Research Article

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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 bivis-bovinus* 8 strain. Therapy with Np5 at doses of 0.01, 0.1, and 1 μg/kg (5 daily injections) decreased the lung damage index compared to untreated controls and to those treated with isoniazid alone. The growth of *M. bivis-bovinus* 8 in spleen culture was decreased. Study of cytokine production showed that on the 24th day after treatment with Np5 the secretion of IL-2 was restored to the level seen in uninfected animals. IFN-γ production be 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.

Keywords : *Protein: Peptide; Receptor; Cytokine; Tuberculosis*

1. 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 (Churchyard et al., 2017; Furin et al., 2019; Natarajan et al., 2020). 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 (Jacobo-Delgado et al., 2023).

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. (Grzegorzewicz et al., 2016; Chiaradia et al., 2017; Singh et al., 2018; Stokas et al., 2020). 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 (Singh et al., 2017).

Several years ago we synthesized the peptide LKEKK corresponding to the sequences 16–20 of human thymosin-α1 (TM-α1) and 131–135 of interferon-α2 (IFN-α2) 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 (Navolotskaya et al., 2019).

The purpose of this work is to study the effect of the peptide LKEKK (Np5) on a mouse model of tuberculosis.

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2. MATERIALS AND METHODS

2.1. Chemicals

IL-17A, TNF-a, IL-10, IL-12 and other chemicals were obtained from Sigma (St. Louis, MO).

2.2. Peptides

Peptide LKEKK (Np5) was synthesized on an Applied Biosystems Model 430A automatic synthesizer (USA) using the Boc/Bzl tactics of peptide chain elongation as described previously (Schnolzer et al, 1992). The peptide was purified to homogeneous state by preparative reverse-phase HPLC (Gilson chromatograph, France) on a Delta Pack C18 column, 100A (39×150 mm, mesh size 5 μm; flow rate 10 ml/min, elution with 0.1% TFA, gradient of acetonitrile 10–40% in 30 min). 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.

2.3. 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 (0.1 mg in 0.2 mL of saline, contained 108 bacterial bodies). Two mice were sacrificed every two days from day 7 after the inoculation, and lungs were inspected. When single submiliary 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 10 μg/kg, started on day 20 after inoculation (when multiple submiliary 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 1 μg/kg, beginning 2 days after the first treatment ended (day 26). Control groups included mace 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 five days course of Np5 therapy, corresponding to the treatment days 28, 34, 41 and 48. At least 5 mice from each group were examined.

2.4. 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 submiliary foci were estimated at 0.5 units (U), multiple submiliary foci (<20) as 1.0 U, multiple submiliary foci (>20) as 1.5 U, single miliary foci as 1.75 U, multiple associated submiliary 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.

2.5. 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 (Lecoeur et al., 1989). If number of colonies was countable (<100) exact count was performed. Many conjugated colonies (but not solid growth) were counted as 200. Solid micobacterial 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 be spleen examination because clearance in spleen was going more quickly the in lunges.

2.6. Peritoneal Macrophage Activity

Phagocytosis was studied in peritoneal macrophages in cell culture. Cells were plated at a concentration of 10^6 cells per Petri dish, and media was added that contained 10^7 *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.

2.7. Cytokine Production and Cytokine ELISAs

For cytokine determination, spleen cell suspensions were diluted to a concentration of 10^7/mL in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin 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 2.5 μg/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 Il-2, Il-4 and IFN-γ in cell supernatants were measured by ELISA kits. Results are expressed as U/ml. ELISAs were carried out using according to the manufacturer's instructions (BD Biosciences, San Jose, CA). Data are presented as mean ± SEM.

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2.8. Statistical Analysis

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

3. RESULTS

3.1. Peptides

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

Table 1

Main characteristics of the peptides

Peptide	Purity, %	Amino acid analysis data	Molecular mass, D
Np5	>98	Glu 1.08, Leu 1.00, Lys 3.32	645.4 (calculated value 644.87)

3.2. 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 0.1 μg/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, 0.01 μg/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-bovinus 8 cultured from spleen: 200 (200 200) CFU vs. 275 (250 300) CFU in isoniazid treated mice, $p<0.05$.

3.3. 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 increases in comparison to those treated with isoniazid alone (41.6 ± 5.6 U/ml and 39 ± 2.8 U/ml vs. 25.2 ± 2.4 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 1 μg/kg (10 injections) returned to the level of uninfected mice.

Table 2

Effect of peptide LKEKK (Np5) on Con A-induced production of IL-21, IL-42, IFN-γ2 by spleen cells

Treatment	Cytokine (U/ml ± SEM)		
	IL-2	IFN-γ	IL-4
Intact mice	74.3 ± 6.5	22.5 ± 2.4	11.2 ± 1.6
Infected mice	12.5 ± 1.6*	10.3 ± 1.4	20.2 ± 2.6
Isoniazid + Np5 (0.01 μg/kg)	39.0 ± 2.8*	12.5 ± 1.7	17.1 ± 1.9*
Isoniazid + Np5 (0.1 μg/kg)	41.6 ± 5.6*	13.7 ± 1.9*	15.7 ± 2.1*
Isoniazid + Np5 (1.0 μg/kg)	45.8 ± 5.0*	14.9 ± 1.5*	14.3 ± 2.0*
Isoniazid + Np5 (10 μg/kg)	53.5 ± 6.2*	18.6 ± 2.0*	13.2 ± 1.4*
Isoniazid treated	25.2 ± 2.4	11.7 ± 1.3	19.0 ± 2.2

* Significant difference from the therapy with isoniazid alone ($p < 0.05$). [1]The evaluation was performed 10 days after the end of Np5 treatment. [2]The evaluation was performed 17 days after the end of Np5 treatment.

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 1 μg/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.

3.4. 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% 9n uninfected mice ($p<0.01$). Phagocytic digestion

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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 3A), Np5 therapy markedly elevated phagocytic activity to 38.5% after a dose of 0.1 μg/kg, compared to 19.4% in the isoniazid control group($p<0.05$). Np5 treatment also increased phagocytic digestion (Table 3B): 228 (83 435) 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 as a result 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 depresse: 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, $p<0.05$.

Table 3

Phagocytic activity of murine peritoneal macrophages, day 4

Treatment	Phagocytic macrophages (%)	Digestion (number of digested yeast during 1.5 h)
Intact mice	64.2 (61.0 66.0)	173 (139 319)
Infected mice	4.6 ((2.0 8.0), *p<0.01	6 (0.0 34.0), *p<0.01
Isoniazid treated	19.4 (6.0 34.0), *p<0.01	58 ((17.0 147.0), *p<0.05
Isoniazid + Np5 (0.01 μg/kg)	21.6 (4.0 54.0), *p<0.01	80 (21.0 215)
Isoniazid + Np5 (0.1 μg/kg)	38.8 (16.0 57.0), **p<0.05	228 ((93.0 435.0), **p<0.01
Isoniazid + Np5 (1.0 μg/kg)	29.8 (11.0 46.0), *p<0.01	184 ((18.0 306)
Isoniazid + Np5 (10 μg/kg)	38.6 (5.0 62.0), *p<0.05	246 (8.0 276.0)

Data is presented as median (min max); *significant difference with uninfected mice; ** significant difference with inoinazid alone.

4. 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 is likely to once again become the leading cause of death in the world, especially since during the COVID-19 pandemic, all health resources were devoted to containing SARS-Cov2 and important anti-tuberculosis programs were ignored. In addition, it should be noted that drug-resistant tuberculosis is currently becoming a serious problem: its treatment is difficult, time-consuming and expensive and often requires the use of toxic and poorly tolerated drugs (Khadela et al., 2022). Compared to existing anti-tuberculosis drugs, peptides have a number of advantages. First, they have a broad spectrum of activity against various strains of M. tuberculosis, including drug-resistant strains, making them potential candidates for the treatment of drug-resistant tuberculosis. Secondly, peptides are fast-acting, which means they can quickly kill germs, which will shorten the duration of treatment. Third, peptides have a low risk of developing drug resistance because they simultaneously target multiple components of bacterial cells and host cells. Finally, peptides are well tolerated by the body. These advantages make potential anti-tuberculosis peptide drugs an attractive option for the development of new treatments for tuberculosis. (Jacobo-Delgado et al., 2023).

The understanding of the innunopathogenesis of the TB infection has significantly increased during the last several years. It has become evident that the imbalance in the functional activity of Th1 and Th2 cells plays a key role in the progression of TB (Kaufmann et al., 1995; Mustafa et al., 2000; Vasiliu et al., 2023) and correlates with the severity of the diseases. This imbalance is accompanied with a decrease in IL-2, IL-12 and IFN-γ production and impairment in the expression of their receptors (Lange et al., 2022). The adequate immune response to Mycobacterium appears to be characterized by greater Th1 activity and production of IFN-γ and IL-2, while a low resistance to infection results from Th2 stimulation and IL-4 production (Baliko et al., 1998; Dieli et al., 2000; Tamburini et al., 2021). In mice, experimental TB infection is more benign in strains that produce high levels of Th1 cytokines, while mouse strains with lower Th1 cytokines levels are more susceptible (Flinn et al., 1993; Actor et al., 1999). Moreover, reactivation of latent TB is accompanied by an elevation of the Th2 cytokine production and by a simultaneous decline in Th1 functions (Shiratsuchi et al., 1987; Dieli et al., 2000); in addition, an evidence of Th1 lymphocyte suppression has been reported (Ellner et al., 1987; Shiratsuchi et al., 1987; Hirsch et al., 1999).

The use of immune-modulating cytokines for therapy of TB has recently been investigated. Recombinant IL-2 (rIL-2) normalized IL-2 receptor expression, lymphocyte proliferation and, partially, IFN-γ production in lymphocyte cultures obtained from patients with active TB (Shiratsuchi et al., 1987; McDyer et al., 1997; 2002) and a positive effect of rIL-2 has been reported in treatment of murine experimental TB induced by M. avium (Bermudez et al., 1989); rIL-2 therapy

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decreased the number of Mycobacterial CFU in organ cultures, and increased endogenous IL-2 production and IL-2 receptor expression in M. tuberculosis H37RV infected mice (Denis, 1991). In patients with severe TB that were infected multi-resistant M. tuberculosis strains, rIL-2 led to clinical improvement and an increase in lymphocyte functional activity (Suárez-Méndez et al., 2004). IFN-γ used in similar patients decreased M. tuberculosis expectoration and cavity size and increased BCG ingesting and killing by alveolar macrophages (Shimokata, 1996; Condos, 1997; Fenhalls et al., 2000, Zhuang et al., 2024). In vitro data suggest that IFN-γ inhibits growth of M. avium inside blood monocytes (Shiratsuchi et al., 1991). And finally, a beneficial effect of IFN-γ on M. tuberculosis clearance was found in multiple drug resistant patients (Giosue et al., 2000). IL-12 efficacy has been observed in mice and in patients (Kobayashi et al., 2000; Greinert et al., 2001).

Our results demonstrate that the Np5 treatment increases the production of Th1 cytokines in experimental murine TB, similarly to the interleukins described above. We used M. bovis-bovinus 8 strain with virulence in mice that was similar to M. tuberculosis strains (submiliary foci appeared on day 12 after inoculation and mortality in untreated mice was observed on day 34-46). The model with relatively slow development of TB was used for a better detection of Np5 effects. Other groups of investigators have also used animal models of TB with M. bovis (Kondo et al., 1977; Kanai et al., 1981; Dahl et al., 2004; Hayashi et al., 2010). Production of IL-2 by spleen cells was decreased in mice with an extensive TB infection. Other reports also described decreased IL-2 levels in animals and humans with TB (Ellner, 1997; Estrada-García et al., 2021; Torres-Juarez et al., 2021). In our experiments IL-2 had significantly less reduction after the treatment with Np5. On the 24th day after the treatment with Np5 production of IL-2 was restored to the level found in the uninfected animals. Production of basal an stimulated IFN-γ by both thymus an spleen cells, as well as circulating serum levels of this cytokine, was increased after the Np5 treatment. At the same time, the IL-2 production by the same cell and in the serum was decreased. These changes, the increase in the IFN-γ production and decrease in IL-4 secretion, suggest that Np5 is elaborating a shit of T helper cell activity towards a Th1-like immune response.

Other immune parameters were improved as well – an increase in Con A-stimulated thymic cell proliferation was observed as early as 4 days after the Np5 treatment. Twenty-four days after treatment the proliferative responses for both thymic and spleen cells were restored nearly to the parameters seen in the uninfected animals.

Yeast phagocytosis is a sensitive marker of macrophage function during experimental TB. Ingestion and digestion functions were decreased in inoculated untreated and isoinazid treated animals with an extensive lung damage. Inhibition of macrophage functions is thought to result from either the Mycobacterium (Phyu et al., 2000; Beltan et al., 2000; Ragno et al., 2001) or from certain anti-TB drugs (Kramer et al., 1976), Np5 treatment actually stimulated the macrophage functions, which had been decreased by TB infection and isoniazid therapy, with an improvement seen in peritoneal macrophage ingesting and digestion ability.

The results presented here suggest that Np5 treatment during isoniazid therapy of TB increases the effectiveness of anti-TB therapy as well as the strength of the immune response. In this study, the treatment with Np5 provided as 5 daily i.p. injections, significantly decreased the lung weight index, the lung damage index, the severity of lung tissue damage, the markers of alteration, and protected the multilayer bronchial epithelium. The growth of M. bovis-bovinus 8 in spleen culture was also decreased.

CONCLUSION

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.

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