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Summary of dissertation

Adjuvant mucosal immunization of mice against influenza virus

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CONTENT

CHAPTERS

Abbreviations

BF	*Bacillus firmus*
DBF	Delipidated *Bacillus firmus*
DC	Dendritic cells
HA	Hemagglutinin
HT	Hemagglutination titre
iNOS	Inducible nitric oxide synthase
LAIV	Live attenuated influenza vaccine
mDC	Myeloid dendritic cells
NA	Neuraminidase
NALT	Nasopharynx-associated lymphoid issue
PCA	Principal component analysis
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
RQ	Relative quantification
TLR	Toll-like receptor

Abstract in Czech

Chřipkové infekce způsobují celosvětově vysokou morbiditu a mortalitu. Současná imunizace proti chřipce je prováděna pomocí parenterálně podávaných vakcín. Tyto vakcíny navozují dobrou systémovou imunitu, ale nevyvolávají imunitu slizniční. Vzhledem k trvalému nebezpečí nového vzniku vysoce patogenních subtypů chřipky typu A a také značně odlišných driftových variant typu B je v současné době žádoucí, vývoj vakcíny, která by indukovala křížovou protekci. Hlavní cíle této práce byly následující: prozkoumat adjuvantní účinek *Bacillus firmus* (BF) a jeho delipidované formy (DBF) po imunizaci myší cestou respiračního traktu, otestovat schopnost BF a DBF navodit křížovou (intrasubtypovou a intersubtypovou) protekci a nakonec charakterizovat adjuvantní mechanismus účinku BF/DBF.

Adjuvantní účinek BF a DBF byl testován po intratracheální nebo intranazální imunizaci myší inaktivovanými viry chřipky typu A a B. Oba typy imunizace stimulovaly jak systémovou tak i slizniční imunitu, přičemž typ B, byl méně immunogennní oproti typu A. Adjuvantní imunizace směsí virus (A nebo B)+DBF vedla ke zvýšení protilátkové odpovědi. Účinek BF a DBF na navození heterosubtypické imunity byl testován v *in vivo* experimentech. Po imunizaci byly myši infikované letální dávkou chřipky typu A (A/PR/8/34) nebo B (B/Lee/40). V našich experimentech jsme zjistili výrazný ochranný efekt adjuvantní imunizace proti homologním virům a také navození výrazné křížové protekce (ochrana proti H1N1 po imunizaci s H3N2 a ochrana proti B/Lee po imunizaci s B/Yamanashi).

Mechanismus adjuvantního účinku byl testován v NALT po intranazální imunizaci myší inaktivovaným virem chřipky typu A, samotným adjuvans (DBF) nebo kombinací virus +DBF. Sledována byla změna exprese genů pro: cytokiny, toll-like receptory a také další geny podílející se na imunitní odpovědi pomocí kvantitativní real-time PCR. Intranazální imunizace samotným DBF, ale hlavně kombinací virus+DBF vedla k zvýšení exprese cytokinů charakteristických pro Th1 odpověď (IFN-γ a IL-2) přičemž exprese genů typu Th2 byla snížená (IL-4). Dále byla detekována zvýšená exprese genů pro IL-6 a IL-10, které jsou důležité pro indukci tvorby IgA. Rozdíly v expresi TLR7, TLR9, CCR7 a interferonů I. typu vyplývající z PCA analýzy ukazují na možnou aktivaci plasmacytoidních dendritických buněk. DBF se jeví jako velmi účinné adjuvans pro stimulaci jak slizniční, tak i systémové imunitní odpovědi, pro indukci křížové protekce proti různým subtypům a driftovým variantám chřipkového viru a pro stimulaci přirozené imunity.

Abstract in English

Influenza virus infections cause considerable morbidity and mortality in the world. Current immunization against influenza is provided using parenterally given influenza vaccines. These vaccines can induce good systemic immunity but they fail to induce a protective mucosal immunity. Because of persisting threat of new highly pathogenic influenza A subtypes development, a vaccination inducing intersubtypic cross-protection is desirable. The principal aims of the present study were: firstly, to explore the effect of *Bacillus firmus* (BF) and its delipidated form (DBF) as mucosal adjuvants for immunization via respiratory tract, secondly to test the potencial of BF and DBF to induce intrasubtypic and intersubtypic protection and finally to characterize the mechanism of adjuvant effect.

The adjuvant effect of BF and DBF was tested after intratracheal or intranasal immunization of mice with inactivated influenza virus type A or B. Both types of immunization stimulated both systemic and mucosal immunity. Inactivated influenza virus type B was less immunogenic in contrast to type A. Adjuvant immunization with mixture of virus (type A or B) + DBF increased both systemic and mucosal antibody response. The effect of BF and DBF on induction of heterosubtypic immunity was tested in *in vivo* protective experiments. After immunization, mice were infected with influenza A (A/PR/8/34) or B (B/Lee/40), both lethal for mice. Our experiments documented a pronounced protective effect of the adjuvant immunization against homologous virus and a conspicuous cross-protection (protection against H1N1 after immunization with H3N2 and protection against B/Lee after immunization with B/Yamanashi).

The mechanism of adjuvant effect was tested in NALT after intranasal immunization of mice by inactivated influenza virus type A, adjuvant alone (DBF) and by mixture of virus+DBF. We tested the expression of selected genes for cytokines, toll-like receptors and other genes participating in immune response by qPCR. Intranasally given DBF and mainly mixture virus+DBF induced expression of cytokines characteristic for Th1 immune response (IFN-γ and IL-2) whereas expression of genes characteristic for Th2 was decreased (IL-4). Increased expressions of IL-6 and IL-10 are important for production of IgA. Differences in expression of TLR7, TLR9, CCR7 and type I IFN followed by PCA analysis indicate activation of plasmacytoid dendritic cells (pDC). DBF has been shown to be very efficient adjuvant for stimulation of both mucosal and systemic immune responses, in induction of heterosubtypic immunity against influenza virus and in stimulation of innate immunity.

Introduction

Influenza virus remains important viral pathogen of significant medical importance causing each year pronounced morbidity and considerable mortality in population [1]. Influenza epidemics continue to infect large numbers of people worldwide, despite the availability of vaccines. These are derived from the current circulating viral strains and continuously modified according to actual epidemiological situation, because of frequent natural variation of the hemagglutinin (HA) and neuraminidase (NA) surface proteins of the virus. This variation allows to the virus to escape neutralization by preexisting circulating antibodies in the blood, present as a result of either previous natural infection or immunization. The current vaccination against influenza succeeded in achieving a considerable lowering of morbidity and mortality of this disease. However, the hitherto predominantly parenteral mode of vaccination cannot be considered optimal. This mode of vaccination induces immunity mostly against a homologous strain of the virus used for vaccination. Although systemic immunity is reached, mucosal immunity is not stimulated efficiently enough. In contrast, mucosal vaccination can cause good mucosal as well as systemic immunity [2]. Mucosal stimulation is generally much more efficient with live than with inactivated virus. The use of live viruses is always connected with certain risk. However, various types of mucosal adjuvants can enhance the stimulatory effect of inactivated virus after mucosal immunization. The attention is focused on vaccines that are able to induce cross-protection and could be effective also in the case of a sudden appearance of a new virus variant. Cross-protection was studied mainly on the experimental mouse model. In addition to the protection against a homologous virus, mice infected with type A influenza virus could display also protection against the drift virus variants under certain conditions. The probability to induce intrasubtype-specific or heterosubtypic cross-protective immunity is much higher after a natural infection than after vaccination [3]. Efforts are therefore made to make the vaccination as close to the natural infection as possible. The use of vaccines containing live viruses (LAIV) that have been used in Russia and the USA seems to be well protective. These vaccines are administered intranasally, what corresponds to the natural entry point of the influenza infection into the organism. Intranasal application induces excellent mucosal immunity, activates not only antibody but also cellular response, and can induce cross-protection [3]. The handicap of these vaccines is the risk of application to immuno-compromised patients (young children, old and immunodeficient individuals). In these recipients, the attenuated live virus could induce infection, though milder than that evoked by

a wild type virus [4]. Another risk is the large variability of the influenza virus A and the ensuing potential risk of reversion of the cold-adapted mutant. The vaccine used in the USA has considerable indication limitations (5-49 years of age) that exclude population groups that are at the highest risk of influenza [5-10]. It is necessary to admit that an ideal anti-influenza vaccine is not available. At any rate it is important to continue in the effort to design optimal, preferably inactivated, vaccines which can raise good systemic as well as mucosal immunity. Inactivated influenza viruses are known to be often insufficiently effective when used for mucosal immunization and for induction of cross-protection. The drawbacks of vaccination with inactivated virus can be overcome by using a suitable adjuvant.

Aims

The main aim of this thesis was to elucidate the effect of *Bacillus firmus* on tuning of innate and adaptive immunity after immunization of mice with inactivated influenza virus type A and B via respiratory tract.

Particular aims:

1. **To explore the effect of *Bacillus firmus* as adjuvant in mucosal immunization of mice with inactivated influenza virus. Impact on systemic and mucosal immunity.**

2. **To test the potential of *Bacillus firmus* to induce intrasubtypic and intersubtypic cross-protection against influenza infection.**

3. **To characterize the mechanism of adjuvant effect of *Bacillus firmus.* Influence on activation of innate immunity (gene expression of TLRs and IFN type I). Influence on tuning of adaptive immunity (gene expression of Th1 and Th2 cytokines).**

Material and methods

Bacterial adjuvans

Bacillus firmus (BF) strain CCM 2212 was aerobically cultivated in a liquid medium composed of peptone, beef extract and urea (pH 7.2–7.4) at 37° C to the late exponential phase. The cultures were washed with distilled water and inactivated by 0.4% aqueous

formaldehyde at room temperature for 30 min. All bacteria were killed under these conditions, as proved by sterility tests. The inactivated biomass was washed three times with distilled water and lyophilized. Delipidated bacteria (DBF) were obtained from semi-dry biomass by overnight extraction with chloroform–methanol 2:1 (v/v). After extraction and drying at 50 °C, the extraction was repeated; chloroform alone was used for the third extraction. The liquid was filtrated out and the mass dried.

Influenza virus

Influenza viruses' type A and B were proliferated in a standard way in the allantoic sac of chicken embryos. The virus was inactivated by formaldehyde and the efficacy was tested by three blind transfers.

Animals

Adult BALB/c female mice (9-14 week-old) were used for experiments.

ELISA

Specific anti-influenza antibodies (IgG, IgM and IgA) were tested by ELISA method using biotinylated anti-Ig antibodies and inactivated viruses as antigens. Cytokine concentrations were detected using paired antibodies (R&D) according to manufacturer's instructions.

Sample collection for real-time PCR

The mice were exsanguinated from abdominal aorta in ether anesthesia. NALT was separated from the upper jaw by peeling away the palate where NALT was localized bilaterally on the posterior side [11]. Samples from individual animals were placed in RNAlater stabilization reagent (Qiagen).

Histology

Samples of Lungs and NALTs were fixed for a minimum of 24 h in 4% formaldehyde were dehydrated by graded ethanol and embedded in paraffin. 7 μm sections were stained with Haematoxylin –Eosin.

RNA isolation

Samples were homogenized with Ultra-Turrax T8 homogenizer (IKA) and total RNA was extracted using RNeasy mini kit (Qiagen) following the manufacturer's instruction.

Real-time PCR

RNA was converted to cDNA using Taq-Man reverse transcription reagents (Applied Biosystems). A reaction mix for real-time PCR was made with TaqMan Universal PCR master mix, water and Assays on demand gene expression products for IL-2, IL-4, IL-6, IL-10, IFN-γ, TLR-2, TLR-3, TLR-7, TLR-9, IFN-α4, IFN-α11, IFN-α12, IFN-β1, CCR7, iNOS and β-actin (all Applied Biosystems). 20 µl of reaction mix was aliquoted to the wells on a real-time PCR plate. Each sample was analyzed in duplicate. A volume of 5 µl of cDNA was added to each well. PCR reaction was run on the 7300 real-time PCR System (Applied Biosystems) using standard conditions.

Data analysis and statistics

Data from real-time PCR (Ct values) were analyzed by relative quantification and by principal component analysis (PCA). The method of principal component analysis PCA was employed to disclose multivariate response to the treatment. PCA involves a mathematical procedure that transforms a number of variables (here expression values of various genes) into a smaller number of uncorrelated variables called principal components. By this the dimensionality of the data is reduced to a number of dimensions that can be plotted in a scatter plot (two dimensions). The Student's t-test was used to determine significant differences between the control and experimental groups.

Results

Immune response after adjuvant mucosal immunization of mice with inactivated influenza virus

P. Zanvit, M. Havlíčková, J. Táčner, M. Jirkovská, P. Petrásková, O. Novotná, D. Čechová, J. Julák, I. Šterzl and L. Prokešová *[Immunology Letters 97 (2005) 251–25]*

In the first study, we investigated the effect of BF on the production of antibodies after intratracheal immunization of mice with inactivated influenza virus type B which is included in trivalent influenza vaccine and which is supposed to be less immunogenic than influenza A

virus. We tested the possibility to increase the immune response against inactivated influenza virus by BF or DBF as adjuvant. Lymphocyte proliferation was tested on splenocytes from immunized animals after stimulation with influenza virus (HT 1:5) via incorporation of ^{3}H thymidine. Cytokines were determined in culture supernatants by ELISA method after 24 h (IL-2), 3-day (IL-4 and IL-10) and 5-day cultivation (IFN-γ). Expression of selected genes: IL-2, IL-4, IL-10, IFN-γ and iNOS were tested by RT-PCR in lungs. Changes in morphology of lungs after intratracheal immunization were examined histologically.

Production of specific anti-influenza antibodies after intratracheal immunization

Production of specific anti-influenza antibodies was measured by ELISA method after intratracheal immunization in serum, BAL and intestinal washings. Immunization with inactivated virus alone evoked only a mild systemic response and no mucosal response. After immunization with virus suspension plus bacteria, the anti-influenza antibodies increase markedly, the adjuvant effect of DBF being higher than that of BF. The largest rise in the levels of serum antibodies appears in the IgG class, which reflects best the status of systemic immunity. A milder rise in serum antibodies occurs in IgA. A rise of the antibody activity of all three classes, especially in IgG and IgA, is evident after adjuvant immunization in BAL, which reflects the state of antibody immunity in the respiratory tract. BAL antibodies mainly that of IgA class are locally produced but IgG antibodies originate mainly from serum and enter respiratory system by transudation in lungs.

Local production of cytokines in the lungs

RT-PCR technique was used to estimate the expression of IL-2, IFN-γ, IL-4 and IL-10 in lungs. Only traces of mRNA for these cytokines were detected in the lungs of non-immunized animals. Immunization with the virus alone led to an increase in mRNA for all cytokines under study, a still greater mRNA production for all cytokines being found after immunization with BF alone or virus+BF.

Local activation of macrophages in the lung

RT-PCR proved the presence of mRNA for inducible NO syntheses (iNOS), which can serve as a marker of macrophage activation. All types of immunization induced an increased production of mRNA for iNOS relative to non-immunized controls, although even in the controls the production was quite sizable.

Immunization with the inactivated influenza virus B alone did not produce a sufficient immune response, while the use of BF or DBF as adjuvants evoked remarkable antibody response, both systemic and mucosal. Intratracheal immunization gave rise to high levels of mucosal antibodies, in particular in the respiratory tract. The immunization increased the local cytokine production in the lungs without any marked Th1 – Th2 polarization. The use of adjuvant lowers inflammatory changes in lungs accompanying intratracheal immunization. Activation of lung macrophages after immunization was proved by production of iNOS.

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Adjuvant effect of *Bacillus firmus* in intranasal immunization of guinea pigs with inactivated type B influenza virus

M. Havlíčková, L. Prokešová, P. Zanvit, J. Táčner, R. Limberková *[Folia Microbiol. 51 (2), 154–156 (2006)]*

Adjuvant effect of BF after intranasal immunization of guinea pigs was tested in this study. Inactivated influenza virus type B + BF as adjuvant in comparison with the virus alone, can stimulate higher titers of serum hemagglutination-inhibiting antibodies and virus-neutralizing antibodies.

This phenomenon could be exploited for preparation of immune sera for *in vitro* diagnosis. High level of neutralizing antibodies after adjuvant immunization of guinea-pigs with inactivated virus suggests the possibility of safety preparation of diagnostic sera against highly pathogenic strains with respect to standard biosafety guidelines.

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Protective and cross-protective mucosal immunization of mice by influenza virus type A with bacterial adjuvant

Zanvit Peter, Havlíčková Martina, Táčner Jaroslav, Novotná Olga, Jirkovská Marie, Čechová Dana, Julák Jaroslav, Šterzl Ivan, Prokešová Ludmila *[Immunology Letters 115 (2008) 144–152]*

Currently used influenza vaccines are strictly homotypic. Persisting thread a development of new pandemic strain requires vaccination inducing intersubtypic cross-

protection. In the next study, we tried to reach this goal by mucosal immunization of mice using delipidated *Bacillus firmus* (DBF) as adjuvant. BALB/c mice were immunized intratracheally with inactivated influenza A H1N1 and H3N2 viruses. The protective and cross-protective effect against infection was tested in *in vivo* experiments after infection with influenza virus A H1N1.

Production of antiviral antibodies detected by ELISA

The type A virus A/PR/8/34 was found to be much more immunogenic than type B viruses. Intratracheal immunization with inactivated virus alone induced perceptible antibody response in contrast to type B viruses used for immunization in the same amount [12]. The levels of virus-specific antibodies were detected after intratracheal immunization in serum, BAL and intestinal washings. The levels of IgG and IgA in serum of control mice immunized with PBS were low and so were also the levels detected in the group immunized with the adjuvant alone. The group immunized with A/PR/8/34 virus alone exhibited a more conspicuous production of virus-specific antibodies of IgG and IgA classes. The highest IgG and IgA levels were detected in the group of mice immunized with a virus+DBF mixture; this indicates a strong induction of systemic immunity. In BAL the lowest levels of virus specific antibodies were detected in mice immunized with PBS or with the adjuvant alone. Increased levels of IgG and IgA were detected in the group immunized with the virus alone. The highest levels of both IgG and IgA were again detected in the group immunized with the virus+DBF.

Local production of cytokines in the lungs

Testing of cellular immunity in the lungs after immunization was performed by real-time PCR determining the local expression of cytokines characteristic for Th1 (IL-2, IFN-γ) and Th2 (IL-4, IL-10) response. The group immunized with virus+DBF displayed a many-times up-regulated expression of IL-10 and of all other cytokines.

Protective experiment *in vivo*

The mice were immunized with the virus A/PR/8/34 (H1N1) and the heterologous type A/California/7/04 (H3N2). Two various concentrations of adjuvant were tested – 50 and 500 μg of DBF. The infection was induced 10 days after the second immunization dose by using a live, mouse-adapted strain A/PR/8/34 (H1N1), and the progress of infection was monitored for 14 days by following body weight loss and mortality. Control mice and mice

immunized with helerologous type H3N2 were not protected against live H1N1 influenza challenge and, surprisingly, also mice immunized with homologous type H1N1 were not well protected. In contrast, mice immunized with combination of H1N1+DBF or H3N2+DBF in lower and higher concentrations of adjuvant were well protected against challenge with live influenza virus H1N1. The challenge with homologous or heterologous virus subtype proved cross-protective effect of adjuvant immunization. Even mice given the adjuvant in lower or higher dose were protected against death but not against disease.

The protective effect of both standard and adjuvant immunization was confirmed in the infection experiment by the immunized mice exhibiting lower weight loss and lower mortality. After the adjuvant immunization the lethal effect of the virus was completely eliminated and the weight loss was minimized. We succeeded in showing a marked cross-protection between heterologous virus A subtypes H1N1 vs. H3N2, which was reflected in lower weight loss and zero mortality (100% survival), and was also demonstrated by the histological picture of the lungs after infection.

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Stimulation of protective and cross-protective immunity against influenza B virus after adjuvant mucosal immunization of mice

L. Prokešová, P. Zanvit, M. Havlíčková, J. Táčner, M. Jirkovská, P. Petrásková, O. Novotná, D. Čechová and J. Julák

In this study we tested the protective and cross-protective effect of BF after immunization of mice with two different and phylogenetically distant strains of influenza B (B/Lee and B/Yamanashi) and subsequent infection of mice with lethal influenza B/Lee/1/40.

Protective experiment in vivo

Mice were pre-immunized either by influenza virus strain B/Yamanashi, B/Lee, DBF alone as adjuvant and by combination of virus+adjuvant (B/Lee+DBF and B/Yamanashi+DBF). 10 days after the 2nd immunization dose, mice were infected by lethal strain B/Lee. Infection was monitored for 14 days by following body weight loss and mortality.

Intratracheal immunization of mice with inactivated influenza B virus and DBF as adjuvant increases protection of mice against infection with the homologous virus strain and induces cross-protection: mice immunized by influenza virus B/Yamanashi 166/98 were protected even against phylogenetically distant virus drift variant B/Lee/40 lethal for mice.

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Adjuvant effect of *Bacillus firmus* on the expression of cytokines and toll-like receptors in mice nasopharynx-associated lymphoid tissue (NALT) after intranasal immunization with inactivated influenza virus type A

Zanvit Peter, Tichopád Aleš, Havlíčková Martina, Táčner Jaroslav, Novotná Olga, Jirkovská Marie, Kološtová Katarína, Čechová Dana, Julák Jaroslav, Šterzl Ivan and Ludmila Prokešová

To evaluate the effects of adjuvant immunization we studied the expression of genes important for the reaction of both innate and adaptive immunity by real-time PCR: toll-like receptors recognizing antigens of G+ and G- bacteria and microbial nucleic acids (TLR2, TLR3, TLR7, TLR9), type I interferons (IFN-α4, IFN-α11, IFN-α12, IFN-β), Th1 and Th2 type cytokines (IL-2, IFN-γ, IL-4, IL-6, IL-10) and some other genes (CCR7 and iNOS) at time points of 3, 6, 12, 24, 48, 72 and 168 hours post immunization. Data obtained from real-time PCR were further analyzed by relative quantification (RQ) and principal component analysis (PCA).

Relative quantification

RQ data imply that the main changes in gene expression occur during the first 24 h after immunization. Groups immunized with DBF alone or with virus+DBF display very fast changes peaking at 3 and 6 h post immunization. Significant changes after immunization with virus alone were detected later - 12 and 24 h after the treatment. Immunization with the virus alone increases mainly the expression of TLR7, IFN-γ and type I interferons. DBF increases significantly the expression of TLR2, IL-6, iNOS, IFN-γ, and CCR7 and lowers the expression of IL-4. Relative to the virus alone, the combination virus+DBF accelerates the expression of TLR2, TLR7, IL-6 and type I interferons; when compared with DBF alone it increases the expression of TLR7, CCR7 and iNOS. Immunization with the combination

virus+DBF has a faster and longer effect (3 - 72 h) on the expression increase of type I interferons (particularly IFN-α4 and IFN-β1) than treatment with DBF alone.

Principal component analysis (PCA)

The results of PCA imply that the largest changes in the group of genes characteristic for pDC were detected in mice immunized with virus+DBF. These exhibit a similarity between the expression of genes of the functional group for pDC and the functional group of genes for type I interferons. Mice immunized with virus alone evince a „probable" activation of pDC only later at 12 to 24 h post immunization, in contrast to the group immunized with virus+adjuvant, in which a marked change in the expression of genes typical for pDC occurred very early at 3 h post immunization – and the increased expression persisted until 24 h. Immunization with DBF alone caused only mild differences in expression of genes characteristic for both pDC and type I interferons. Mice immunized with the virus alone showed a Th1 polarization of the immune response 3 – 12 h post immunization; 24 h after immunization, the response had rather mixed Th1/Th2 character. In the groups immunized with DBF or virus+DBF, the type of immune responses was mixed (Th1/Th2) in the intervals of 3 – 12 h post immunization it was slightly polarized towards Th1 12 h after immunization and it was Th2-skewed at the interval of 24 h. The most efficient in terms of protection of the organism against virus infection appears to be immunization with virus+DBF, which leads to a fast antiviral immune response based on pDC activation, production of interferons and activation of a mixed Th1/Th2 immune response.

Expression of transcription factor IRF7

In addition to the results described in above mentioned manuscript, the expression of transcription factor IRF7 was tested. It is known that increased gene expression of TLR7 and TLR9 activate MyD88-dependent signaling pathway and trigger expression of IRF7. IRF7 regulates the production of type I interferons and could be involved in the process of pDC activation after immunization with virus+DBF. We have found revealed a pronounced differences in expression of genes for TLR7, TLR9 and CCR7 by PCA analysis. These changes are characteristic for pDC activation. In a group of mice immunized with virus+DBF, a significant difference has been confirmed in IRF7 expression after 6 h ($p=0.0451$), 12 h ($p=0.0009$) and 24 h ($p=0.0062$) after immunization by relative quantification and cluster

heatmap analysis. It is apparent that in these time intervals the changes are the most relevant for the pDC activation process. We found the biggest increase of the IRF7 expression between the 3 and 6 hours after immunization. The increase remains stable from 6 to 24 hours.

Discussion

Our studies of immunomodulating properties of microorganisms revealed that the G+ bacterium of outer environment *Bacillus firmus* has pronounced immunostimulatory properties and, live or inactivated, it is quite harmless to mice *in vivo*; in addition, even high doses of inactivated bacterium do not suppress the viability of cells in culture [13-18].

Excellent adjuvant properties of this bacterium, which were proved in immunization of mice with ovalbumin via the respiratory tract [19], were also seen after intranasal immunization of guinea pigs with inactivated type B influenza virus (B/Lee/1/40). High levels of anti-influenza neutralizing antibodies were detected after adjuvant immunization with virus+BF in contrast to immunization with virus alone. Production of anti-influenza diagnostic sera is often performed by mucosal immunization of guinea pigs or ferrets and this mode of immunization is usually efficient when live viruses are used. High level of neutralizing antibodies after adjuvant immunization of guinea-pigs with inactivated virus demonstrates the possibility of preparation of diagnostic sera against highly pathogenic strains with respect to standard biosafety guidelines [20]. Adjuvant properties of BF were further tested in mouse model after intratracheal immunization of mice with inactivated influenza virus both of type B [21] and type A [22]. Influenza type B has a relatively low immunogenicity and, in the immunization with the virus alone, the level of specific antibodies in the serum and in secretions does not differ much from antibodies in non-immunized control animals. In contrast to influenza type B, influenza type A was found to be much more immunogenic. Intratracheal immunization with inactivated influenza virus type A (A/PR/8/34) alone induced a perceptible antibody response in contrast to influenza type B. Intratracheal immunization with virus (influenza type A or type B) + adjuvants (BF or DBF), strongly stimulates the production of systemic antibodies of IgG class detectable in serum and the production of mucosal IgA antibodies in BAL. Apparently, due to the higher immunogenicity of influenza type A after intratracheal immunization with virus alone, the effect of the adjuvant (DBF) was not as evident as with type B virus. Intratracheal immunization by both influenza type A or type B in combination with adjuvants (BF or DBF)

had a weak (type B) or no effect (type A) on IgA antibody induction in the intestine. This implies that the choice of mucosal site for immunization is important because of subcompartmentalization of mucosal immune system. In contrast, adjuvant immunization with influenza type B affected production of anti-influenza antibodies of IgG class in BAL as well as in intestine. Immunization of mice with influenza type A virus+DBF led to induction of high levels of IgG in BAL, but only low levels of anti-influenza IgG antibodies in intestine. The increased levels of IgG antibodies in BAL are mainly caused by serum transudation in lungs. So that BAL antibodies reflect both mucosal and systemic immunity. In regard to intestinal antibodies, some transudation from serum could be supposed as well. Stimulation of cellular immunity in the lungs after intratracheal immunization was examined by determining the local expression of cytokines characteristic for Th1 (IL-2, IFN-γ) and Th2 (IL-4, IL-10) responses by RT-PCR after immunization with influenza virus type B [23] and by real-time PCR after immunization with influenza virus type A [22]. The results indicate that expression of cytokines was increased without a perceptible Th1 or Th2 polarization after adjuvant immunization with influenza virus type B. Adjuvant immunization of mice with influenza virus type A resulted in a mixed Th1/Th2 response as well. The predominant Th2 response was recorded after immunization with the virus alone. The increased expression of cytokines, mainly those of Th1 type (especially of IFN-γ) points to an increased activation of cellular immune response after adjuvant immunization. The protective effect of a standard and an adjuvant intratracheal immunization was confirmed in protective *in vivo* experiment after challenge of mice with live influenza A/PR/8/34 highly pathogenic for mice. After the adjuvant immunization, the lethal effect of the virus was completely eliminated and the weight loss signalizing disease was minimized. We succeeded in showing a marked intersubtypic cross-protection between heterologous virus A subtypes H1N1 vs. H3N2, which was reflected in lower weight loss and zero mortality (100 % survival) after infection with heterologous virus. The protective effect was also demonstrated by the histological picture of the lungs after infection. It is noticeable that, despite the large increase in total antivirus antibodies and the excellent protective effect, no perceptible increase has been detected in the levels of virus-neutralizing antibodies. This indicates that the role of antibodies acting against conserved internal antigens or the role of cellular immunity against internal antigens may be more pronounced in the protection against influenza infection than generally thought. DBF exerted conspicuous protective effect even in infection of mice immunized with the adjuvant alone; this is in concordance with its stimulatory effect on innate immunity.

The main effort is currently exerted on the induction of protective immunity against influenza A which is more significant from the point of view of public health. Much less attention is paid to vaccination against influenza B. The choice of a suitable virus B strain for vaccination can be difficult. Influenza virus B is not split in various subtypes, nevertheless it exist in many antigenically different variants which developed during phylogeny of the virus and can be clustered into three main developmental groups with very limited cross-reactivity: early strains (1940-1970) including strain B/Lee/40, lineage B/Yamagata-like (1972-2005) including strain B/Yamanashi 166/98 and lineage B/Victoria-like (1975-2007) [24]. We were able to induce cross-protection of mice against lethal influenza B/Lee/40 by adjuvant immunization with phylogenetically and antigenically distant strain B/Yamanashi (B/Yamagata-like). Immunization with virus alone did not have cross-protective effect. The study of cross protection among various strains of both influenza A and B viruses reveal the potency of our adjuvant to support the induction of immune response against cross-reactive epitopes of influenza viruses.

The effect of BF and DBF was compared in our first experiments. Our data confirm slightly stronger stimulatory potencial of DBF than BF. Therefore, DBF was used in majority of further experiments.

The mechanism of adjuvant effect of DBF was followed by studying the changes in the gene expression in the NALT after intranasal immunization of mice. The first defense line against influenza is an innate immunity with its essential component, type I interferons, which are mainly produced by pDC. This function is closely connected to their ability to express TLR7 and TLR9 in early endosomes, which enable them to recognize foreign viral or bacterial nucleic acids. To evaluate the effects of adjuvant immunization, we studied the expression of genes important for the reaction of both innate and adaptive immunity by qPCR: genes for toll-like receptors recognizing antigens of G+ bacteria and microbial nucleic acids (TLR2, TLR3, TLR7, TLR9), type I interferons (IFN-α4, IFN-α11, IFN-α12, IFN-β), type Th1 and Th2 cytokines (IL-2, IFN-γ and IL-4, IL-6, IL-10, respectively) and some other genes (CCR7 and iNOS) at different time points (3, 6, 12, 24, 48, 72 and 168 hours) post immunization. For evaluation of data, relative quantification method (RQ) and principal component analysis (PCA) were used. PCA involves a mathematical procedure that transforms a number of variables (expression values of various genes) into a smaller number of uncorrelated variables called principal components. In this way, the dimensionality of the data is reduced to a number of dimensions that can be plotted in a scatter plot in two

dimensions [23]. Our results showed that immunization with DBF alone had a very fast effect; it has markedly influenced the gene expression already 3 h after immunization. This effect decreased at later time points. This is in agreement with the fact that bacterial adjuvants support mainly innate immunity. Affecting innate immunity then influences adaptive immunity. DBF causes a highly significant early increase in expression of IFN-γ, which can considerably support the immune response of Th1 type, what is important in the defense against viral infection. Marked change in type Th2 cytokines (IL-4, IL-10 and IL-6) after immunization with DBF was evident from PCA analysis as well. However, this cannot be ascribed to stimulation of Th2 response because the expression of IL-4 was not significantly increased at any time point – in fact, at the first time points of 3 and 6 h the expression of IL-4 was even significantly lowered. The large changes in PCA are apparently due to a markedly increased expression of IL-6 which, along with the concomitantly increased expression of iNOS, is mainly caused by the inflammatory effect of DBF. DBF alone increased the expression of IL-10 as well. An environment with increased concentrations of IL-6 and IL-10 is known to support the production of IgA. In contrast to the group immunized with DBF, mice immunized with virus alone exhibited delayed and short-term changes in expression of genes followed. A significant increase in expression of both IL-4 and IFN-γ was demonstrated only 24 h after immunization. Comparison of the results of RQ and PCA indicates that the onset of the Th1 type response occurs 12 h after immunization. It therefore appears to be a mixed Th1/Th2 response with a slight Th1 accent. After adjuvant immunization with virus+DBF, the response was fast, protracted and has a mixed Th1/Th2 character, the Th1 response being the strongest after 3 and 6 hours while the Th2 one after 12 and 24 hours. The DBF effect on stimulation of innate immunity was tested by TLRs expression. TLR2 recognizes different bacterial components such as lipoproteins, lipopeptides and peptidoglycans and is the principal receptor for recognition of G+ bacteria [25]. TLR2 is thus important for recognition of DBF obtained from G+ *Bacillus firmus.* The results point to a significantly increased expression of TLR2 at the time interval of 3 - 6 h after immunization either with DBF alone or with the combination virus+DBF. TLR3 and TLR7 participate in the recognition of influenza virus in certain cell populations. A strong production of type I interferons sets in after the recognition of the influenza virus by TLR7 present in early endosomes of pDC. [26]. TLR9, which is also expressed in pDC, is able to recognize nonmethylated CpG regions in viral and bacterial ssDNA [27;28]; this receptor could thus also participate in the recognition of the bacterial adjuvant. Mature pDC are further

characterized by an increased expression of the chemokine receptor CCR7, which plays a key role in the migration of pDC to lymph nodes. Our data demonstrate strong activation of genes characteristic for pDC (TLR7, TLR9, CCR7), mainly in the group immunized with virus+DBF, at early time points after immunization. DBF alone does not cause any marked increase in TLR7 expression. The group immunized with the virus alone evinced only a non-significant increase in TLR7 expression 3 and 12 h after immunization whereas the increase after 24 h was already significant. These data imply that immunization with the virus alone and with virus+DBF activates pDC, the immunization with virus+DBF causing a stronger, faster and longer activation of genes typical for pDC. The increase in TLR3 (recognizing viral dsRNA) expression was at the limit of significance especially in the group immunized with virus+DBF in the interval of 24-72 hours whereas groups immunized with the virus alone or with DBF exhibited a relatively weaker increase. pDCs are the main producers of type I interferons, which ensure an early innate protection against viral infection. The mouse genome contains 14 known IFN-α genes and 3 IFN-α pseudogenes. The highest anti-proliferation and antiviral activity relative to IFN-α1 is exhibited especially by IFN-α4, IFN-α11, INF-α12 and IFN-β [29]. For instance, the activity of IFN-α4 is 5-10-fold higher than that of IFN-α1 [30;31]. A two-step mechanism of expression has been described in interferons [32;33]. Transcription of genes encoding IFN-α4 and IFN-β takes place very early after viral infection and is governed by the transcription factor IRF-3. Transcription of further genes of interferons is then controlled by the transcription factor IRF-7. Viral infection first activates the expression of IFN-α4 and IFN-β; this activation is then followed by an increase in the expression of other interferon types. Our data indicate that the increase in expression of interferons IFN-α4, IFN-α11, IFN-α12 and IFN-β begins early after immunization with virus+DBF and can be detected at all time points from 3 to 72 hours. Type I interferons released by pDC not only prevent viral infection but also activate NK cells, myeloid dendritic cells (mDC) and also B and T lymphocytes, and participate therefore in the regulation of both innate and adaptive parts of immunity. We have found a pronounced difference in expression of genes TLR7, TLR9 and CCR7 by PCA analysis in group of mice immunized with virus+DBF when compared to other treatments (virus alone or DBF alone). I has been reported that increased gene expression of TLR7 and TLR9 activate MyD88-dependent signaling pathway and trigger expression of IRF7 [34;35]. This change is characteristic for pDC activation. We confirmed significant difference in IRF7 expression after 6, 12 and 24 hours in group immunized with virus+DBF. The up-regulated expression of IRF7 is in

concordance with the increased expression of the type I interferons. These results indicate that immunization with inactivated influenza virus type A together with adjuvants trigger the activation of pDC.

Bacillus firmus has been shown to be very efficient adjuvant with strong effect on activation of both mucosal (induction of high levels of IgA in secretions) and systemic (induction of high levels of IgG in serum) antibody responses. Adjuvant immunization protected mice against lethal infection by both homologous and heterologous strains of influenza virus (intrasubtypic and intersubtypic cross-protection).

Conclusions

In relation to the aims of this work we tested impact of adjuvant effect of *Bacillus firmus* on stimulation of mucosal and systemic immunity after immunization of mice via respiratory tract. Intratracheal immunization gave rise to high levels of mucosal antibodies, in particular in the respiratory tract, whereas intranasal immunization enhanced the local production of antibodies both in the respiratory tract and in the intestine. Both types of immunization stimulate systemic immunity as well. Inactivated influenza virus type B alone was less immunogenic but high levels of mucosal and systemic antibodies were detected after adjuvant immunization. In contrast to inactivated influenza type B, inactivated influenza virus type A alone induced a satisfactory systemic and mucosal antibody response which was still increased by adjuvant.

Currently used influenza vaccines are strictly homotypic and they fail in induction of cross-protection. Our *in vivo* experiments documented a pronounced protective effect of the adjuvant immunization against homologous virus and a conspicuous cross-protection was observed in protective experiments (protection against H1N1 after immunization with H3N2 and protection against B/Lee after immunization with B/Yamanashi). In addition, a distinct protective effect against infection was observed even after preimmunization with BF alone. Mice given only delipidated BF (DBF) were protected against death but not against disease. Their lungs were more afflicted than in mice immunized by virus+adjuvant. Partial protective effect of DBF was probably caused by stimulation of innate immunity.

On the basis of the previous results we tried to characterize the mechanism of action of BF in NALT after intranasal immunization. Intranasally given DBF and mainly mixture

virus+DBF induced elevated expression of cytokines characteristic for Th1 immune response (IFN-γ and IL-2). Expression of typically Th2 cytokine IL-4 was lowered in contrast to increased expression of IL-6, and IL-10. Th1 polarization of immune response after immunization with DBF alone or virus+DBF is important in the defense against viral infection. Increased concentrations of IL-6 and IL-10 are important for production of IgA. Differences in expression of TLR7, TLR9, CCR7 and type I IFN followed by PCA analysis leads us to the idea of pDC activation. IRF7 regulates the production of type I interferons and its increase after adjuvant immunization could be involved in the process of pDC (IFN-α4, IFN-α11 and IFN-α12) and mDC (IFN-β) activation. Adjuvant immunization had also influenced inflammation in respiratory lymphatic tissue which was evident from the increased iNOS expression in NALT. Increased gene expression found is in accordance with stimulation of both innate and adaptive immunity and elucidates adjuvant effect of *Bacillus firmus.*

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Pavel Drastich, Lenka Frolova, **Peter Zanvit**, Julius Spicak, Helena Tlaskalova-Hogenova: Mucosal IL-6 production in various parts of intestine in IBD patients.
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