Action of Synthetic Peptide LKEKK in Experimental Tuberculosis

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Abstract

In the present study, we investigated the activity of the synthetic peptide LKEKK (Np5) in murine model of tuberculosis induced by Mycobacterium bovis-bovis strain. Therapy with Np5 at doses of 0.01, 0.1, and 1µg/kg (5 daily injections) decreased the lung damage index compared to untreated controls and to those treated with isoniazid alone. The growth of M. bovis-bovis 8 in spleen culture was decreased. Study of cytokine production showed that on the 24th day after treatment with Np5 (doses of 0.01, 0.1, 1µg/kg) the secretion of IL-2 was restored to the level seen in uninfected animals. IFN-γ production both thymus and spleen cells, as well as its circulating levels in serum, was increased by the Np5 treatment. Concurrently, IL-4 production was decreased in the same cell types and in the serum. The Np5 treatment also stimulated the macrophage functions, which had been decreased by tuberculosis infection and isoniazid therapy, with an improved phagocytosis activity of peritoneal macrophages. Thus, the Np5 treatment increased the efficacy of anti-tuberculosis therapy as well as strength of the immune response.

INTRODUCTION

Tuberculosis is a widespread in the world chronic infectious disease of humans and animals, which is caused by various types of mycobacteria from the Mycobacterium tuberculosis complex group (M. tuberculosis, M. bovis, M. africanum, M. microti, M. canettii); it causes more than 1.5 million deaths annually [1-3]. Experts note that over the past few decades, drug resistance of mycobacterial strains has increased significantly. Currently, patients are increasingly becoming infected with strains of mycobacteria that are resistant to almost all antibiotics, that requires an urgent search for new drugs and treatment methods [4].

A unique feature of mycobacteria is the complexity of its cell wall. This structure consists of a plasma membrane, a cell wall core and an outer envelope, including many complex lipids, peptidoglycans and mycoic acids [5-8]. Mycolic acids, long-chain branched fatty acids, containing 60-90 carbon atoms per molecule, are an exclusive component of the cell wall of mycobacteria; they make the surface of the bacilli waxy and highly hydrophobic, providing protection against hydrophilic antibiotics, oxidative damage and the host immune response [9].

Several years ago, we synthesized the peptide LKEKK corresponding to the sequences 16-20 of human thyroxin-α1 (TM-α1) and 131-135 of interferon-α1 (IFN-α1) and showed that it binds with high affinity to murine macrophage-like cells of line RAW 264.7. In the 10-1000 nM concentration range, the peptide dose-dependently increased the Nitric Oxide (NO) production, the activity of soluble guanylate cyclase (sGC), as well as the adhesion, spreading, and capacity to digest bacteria of Salmonella typhimurium virulent strain 415 in vitro by the cells. The synthetic peptide with inverted KKEKL sequence tested in parallel was inactive. Thus, the peptide LKEKK binding to RAW 264.7 cells leads to an increase in NO-synthase, guanylate cyclase and phagocytic activity [10]. The purpose of this work is to study the effect of the peptide LKEKK (Np5) on a mouse model of tuberculosis.

MATERIALS AND METHODS

Chemicals

IL-2, IL-4, IFN-γ and other chemicals were obtained from Sigma (St. Louis, MO).

Peptides

Peptide LKEKK (Np5) was synthesized on an Applied Bio systems Model 430A automatic synthesizer (USA) using...
the Boc/Bzl tactics of peptide chain elongation as described previously [11]. The peptide was purified to homogeneous state by preparative reverse-phase HPLC (Gilon chromatograph, France) on a Delta Pack C18 column, 100A (39×150 mm, mesh size 5µm; flow rate 10ml/min, elution with 0.1%TFA, gradient of acetonitrile 10-40% in 30min). The molecular masse of peptide was determined by fast atom bombardment mass spectrometric analysis (Finnigan mass spectrometer, San Jose, CA). The data of amino acid analysis (hydrolysis by 6 M HCl, 22h, 110°C; LKB 4151 Alpha Plus amino acid analyzer, Sweden) and mass spectrum analysis are presented in (Table 1).

Animal Infection and Treatment

Infection of 200 white wild type mice, obtained from Lab. Animal Nursery, Rappolovo, Russia, was performed with disseminated tuberculosis (TB) by injection of M. bovis-bovinus 8 suspension (0.1mg in 0.2mL of saline, contained 100 bacterial bodies). Two mice were sacrificed every two days from day 7 after the inoculation, and lungs were inspected. When single subsidiary foci (< 1 mm) in the lungs were seen in the sacrificed mouse (day 12 after inoculation), all other animals were selected to one of the groups and isoniazid therapy (at a sub-therapeutic dose of 10 daily, subcutaneous) was started. Np5 treatment consisting of 5 daily intraperitoneal (i.p.) injections of doses of 0.01, 0.1, 1.0 and 10µg/kg, started on day 20 after inoculation (when multiple subsidiary foci were found on the autopsy of untreated mice). One group of animals was treated with a second course of Np5 treatment at a dose of 1µg/kg, beginning 2 days after the first treatment ended (day 26). Control groups included mice without therapy (inoculation control) and mice on isoniazid therapy alone (therapy control). Samples were harvested on day 4, 10, 17 and d 24 after the end of 5 days course of Np5 therapy, corresponding to the treatment days 28, 34, 41 and 48. At least 5 mice from each group were examined.

Severity of Experimental TB

Severity of experimental TB was evaluated by visual examination and calculated as a “Lung Damage Index”. The following criteria were used single subsidiary foci were estimated at 0.5 units (U), multiple subsidiary foci (< 20) as 1.0 U, multiple subsidiary foci (> 20) as 1.5 U, single miliary foci as 1.75 U, multiple associated subsidiary and single military foci as 2.0 U, military foci (< 10) as 2.25 U, multiple associated military foci as 2.75 U, small caseous foci as 3.0 U, disseminating caseous as 4.0 U, damage to the entire lung as 5.0 U. In the case of lung maceration by serous liquid, the index was increased (by 0.25 1.0 U), depending on the extent of damage. Lung and spleen weight were also measured and compared to the total weight of the mouse to provide an organ weight index.

Mycobacterium Contamination

Mycobacterial contamination was assessed by bacteriological investigation of spleen tissue homogenate cultured on solid egg Lowenstein-Jensen media with the growth density of M. bovis-bovinus 8 expressed in Colony Forming Units (CFU). Colony count was performed by visual examination of solid media surface [12]. If number of colonies was countable (< 100) exact count was performed. Many conjugated colonies (but not solid growth) were counted as 200. Solid mycobacterial growth was counted as approximately 300 colonies. Two cultures for each sample were performed and then median was counted. Data were presented as median (min-max). We estimated mycobacterial contamination by spleen examination because clearance in spleen was going more quickly the in lungs.

Peritoneal Macrophage Activity

Phagocytosis was studied in peritoneal macrophages in cell culture. Cells were plated at a concentration of 106 cells per Petri dish, and media was added that contained 107 Saccharomyces cerevisiae cells, opsonized by mouse serum. Phagocytic activity, the percent macrophages involved in phagocytosis, and phagocytic digestion, the number of yeast digested be macrophages after 1.5 h of incubation, were counted by microscopy.

Cytokine Production and Cytokine ELISAs

For cytokine determination, spleen cell suspensions were diluted to a concentration of 107/mL in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 1 mM PMSF, 10µg/ml aprotinin, 10µg/ml leupeptin, and 10µg/ml peptatin A. 100 µl of cell suspension was added to each well of 96-well cell culture plates. RPMI 1640 alone was added to control wells. Cytokine production was induced by concanavalin A (Con A, final concentration of 2.5µg/ml). Cells were incubated for 24 h at 37°C in a humidified atmosphere 5% CO2. After incubation 150 µl of supernatant was removed from each well. Supernatants were stored at -70°C. Concentrations of II-2, II-4 and IFN-γ in cell supernatants were measured by ELISA kits. Results are expressed as U/ml. ELISAs were carried out according to the manufacturer’s instructions (BD Biosciences, San Jose, CA). Data are presented as mean ± SEM.

Statistical Analysis

The data were evaluated using the Mann-Whitney test. The results are presented as mean ± SEM or as median (min-max).

RESULTS

Peptides

The main characteristics of the peptide LKEKK (Np5) (purity, amino acid content, and molecular mass) are shown in (Table 1).

Severity of Experimental TB

Therapy with Np5 rapidly changed the progression of TB infection in mice: at all doses, the peptide decreased the lung damage index when measured 4 days after the end of 5 days of the therapy (day 28 of the infection). Significant differences were
seen between the animals treated with Np5 doses of 0.01, 0.1, 1 µg/kg and untreated controls (1.62 ± 0.37, 1.81 ± 0.12 and 1.97 ± 0.28 compared to 2.73 ± 0.15, respectively, p < 0.05). A significant difference was also seen between animals treated with a dose of 0.1µg/kg and those treated with isoniazid alone: on day 24 after the end of 5 days of the Np5 therapy, the lung damage index was also significantly lower at Np5 doses of 1.0, 0.1, 0.01µg/kg (2.56 ± 0.15, 2.58 ± 0.14 and 2.60 ± 0.16 compared to 3.32 ± 0.26 in the control group, respectively, p < 0.05).

At the dose 0.1 µg/kg other beneficial effects of Np5 were also seen. There were significant increases in body weight (30.7% vs. 20.5% in isoniazid treated mice, 24 days after therapy) and decreases in lung weight index (1.37 ± 0.06 vs. 1.97 ± 0.28, p < 0.05) and spleen weight index (1.59 ± 0.25 vs. 1.94 ± 0.32). There was also a significant decrease in the growth of M. bovis-bovis B cultured from spleen: 200 (200-200) CFU vs. 275 (250-300) CFU in isoniazid treated mice, p < 0.05.

**Cytokine Production**

A decrease of production of IL-2 in Con A-stimulated spleen cells was seen after TB infection with extensive lung damage (Table 2). The Np5 treatment led to an increase in IL-2 production in all treated groups, at all-time points after the therapy. IL-2 production in animals treated with Np5 doses of 0.1 and 0.01 µg/kg was markedly increased in comparison to those treated with isoniazid alone (4.16 ± 5.9 U/ml and 13.2 ± 4.5 U/ml, respectively) as early as 4 days after Np5 therapy. Twenty-four days after the start of therapy, the IL-2 level in animals treated with Np5 doses of 0.1 or 1µg/kg (10 injections) returned to the level of uninfected mice.

Basal IFN-γ production in spleen cells was not different from isoniazid-treated control mice as determined 4 days after Np5 therapy. Ten days after the therapy, however, there was a significant elevation of basal IFN-γ production in animals treated with Np5 at doses of 0.1 and 0.01µg/kg i.p. (5 or 10 injections), and by 17th day this increase was seen in all Np5 treated animals. No significant changes in basal IL-4 production were seen after treatment with Np5.

Con A-stimulated production of IFN-γ and IL-4 in spleen cells was significantly affected by Np5 treatment (Table 2). It is interesting to note that changes in the production of IL-4 and IFN-γ were opposite in direction: the IFN-γ production was increased, whereas the IL-4 production was decreased.

**Peritoneal Phagocytes Function**

Extensive and wide spread lung damage with TB lead to a decrease in the activity of the peritoneal phagocytes and to a poor phagocytic digestion of yeast cells. The average phagocytic activity 28 days after infection was 4.6% compared to 64.2% in uninfected mice (p < 0.01). Phagocytic digestion decreased similarly. Isoniazid therapy caused an increase in the parameters of the phagocytic activity, but not to the level of uninfected mice. When measured 4 days after the treatment (Table 3), Np5 therapy markedly elevated phagocytic activity to 38.5% after a dose of 0.1µg/kg, compared to 19.4% in the isoniazid control group (p < 0.05). Np5 treatment also increased phagocytic digestion (Table 3): 228 (83-435) yeast killed per 1.5 h vs. 173 (139-319) in intact mice. Np5 treatment, particularly at 0.1µg/kg and 1.0 µg/kg, significantly increased the digestion: 194 (110-346) yeast killed per 1.5 h vs. 138 (63-290) in untreated mice, p < 0.05. Np5 treatment, particularly at 0.1µg/kg and 1.0 µg/kg, significantly improved the digestion: 199.5 (86-375) yeast killed per 1.5 h vs. 173 (139-319) in intact mice, (p < 0.05).

Ten days after Np5 therapy, we observed a weakening of the drug effect, perhaps because of improvement due to the isoniazid therapy. In fact, 24 days after the treatment the phagocytic activity in the isoniazid treatment group had returned to the level of uninfected mice. However, phagocytic digestion was still depressed: 60 (21-107) vs. 138 (63-290) in uninfected mice, p < 0.05. Np5 treatment, particularly at 0.1µg/kg and 1.0 µg/kg (10 injections), significantly improved the digestion: 199.5 (86-375) and 167 (64-291), respectively, vs. 60 (21-107) in isoniazid alone treated mice, p < 0.05.

**DISCUSSION**

Despite growing global efforts to eradicate tuberculosis, it killed a total of about 5 million people between 2021 and 2023, and was in fact the second biggest infectious killer after COVID-19. Thanks to vaccination and control of the pandemic, tuberculosis was in fact the second biggest infectious killer after COVID-19. Thanks to vaccination and control of the pandemic, tuberculosis and was in fact the second biggest infectious killer after COVID-19. Thanks to vaccination and control of the pandemic, tuberculosis and was in fact the second biggest infectious killer after COVID-19.
Peptide Np5 with simple structure LKEKK has significant anti-TB activity and is suitable as a basis for the development of complex anti-TB therapy.

DECLARATION OF INTEREST

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
REFERENCES


