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Role of T Cells in the Adjuvant Effect of *Bacillus firmus* on the Immune System of Mice: Intranasal and Intratracheal Immunization Study with Ovalbumin

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ABSTRACT. Functions of T cells were determined after intranasal and intratracheal immunization of mice with ovalbumin (Ova) and *Bacillus firmus* (*Bf*), a Gram-positive nonpathogenic bacterium of the external environment, or delipidated *Bf* (d*Bf*) as adjuvants, with the aim to elucidate the mechanism of support of Ova-specific antibody production caused by *Bf* that had been observed in an identical experiment. Neither *Bf* nor d*Bf* in a mixture with Ova stimulated Ova-specific T-cell response tested as antigen-specific blast transformation. By contrast, a mild polyclonal stimulation was observed in splenocytes from mice given d*Bf*. *In vitro* incubation of splenocytes with 100 μg (but not 10 μg) of *Bf* or d*Bf* led to a highly significant inhibition of proliferation below the control level in all groups of animals. Supernatants of splenocyte cultures were further tested for cytokine production. IL-10 and IFN-γ were released after *in vitro* challenge with d*Bf* and in some cases also with *Bf*. Analysis of sera demonstrated that administration of Ova + adjuvant brought about an increase in anti-Ova IgG_1, IgG_{2a} and IgG_{2b} whereas treatment with Ova alone caused a rise in IgG_1 only. The role of *Bf* or d*Bf* in the enhancement of antigen-specific antibody production could be in influencing macrophages and inducing cytokine milieu composed of IL-10, IFN-γ and other factors that leads to a bystander stimulation of specifically activated Ova-B cell receptor (Ova-BCR)-bearing cells.

Abbreviations

Bf	*Bacillus firmus*
BCR	B-cell receptor
ConA	concanavalin A
CT	cholera toxin
d*Bf*	delipidated *Bacillus firmus*
FCS	fetal calf serum
i.n.	intranasal
i.t.	intratracheal
LPS	lipopolysacharide
LT	heat-labile toxin of *E. coli*
Ova	ovalbumin
PBS	phosphate-buffered saline
PRR	pattern recognition receptors
PAMP	pathogen-associated molecular patterns
ROI	reactive oxygen intermediates
RNI	reactive nitrogen intermediates
RTDC	respiratory tract dendritic cells
SI	stimulation indices
TCR	T-cell receptor

The majority of pathogenic microorganisms initiate an infection at the mucosal surfaces of the respiratory or gastrointestinal tract. The benefit of mucosal immunization is in supporting the of defense mechanisms at the site of infectious agent invasion and also in the systemic lymphoid tissue while classical parenteral vaccination influences mainly the systemic compartment. It should be noted that the natural response of healthy mucosa to non-replicable protein antigen is unresponsiveness. Thus, search for safe mucosal adjuvants is required not only to boost mucosal and systemic immunity but also to prevent the induction of mucosal tolerance (McGhee *et al*. 1992).

An important advantage of the use of bacterial adjuvants in vaccination is the involvement of non-specific immunity in the initial phase of the immunization. Admixed bacterial adjuvants are recognized by germ-line-encoded pattern recognition receptors on cells of innate immunity. Binding of pathogen-associated molecular patterns to PRR induces the production of ROI and RNI, pro-inflammatory cytokines, and up-regulates the expression of costimulatory molecules, subsequently initiating adaptive immunity (Werling *et al*. 2003). Co-administration of complex bacterial antigens together with the protein responsible for the specificity of immunization should provide an effective defense. On the other hand, bacterial adjuvants are often potent polyclonal stimulator of lymphocytes and can cause harmful systemic immunological reactions. In summary, the development of bacterial adjuvants is focused on both effectiveness and safety.

The most extensively studied mucosal adjuvants are CT derived from *Vibrio cholerae* and LT of *Escherichia coli* (Elson *et al*. 1984). Mucosal exposure to them results in antibody response restricted to CT or LT and to concomitantly given protein antigen, and stimulates also antigen-specific T cells (Xu-Amano *et al*. 1993; Takahashi *et al*. 1996). Unfortunately, the most efficient forms of these adjuvants are toxic in humans and so the bulk of current work is focused on construction of non-toxic mutants with preserved adjuvanticity (Yamamoto *et al*. 1997; Kweon *et al*. 2002).

Gram-positive bacteria and their components were found to be attractive candidates for immunomodulators or adjuvants because of their interesting immuno-active properties and lack of potentially damaging effects mediated by LPS. *Bacillus firmus*, a G^+ nonpathogenic bacterium of the external environment, appeared to excel among other G^+ microrganisms in the ability to stimulate macrophages *in vivo* and *in vitro* (Mára *et al.* 1992; Štěpánková *et al*. 1995; Zídek *et al.* 1998) and in polyclonal activation of B cells (Prokešová *et al.* 1994, 1995). *Bf* appeared to influence specific antibody production in mice after both parenteral (subcutaneous and intraperitoneal) and mucosal (intragastric, rectal, intranasal and intratracheal) immunization if given as an adjuvant in a mixture with a protein antigen (Prokešová *et al.* 1998; Mlčková *et al*. 2001).

An immunization study with ovalbumin as a model antigen and *Bf* as an adjuvant *via* respiratory tract (intranasal and intratracheal) in mice was particularly interesting since it demonstrated a good efficiency of the adjuvant in stimulation of Ova-specific antibody formation without any stronger polyclonal activation of B cells (Mlčková *et al*. 2001). There was an increase of anti-Ova IgG in sera, bronchoalveolar lavage and intestinal washings. A significant increase of anti-Ova IgA was observed in intestinal washings after i.n. and in bronchoalveolar lavage after i.t. immunization. Analysis of anti-Ova IgG subclasses in sera did not demonstrate any prevalent T_H1 or T_H2 cell impact (Mlčková *et al*. 2001).

In this study we focused on the role of T cells in i.n. and i.t. immunization of mice with *Bf* as an adjuvant admixed to Ova. Localization of the immunomodulatory activities of *Bf* in individual subcellular fractions of the bacterium has been under intense investigation; according to some authors, the complex structure of a G^+ cell is often important for the adjuvant effect (*e.g.*, Hessle *et al*. 2000). Interestingly, delipidated bacteria exceeded whole formolized lyophilized bacteria in polyclonal stimulation of B cells *in vitro* (*unpublished data*). Also the effectiveness of d*Bf* in antigen-specific antibody production support was better than with whole bacteria and so d*Bf* was used as an alternative adjuvant in i.n. and i.t. immunization with Ova and its effect on T cells were compared with that of *Bf*.

MATERIAL AND METHODS

Animals. Adult BALB/c female mice (8–12-week-old) were purchased from *AnLab* (Czechia) and were fed *ad libitum* with Ova free diet.

Bacteria. *Bacillus firmus* strain CCM 2212 was cultivated and formolized according to Prokešová *et al.* (1998).

Delipidated *B. firmus* was prepared from semi-dry inactivated biomass by overnight extraction with chloroform–methanol 2 : 1 (*V*/*V*). After filtration and drying at 50 °C, the extraction was repeated. For the third extraction chloroform alone was used; the mass was filtrated and dried (Mára *et al.* 1992).

Immunization. BALB/c mice were intraperitoneally anesthetized with Ketamine (120 μg/g body mass). Immunization solution (5 μL for i.n. and 25 μL for i.t. immunization) was applied into each nostril with an automatic micropipette. There were 6 groups of mice (5 animals per group) in the experiment:

Group of mice	Immunization solution/suspension (all in PBS)
Control	PBS
Ova	100 μg of ovalbumin (*Sigma-Aldrich*, USA)
Bf	500 μg of formolized lyophilized *Bf*
Ova + *Bf*	100 μg of Ova + 500 μg of *Bf*
d*Bf*	500 μg of d*Bf*
Ova + d*Bf*	100 μg of Ova and 500 μg of d*Bf*

The mice were immunized three times at 1-week intervals and were sacrificed by exsanguination under ether anesthesia 1 week after the last dose. Sera were used for anti-Ova IgG subclass analysis. Spleens and Peyer's patches were collected.

Isolation of cells. The spleens were homogenized in a glass tissue grinder and the cell suspension was filtered through sterile cotton tissue. Isolated splenocytes were washed three times, incubated for 1 h at 37 °C to remove cytophilically bound Ig and washed again three times.

Peyer's patches were pooled from one-half of animals in each group to gain two samples per group, washed twice in minimum essential medium with gentamicin (40 mg/L) and 5 % FCS and teased apart with forceps in a Petri dish. Released cells were filtered through nylon tissue.

Both splenocytes and Peyer's patch cells were resuspended in RPMI 1640 with 5 % FCS for cultivation.

Blastic transformation. Isolated cells (2×10^4 cells per well) were stimulated with Ova at a final concentration of 100 mg/L or 10 mg/L, with formolized lyophilized *Bf* at 100 mg/L or 10 mg/L, or with concanavalin A at 5 mg/L, and incubated for 4 d in a humidified atmosphere with 5 % CO_2 at 37 °C in polystyrene plates (*Gama*, Czechia). On day four ^{3}H-thymidine (*Lacomed*, Czechia) was added at 37 kBq per well. After 18 h of incubation, the cells were harvested and incorporation of ^{3}H-thymidine was measured using a 1450 Micro Beta (*Wallac*, Finland) liquid scintillation counter.

Cultivation of cells for cytokine production. Splenocytes (1/nL, *i.e.* 10^6 cells per mL) were incubated with Ova, *Bf*, d*Bf* or ConA in a humidified atmosphere with 5 % CO_2 at 37 °C in polystyrene tissue culture tubes (*Gama*, Czechia). Production of IL-2 was measured by ELISA after a 1-d incubation; IL-4 and IL-10 were assayed after a 3-d, and IFN-γ after a 5-d incubation.

Cytokine detection. IL-2, IL-4, IL-10 and IFN-γ were detected in culture supernatants by ELISA. Immunoreagents from *R&D System* were used for IL-2, IL-10 and IFN-γ detection and from *Serotec* for IL-4 detection. The procedure was performed according to manufacturers' instructions using high adsorption 96-well tissue culture-treated *Costar* plates (USA).

ELISA for anti-Ova IgG subclass detection. Anti-Ova IgG subclasses were measured in sera by ELISA according to Mlčková *et al*. (2001) using the same immuno-reagents.

Statistics. The results were evaluated by Student's *t*-test.

RESULTS

Blastic transformation. Splenocytes obtained from nonimmunized controls after i.n. immunization displayed a low proliferation level. Those derived from immunized animals (Ova, *Bf*, d*Bf*, Ova + *Bf*, Ova + d*Bf* administered groups) exhibited an increase of proliferation without any *in vitro* stimulation (Table I). A modest increase ($p < 0.05$) was detected in animals immunized with Ova, *Bf* or their combination, in comparison with nonimmunized mice. Administration of d*Bf* or Ova + d*Bf* caused a higher increase ($p < 0.01$) of spontaneous proliferation *in vitro*.

Splenocyte proliferation after i.t. immunization was similar to that after i.n. treatment but the level of proliferation in non-immunized, PBS-administered mice was higher than after i.n. immunization. Proliferation of splenocytes from Ova-administered animals did not differ from non-immunized controls. Splenocytes derived from Ova + *Bf*- or Ova + d*Bf*-treated mice proliferated significantly better than splenocytes isolated from animals immunized only with Ova ($p < 0.03$) (Table I). Proliferation of splenocytes was not significantly influenced by further *in vitro* stimulation with Ova in either i.n. or i.t. immunization regime.

In vitro incubation of splenocytes of all groups of mice with high doses of *Bf* or d*Bf* (100 mg/L culture) caused a highly significant suppression of proliferation in both i.n. and i.t. regimes (i.n. – $p < 0.01$, i.t. – $p < 0.001$, *in vitro* nonstimulated splenocytes *vs*. splenocytes of the same group incubated with 100 μg of *Bf* or d*Bf*, respectively). The inhibition of mitogenic activity was not due to the toxic effect of a high dose of bacterial antigens because the viability of splenocytes incubated even with 200 mg/L of *Bf* or d*Bf* under the same conditions was very good (*unpublished data*). Also the level of IL-10 and IFN-γ in samples stimulated with 100 μg of *Bf* or d*Bf* was significantly elevated compared to controls (cf. Tables III and IV). The suppressive effect on splenocyte proliferation was dose-dependent. Cultivation with low doses of *Bf* or d*Bf* (10 mg/L culture) was either without effect (in Ova-immunized animals) or caused an actual proliferation increase in cells of d*Bf* or Ova + d*Bf* administered animals (i.n. – p = 0.014 for control *vs*. d*Bf*-administered group, $p = 0.012$ for Ova *vs*. Ova + d*Bf*-immunized group) (Table I). Similar results were obtained for i.t. immunization.

No changes of either spontaneous or Ova-stimulated mitogenic activity of Peyer's patch cells were observed after either i.n. or i.t. immunization (*data not shown*).

Detection of anti-Ova IgG subclasses. Anti-Ova IgG subclass profile was very similar after both i.n. and i.t. immunization. There were very low levels of anti-Ova IgG in sera of nonimmunized controls. Anti-Ova IgG increased after immunization with Ova. The main rising subclass was IgG_1 ($p < 0.0001$). The level of IgG_1 remained about the same after addition of *Bf* or d*Bf* into the immunization solution but IgG_{2a} and IgG_{2b} rose in both cases, Ova + *Bf* or Ova + d*Bf* (for IgG_{2a} $p = 0.0002$, Ova *vs*. Ova + *Bf*; for IgG_{2b} $p < 0.0001$, Ova *vs*. Ova + *Bf*; for IgG_{2a} $p = 0.0002$, Ova *vs*. Ova + d*Bf*; for IgG_{2b} $p < 0.0001$, Ova *vs*. Ova + d*Bf*). There

were no significant differences in the administration of Ova + *Bf* or Ova + d*Bf* in terms of IgG subclasses (Table II).

Table I. *In vitro* proliferation[a] of splenocytes[b] from intranasally and intratracheally immunized animals[c]

Immunization antigen	None	Ova[d]		*Bf*[d]	d*Bf*[d]		ConA[d]
		10	100	100	10	100	5
Intranasal dosing							
None (control)	1.0 ± 0.1	1.2 ± 0.2	1.3 ± 0.2	0.5 ± 0.1	2.2 ± 0.8	0.6 ± 0.1	5 ± 1
Ova	2.5 ± 0.7	2.4 ± 0.8	2.5 ± 0.7	0.5 ± 0.1	2.1 ± 0.3	0.7 ± 0.1	19 ± 7
Bf	2.3 ± 0.8	2.7 ± 1.0	3.0 ± 1.1	0.9 ± 0.3	–	0.5 ± 0.1	34 ± 28
Ova+*Bf*	2.6 ± 0.5	3.8 ± 0.5	3.3 ± 0.8	0.7 ± 0.1	–	0.9 ± 0.1	39 ± 13
d*Bf*	4.8 ± 1.2	5.4 ± 1.8	5.8 ± 1.6	1.4 ± 0.4	9.3 ± 2.3	1.0 ± 0.2	34 ± 24
Ova+d*Bf*	4.6 ± 1.1	5.1 ± 1.2	6.5 ± 2.2	1.3 ± 0.4	8.0 ± 2.0	0.7 ± 0.1	134 ± 60
Intratracheal dosing							
None (control)	1.0 ± 0.2	0.8 ± 0.2	0.9 ± 0.2	0.2 ± 0.06	–	0.2 ± 0.05	34 ± 33
Ova	1.1 ± 0.2	0.9 ± 0.1	0.8 ± 0.2	0.1 ± 0.02	–	0.2 ± 0.03	14 ± 10
Bf	1.9 ± 0.5	1.3 ± 0.4	1.6 ± 0.5	0.2 ± 0.03	–	0.7 ± 0.40	70 ± 23
Ova+*Bf*	1.9 ± 0.3	1.6 ± 0.3	1.5 ± 0.2	0.2 ± 0.03	–	0.3 ± 0.05	9 ± 6
d*Bf*	1.4 ± 0.3	1.4 ± 0.4	1.3 ± 0.3	0.2 ± 0.04	–	0.2 ± 0.04	19 ± 9
Ova+d*Bf*	2.0 ± 0.3	1.6 ± 0.3	1.4 ± 0.3	0.2 ± 0.05	–	0.3 ± 0.05	25 ± 12

[a]Expressed as mean of stimulation indices ±SEM; SI of nonimmunized *in vitro* nonstimulated control was arbitrarily given the value 1 that responds to 1.6 ± 0.2 Bq; detected by ^{3}H-thymidine incorporation.

[b]Splenocytes of each group were incubated with 5 μg of ConA as a positive control; mean values ±SEM of two independent experiments (five animals per group in each experiment).

[c]Animals were immunized intranasally or intratracheally with 100 μg of Ova, 500 μg of *Bf*, 500 μg of d*Bf*, with a mixture of Ova + *Bf* and Ova + d*Bf*; splenocytes and Peyer's patch cells were isolated and proliferation assay was performed after *in vitro* stimulation with Ova, *Bf*, d*Bf* and ConA; aliquots of splenocytes were incubated with Ova, *Bf*, d*Bf* and ConA for cytokine production; the concentration of IL-2, IL-4, IL-10 and IFN-γ was measured; sera were analyzed for anti-Ova IgG subclasses.

[d]In μg/mL.

Table II. Anti-ovalbumin IgG subclasses (IgG_1, IgG_{2a}, IgG_{2b}, IgG_3) in sera after i.n. immunization[a]

Immunization antigen	IgG_1	IgG_{2a}	IgG_{2b}	IgG_3
None	0.24 ± 0.05	0.43 ± 0.07	0.42 ± 0.05	0.240 ± 0.04
Ova	2.00 ± 0.24	0.86 ± 0.14	1.13 ± 0.15	0.240 ± 0.02
Bf	0.27 ± 0.05	0.44 ± 0.10	0.38 ± 0.04	0.170 ± 0.01
Ova + *Bf*	2.57 ± 0.01	1.86 ± 0.16	2.48 ± 0.11	0.390 ± 0.12
d*Bf*	0.37 ± 0.06	0.73 ± 0.21	0.53 ± 0.09	0.160 ± 0.02
Ova + d*Bf*	2.50 ± 0.03	2.05 ± 0.21	2.51 ± 0.07	0.480 ± 0.12

[a]Antibody levels were detected in sera diluted 1/10 by ELISA and expressed as absorbance A_{490}; mean values ±SEM of two independent experiments (five animals per group in each experiment).

Cytokine detection. Small changes in spontaneous cytokine production in all groups of mice after both i.n. and i.t treatments point to the absence of significant polyclonal stimulation *in vivo* during immunization. The cytokine levels in culture fluids were not influenced by *in vitro* stimulation with specific Ova antigen either after i.n. or i.t. doses. *In vitro* incubation with *Bf* and d*Bf* appeared to be stimulatory for both IL-10 and IFN-γ.

The results after i.n. doses demonstrated that IL-10 slightly but significantly increased after incubation with d*Bf*. The i*n vitro* stimulatory effect of d*Bf* on IFN-γ production was even more pronounced (Table III). A similar effect was achieved when using *Bf* as stimulator *in vitro* but the significance of this effect could not be assessed because of large differences among samples.

Previous immunization with *Bf* or d*Bf* enhanced the production of both IL-10 and IFN-γ following incubation with these bacterial adjuvants *in vitro* in i.t. study. There was a significantly higher production of IL-10 in splenocytes incubated with d*Bf* as compared to *in vitro* unstimulated splenocytes of the same group

($p \leq 0.009$). Splenocytes of groups immunized with *Bf* or d*Bf* alone or in a mixture with protein responded by IL-10 secretion also to incubation with *Bf* ($p \leq 0.03$, *in vitro* nonstimulated splenocytes *vs*. *Bf*-stimulated splenocytes of the same group). Incubation with d*Bf* led to a significant enhancement of IFN-γ production in all groups of animals ($p \leq 0.01$, *in vitro* nonstimulated splenocytes *vs*. d*Bf* stimulated splenocytes of the same group) (Table IV).

Production of IL-2 was not proved and secretion of IL-4 was only very low in both i.n. and i.t. immunization approaches (*data not shown*).

Table III. *In vitro* secretion[a] of IL-10 and IFN-γ (both pg/mL) by splenocytes of i.n. immunized mice

Immunization antigen	None	d*Bf*[b]		ConA[b]
		10	100	5
IL-10				
None (control)	36 ± 18	80 ± 38	99 ± 34	58 ± 18
Ova	38 ± 6	148 ± 16	151 ± 1	104 ± 21
Bf	40 ± 6	67 ± 6	138 ± 36	75 ± 11
Ova + *Bf*	65 ± 9	136 ± 19	190 ± 17	266 ± 60
d*Bf*	33 ± 6	245 ± 132	132 ± 60	79 ± 10
Ova + d*Bf*	77 ± 39	177 ± 24	225 ± 72	210 ± 26
IFN-γ				
None (control)	168 ± 116	929 ± 502	811 ± 467	3371 ± 1130
Ova	217 ± 130	1320 ± 4 13	1393 ± 488	4440 ± 94
Bf	159 ± 88	1048 ± 26	938 ± 77	3195 ± 211
Ova + *Bf*	243 ± 83	1321 ± 297	1344 ± 326	5205 ± 404
d*Bf*	133 ± 24	1787 ± 1263	1992 ± 1056	3538 ± 1188
Ova + d*Bf*	369 ± 285	1338 ± 32	1555 ± 166	3147 ± 292

[a]Mean values ±SEM of two independent experiments (two values per one experiment).
[b]In μg/mL.

Table IV. *In vitro* secretion of IL-10 and IFN-γ (both in pg/mL) by splenocytes of i.t. immunized mice

Immunization antigen	None	*Bf*[b]		d*Bf*[b]		ConA[b]
		10	100	10	100	5
IL-10						
None (control)	38 ± 22	62 ± 13	54 ± 25	150 ± 60	148 ± 56	261 ± 141
Ova	23 ± 8	100 ± 34	100 ± 38	246 ± 21	101 ± 30	240 ± 187
Bf	20 ± 6	174 ± 40	102 ± 63	113 ± 31	125 ± 23	419 ± 31
Ova + *Bf*	30 ± 12	122 ± 46	108 ± 49	102 ± 3	131 ± 32	336 ± 21
d*Bf*	19 ± 5	151 ± 26	103 ± 50	324 ± 20	186 ± 43	240 ± 66
Ova + d*Bf*	17 ± 6	43 ± 8	86 ± 2	110 ± 17	94 ± 10	102 ± 14
IFN-γ						
None (control)	285 ± 151	1434 ± 567	718 ± 301	2608 ± 903	2007 ± 267	7896 ± 23
Ova	35 ± 26	1096 ± 454	377 ± 205	1716 ± 301	1658 ± 557	4568 ± 3154
Bf	111 ± 65	1083 ± 656	810 ± 699	749 ± 126	366 ± 27	2665 ± 304
Ova + *Bf*	104 ± 47	1662 ± 1092	1777 ± 1634	837 ± 350	637 ± 275	3161 ± 237
d*Bf*	75 ± 25	1009 ± 705	632 ± 557	2270 ± 512	1872 ± 170	3703 ± 554
Ova + d*Bf*	252 ± 132	1211 ± 654	893 ± 337	1742 ± 563	2186 ± 1165	5937 ± 190

[a]*See* footnotes to Table III.

DISCUSSION

The results of i.n. and i.t. immunization were similar in most of the parameters studied. The established differences were caused by different administered volumes and different character of the upper and lower respiratory tract-associated lymphoid tissue (Ryan *et al.* 1999; Hodge *et al*. 2001). Larger volumes of immunization solution, import of commensal microflora of upper airways into the lungs and the bronchoalveolar space as the target site in i.t. immunization may have led to a more extensive irritation than in i.n. treatment and gave rise to a higher level of splenocyte stimulation at the end of the experiment (Simecka *et al.* 2000; Hodge *et al*. 2001). Also the close relation of the alveolar space and the vascular network of lungs probably resulted in a stronger systemic response after i.t. immunization. The data obtained from tests on splenocytes reflect mainly the level of stimulation of systemic immunity. This could also be the reason why a higher activation of splenocytes was observed after i.t. than after i.n. immunization.

The mild spontaneous stimulation of splenocytes *in vitro* demonstrated after *in vivo* administration of any antigen (protein, adjuvant or combination) was at least partly caused by manipulation in the respiratory tract during immunization (Simecka *et al.* 2000; Hodge *et al*. 2001). The higher level of proliferation of splenocytes derived form d*Bf* and Ova + d*Bf* administered group points to the capacity of d*Bf* to modestly polyclonally stimulate T cells *in vivo*. The ability of mild polyclonal stimulation of T cells was observed also in *Bf*- and Ova + *Bf*-immunized animals in case of i.t. immunization. On the other hand, the absence of spontaneous production of IL-10 and IFN-γ in d*Bf*- and *Bf*-treated mice refutes the effectivity of the adjuvants used as polyclonal activators *in vivo*.

No significant effect was observed of *in vitro* restimulation with Ova on splenocyte proliferation or cytokine production. The study of naive T-cell activation *in vivo* is technically demanding because of the low precursor frequency of Ag-specific T cells (Stumbles *et al.*1998; Tsitoura *et al.*1999; Lambrecht *et al.* 2000). The small expansion of the TCR-Ova positive population did not allow us to follow either the Ova-specific cytokine secretion or proliferation. The reason for low proliferation of antigen specific T-cells could be in the response of lung macrophages and predominant antigen presenting cells of the respiratory tract, RTDC, to *Bf* and d*Bf*. Both *Bf* and d*Bf* were demonstrated to be potent stimulators of nitric-oxide synthase in peritoneal macrophages (Zídek *et al.* 1998). The effect on alveolar macrophages is very probably similar. *Bf* and d*Bf* further induced the secretion of IFN-γ in mononuclear cell culture (Tables III and IV; Prokešová *et al.* 2002). The production of high levels of NO in the presence of IFN-γ in rodent lung macrophages was in most cases inhibitory for T-cell activation. The mechanism was explained as NO-mediated transient inhibition of tyrosine phosphorylation by intracellular kinases in T cells that probably protects the mucosa against a harmful immune response in the case of a rapid release of proinflammatory cytokine (Kawabe *et al*. 1992; Upham *et al*. 1995; Strickland *et al*. 1996).

The highly significant inhibition of proliferation of splenocytes rich in macrophages after *in vitro* incubation with 100 μg of *Bf* or d*Bf* (Table I) could also demonstrate the suppressive effect of macrophage-derived NO on T-cell mitosis. Further NO released from alveolar macrophages holds RTDC in an immature state with low T-cell priming activity (Holt *et al.* 1993; Stumbles *et al.* 1999) promoting the T_H2 type of immune response (Liew *et al.*1991; Stumbles *et al.* 1999; Tang *et al.*2001).

Analysis of anti-Ova IgG subclasses and cytokine screening demonstrated that T cells were engaged in reactions induced by immunization with Ova and *Bf* or d*Bf*, respectively, but their proliferation was limited and neither T_H1 nor T_H2 cytokine pattern seemed to dominate. Immunization with Ova + *Bf* or Ova + d*Bf* caused an elevation of anti-Ova IgG_1, IgG_{2a} and IgG_{2b} whereas administration of Ova alone occasioned a rise in anti-Ova IgG_1 only. These results provided evidence of an immunoglobulin switch dependent on the help of T cells. Cytokine screening demonstrated no IL-2 or IL-4 production in splenocytes incubated with *Bf* or d*Bf*. d*Bf* in both doses (and in i.t. regime also *Bf*) was stimulatory for IL-10 and IFN-γ.

Nitric oxide released from macrophages in response to *Bf* or d*Bf* could inhibit T-cell proliferation and influence RTDC to promote T_H2 type of response with a low level of TCR-Ova + cell priming activity (Murray *et al.* 1999; Stumbles *et al.* 1999; Van der Veen *et al.* 2000). *Bf* and d*Bf* further support the production of IFN-γ and IL-10. The final cytokine milieu could support the secretion of anti-Ova antibodies from activated Ova-BCR bearing lymphocytes.

There are scant literature data about the adjuvanticity of G^+ bacteria and T cells. The most extensively studied G^+ bacterium is *B. subtilis.* Research focused on its effect on T cells (Rasanen *et al.* 1982; Caruso *et al.* 1993; Gao *et al.* 1996) is highly heterogeneous in both experimental conditions and it is therefore difficult to compare data of these authors with our investigation. The important contribution of Hessle *et al.* (2000) (who made a screening of the capacities of G^+ and G^- bacteria to induce IL-10 and IL-12) suggested that G^+ bacteria induce cytotoxicity and IFN-γ secretion by T cells and NK cells, a cytokine pattern that promotes T_H1 effector function. IL-10 was also stimulated by G^+ bacteria but much less than IL-12. The cyto-

kines preferentially induced by G^+ bacteria (IL-12, IFN-γ and TNF-α) should synergize to increase the capacity of macrophages to kill and digest the bacteria that they had phagocytosed. It is probable that this type of response has evolved to cope with the exceptional sturdiness of the G^+-cell wall. On the other hand, production of antigen-specific antibodies supported by IL-10 can play an important role in opsonization facilitating phagocytosis of G^+ bacteria (Hessle *et al.* 2000; *this work*). In addition to activation of macrophages, *Bf* provokes immune reactions supporting the maturation of B cells as demonstrated by polyclonal stimulation of B cells *in vitro* (Prokešová *et al.* 1994, 1995) and influence on antigen-specific antibody production *in vivo* (Prokešová *et al.* 1998; Mlčková *et al*. 2001).

Results of mucosal immunization studies with *Bf* and protein antigen plus experimental work done with this bacterium *in vitro* illustrate the fact that a bacterial adjuvant with a major impact on nonspecific immunity may importantly influence specific response; the final effect is then essentially dependent on the conditions of immunization (target site, antigen presentation, character of the vaccine).

Although we observed a remarkable support of antigen-specific antibody production after i.n. and i.t. immunization with Ova and *Bf* (Mlčková *et al*. 2001), we have not proven the antigen-specific cytokine production and proliferation of T cells in identically designed experiments. The expansion of TCR-Ova bearing cells after treatment with both Ova alone or Ova + adjuvant was probably so low that it could not be detected by the methods used. *Bf* or d*Bf* could, at first, activate lung macrophages that produced NO temporarily inhibitory for T-cell division and T-cell priming activity of RTDC and, secondly, could induce cytokine milieu composed of IL-10 and IFN-γ stimulatory to activated Ova-BCR bearing cells. Finally, a mild spontaneous proliferation of splenocytes (enhanced by d*Bf*) was demonstrated (caused probably by immunization *via* the respiratory tract). Despite the high efficiency of d*Bf* in supporting the antibody production, the use of this adjuvant *in vivo* should be considered carefully in view of the possibility of polyclonal activation of lymphocytes.

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