concentration, probably due to a toxic effect.

As the anti-viral assay does not identify the type of IFN, we characterized the antiviral activity present in the supernatant of PBMC suspensions treated with different molar concentrations of r.i. tuftsin, as described in Materials and Methods. It was demonstrated (Table 2) that the activity was referable to a pH 2 labile protein, sensitive to trypsin and not sedimentable at 110000 × *g*. More importantly, the activity was completely neutralized after addition and incubation with goat antiserum against human IFN-γ, while antisera against human IFN-α and -β were either ineffective or barely effective, respectively.

5. Discussion

In this study we have shown that a synthetic analogue of tuftsin, obtained by retro-inversion of Thr-Lys bond, induces the synthesis of IFN-γ and TNF-α by PBMC in culture. The most effective concentration of r.i. tuftsin for the induction of the two cytokines was 10^{-10} M, but even lower concentrations were effective. Interestingly, under similar experimental conditions, natural tuftsin never showed any effect at all on the doses tested.

These results confirm previous data [13,17] showing that the r.i. analogue possesses a higher

immunostimulatory activity in different experimental systems, when compared with natural tuftsin. The mechanisms responsible for the enhanced stimulation by r.i. tuftsin have yet to be elucidated. However a possible explanation could be found in the high stability of the Thr-Lys bond to enzymatic degradation [13]. It is well known that natural tuftsin is rapidly degraded in plasma in two different biologically active tripeptides which actually inhibit tuftsin activity [18–20]. In contrast r.i. tuftsin has been shown to be resistant either against isolated aminopeptidases, or human plasma proteolytic enzymes [13].

The failure to induce cytokines by natural tuftsin is also in agreement with the results of Dagan et al. [21] who reported that tuftsin stimulates the secretion of IL-1 from macrophages only if conjugated with antigens. More recently it has also been reported that the release of IL-1 and TNF may be modulated by glyco-tuftsin derivatives in mouse peritoneal macrophages and in human monocytes [10].

Our observation on the stimulatory effect of an analogue of tuftsin with a reversed amine bond direction maintaining the overall topology of the original peptide, confirms the immune adjuvant potential of tuftsin. The release of the two cytokines by PBMC is novel and interesting and can help to explain the pleiotropic tuftsin activities on a new basis. Even though the production of cytokines is small it has a significant value because cytokines act at femtomolar concentrations and actually high levels can elicit toxic effects. IFN-γ and TNF-α are known to possess antiviral and immunomodulatory properties and therefore their release by PBMC is worth mentioning particularly in view of tuftsin or r.i. analogue therapeutical application.

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