Effect of chicken antibodies on inflammation in human lung epithelial cell lines

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Abstract

OBJECTIVES: As an alternative therapeutic approach for the prevention and treatment of bacterial infections with *P. aeruginosa* of cystic fibrosis (CF) patients, chicken yolk antibodies (IgY) could be used. The most significant advantage of IgY, in contrast to mammalian IgG, consists in the fact, that when bound to the antigen, they usually do not induce inflammatory reaction. In addition, the simplicity of egg production and the ease of IgY preparation makes this kind of antibody an excellent tool for passive immunization. Thus, the aim of our project was to study the effect of IgY and its Fab fragment on the potential induction of pro-inflammatory reactions in lung epithelial cells.

METHODS: Chicken IgY were prepared from pooled egg yolks. Fab fragmens of IgY were purified from the papain digest of IgY using DEAE-Sephacel ion exchange chromatography. Their purity was verified by SDS electrophoresis in polyacrylamide gel. Immortalized human cell lines, CuFi (CF patient) and NuLi (healthy subject), and A549 (human adenocarcinoma cells) were exposed to IgY, Fab, OVA, LPS (positive control), PBS (negative control), and human and goat IgG for 24 hours. The concentration of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and GM-CSF were determined in cell media using the BioPlex method, which enables the quantification of multiple analytes simultaneously in one sample.

RESULTS: Our results show that i) the Fab fragment induced levels of some pro-inflammatory cytokines, when compared to the PBS control, whereas ii) chicken IgYs did not induce any notable production of pro-inflammatory cytokines in contrast to intense effect of LPS on TNF-α and GM-CSF. In summary, our data show that levels of all cytokines are comparable with physiological values in human serum except of IL-1β, which concentration in cell medium was markedly elevated by Fab fragment.

CONCLUSIONS: The present data indicate that IgY are not inflammatory for lung cells and thus they are possibly applicable for prevention of airway bacterial infections.

ORIGINAL ARTICLE

Abbreviations:

A549	- cell lines derived from lung adenocarcinoma (58-year old patient)
BCIP	- 5-bromo-4-chloro-3′-indolyphosphate
BEGM	- bronchial epithelial cell growth medium
BSA	- bovine serum albumin
CuFi	- immortalized human epithelial cells isolated from lungs (14-year-old female patient with cystic fibrosis)
CF	- cystic fibrosis
Fab	- antigen-binding fragment
Fc	- crystallizable fragment
gIgG	- goat IgG
GM-CSF	- granulocyte macrophage-colony stimulating factor
hIgG	- human IgG
IgY	- chicken yolk antibody
IL	- interleukin
LPS	- lipopolysaccharide
NBT	- nitro-blue tetrazolium
NuLi	- immortalized human epithelial cells isolated from the lungs (healthy 36-year old man)
OVA	- ovalbumin
PBS	- sodium phosphate buffered isotonic saline pH 7.4
PBS-Tw	- PBS containing 0.05% Tween 20
SDS-PAGE	- sodium dodecyl sulfate polyacrylamide gel electrophoresis
TNF-α	- tumor necrosis factor α
TMB - 3,3′,5,5′	-tetramethylbenzidine

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in the gene coding for the CF transmembrane conductance regulator. The major clinical problem for patients with CF has been over many years caused by chronic microbial infections (e.g. *Pseudomonas aeruginosa, Burkholderia cepacia* complex, *Aspergillus spp., Staphylococcus aureus*), which are facilitated by decreased mucociliary clearance. These infections result in progressive loss of pulmonary function and consequently in early death of CF patients (Campodónico *et al.* 2008). The cure is frequently based on antibiotic prophylaxis in order to suppress the infection. Antibiotic therapy is often complicated by the bacterial resistance and thus new ways of therapy are considered.

Chicken yolk antibody (IgY) may provide an alternative therapeutical approach for prevention and treatment of *P. aeruginosa* infections. These IgYs are superior to mammalian IgGs in several aspects. Avian IgY antibodies do not fix mammalian complement components nor bind human Fc receptors, hence do not trigger antibody mediated biological responses such as phagocytosis, endocytosis, antibody-dependent cellular cytotoxicity, release of inflammatory mediators, and enhancement of antigen presentation, which might endanger CF patients (Carlander *et al.* 1999). Avian IgYs are generally suggested to neutralize or inactivate bacterial virulence factors in a similar way as mammalian IgGs do. Thus, chicken IgYs have been examined as a tool for oral immuno-prophylaxis, as well as medication for therapy of infectious diseases. For instance, the oral administration of IgY prevented infection due to rotavirus infection in mice (Ebina *et al.* 1990). Data of other studies indicate a dose dependent effect of specific egg yolk antibodies on bovine coronavirus-induced diarrhea of newborn calves. Moreover, the orally administered IgY raised against enterotoxigenic *E. coli* provided protection from diarrhea of neonatal pigs (Dias da Silva *et al.* 2010). In addition, production of avian IgY antibodies and their purification is quite simple which makes their extensive production and application feasible (Hodek *et al.* 2013).

In this study, we examined the potential pro-inflammatory properties of IgYs on the basis of cytokine production. To asses IgY as a tool for passive immunization of CF patients against bacterial airway infections, we have tested IgY and IgY-derived Fab fragments *ex vivo* in a cell model system derived of lung epithelium of healthy subject (NuLi) and of CF patient (CuFi). The data presented in this article prove that the IgYs (possible Fab) are not inflammatory and might be used for anti-bacterial prophylaxis.

MATERIAL AND METHODS

Production and purification of chickem antibodies IgY and IgY-derived Fab fragments

Production of IgY from conventional eggs

A two-step procedure, consisting of yolk extraction by tap water (8-fold dilution, freezing, and filtration) followed by IgY precipitation at pH 4 with sodium chloride in the final concentration of 8.8% (Hodek *et al.* 2013), was used for the IgY preparation. IgY fractions were stored in PBS, pH 7.2 (containing 0.02% sodium azide). The purity and recovery of IgY in water-soluble fractions was monitored by the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Proteolytic digestion with papain

The proteolytic digestion with papain was carried out as described by Akita *et al.* (1993) and Bereli *et al.* (2005). It yielded providing three cleavage products of IgY, two 44 kDa Fab (antigen-binding) fragments, and one Fc (crystallizable) fragment – size 55 kDa. Papain at the final concentration 0.028 mg/ml was activated with cysteine (the final concentration 0.011 mg/ml) and dithiothreitol (the final concentration 0.030 mg/ml) in 50 mM TRIS/HCl buffer (pH 7.2) under stirring for 25 min at 37 °C. IgY was digested at the ratio of 23:1 (IgY:enzyme) for 15 hours at 37 °C under stirring. The reaction was stopped by addition of iodoacetamide to a final concentration of 15 mM.

Chromatographic separation of cleaved IgY

Products of IgY cleavage were separated by ion exchange chromatography using a weak anion exchanger diethylaminoethyl-cellulose (DEAE-Sephacel). Before separation, the sample was dialyzed against the equilibration buffer (10 mM TRIS/HCl, pH 8.6). After sample appli-

cation, the column was washed with the equilibration buffer (washing away non-bound antibody fragments) and the Fab fragment was eluted with 100 mM TRIS/HCl buffer (pH 8.0). The Fc fragment was released with 100 mM TRIS/HCl containing 200 mM NaCl (pH 8.0). The elution profile was monitored at 280 nm.

Western blots

The isolated Fab and Fc fragments were examined by Western blotting on the Immobilon-P membrane (Millipore, Bedford, MA) as described earlier (Krizkova *et al.* 2009). Five μg of Fab, Fc and IgY in reduced buffer were applied in triplicates onto SDS-containing 12% polyacrylamide separation gel. The blot after the electrotransfer was blocked in a solution of 5% skim milk in PBS containing 0.05% Tween 20 (PBS-Tw) and cut into 3 separate strips, each containing Fab, Fc and IgY. The strips were incubated with concanavalin A (0.05% in 3% BSA in PBS-Tw), anti-chicken IgY Fab goat IgG (1 μg/ml of 3% BSA in PBS-Tw), or PBS-Tw, respectively. The strip treated with concanavalin A was subsequently incubated with horseradish peroxidase (0.1 mg/ml of 3% BSA in PBS-Tw) and the strip treated with PBS-Tw was then incubated with anti-chicken IgY rabbit IgG-peroxidase conjugate (1:1 000 diluted in 5% skim milk). Subsequently, both strips were developed with the peroxidase substrate, SigmaFast 3,3´-diaminobenzidine tablets. The third strip, treated with anti-IgY Fab primary antibody, was incubated with alkaline phosphatase-conjugated rabbit anti-goat IgG (0.08 μg/ml of 5% skim milk in PBS-Tw) and protein bands were visualized with the alkaline phosphatase substrate, BCIP/NBT tablet.

Cell exposure and cytokine assay

Three types of lung epithelial cells were used in this study: i) NuLi-immortalized cells originally isolated from the lungs of healthy 36-year old man, ii) CuFi – immortalized cells from lungs of 14-year-old female patient suffering from cystic fibrosis and iii) A549 – cell lines derived from lung adenocarcinoma of 58-year old patient (from alveolar type II cells).

Immortalized epithelial cell lines NuLi and CuFi were cultivated in a BEGM medium (Bronchial epithelial cell growth medium, Lonza). For the cultivation of an A549 cell line, the DMEM medium (PAA Laboratories, glucose free) with addition of amino acids 0.28 mM L-alanine, 0.25 mM L-asparagine, 0.225 mM L-aspartic acid, 0.51 mM L-glutamic acid, 0.35 mM L-proline and 2 mM L-glutamine was used. The medium was further supplemented with 10% (v/v) fetal bovine serum and gentamycine (50 μg/ml). The cells were cultured at 37 °C with 5% CO_2. The cells were transferred from cultivation flasks to 24-well tissue culture plates CellBIND (Corning, U.S.A.) and grown to confluence. Cells in wells were exposed to chicken IgY, its fragment Fab, human (hIgG) and goat (gIgG) IgG (Sigma Chemical Co., USA), OVA (Serva, Germany), respectively, or PBS (negative control) in final concentrations of 1 mg/ml for 24 hours. As a positive control, 80 μg/ml LPS was used in the exposition experiments. Concentrations of cytokines TNF-α, IL-1β, IL-6 and GM-CSF were determined in cell media using BioPlex method, which enables the quantification of multiple analytes simultaneously in one sample. Fluorescent detection with dual laser allows determining both the type and concentrations of cytokine based on the detection of micro particles carrying specific anti-cytokine antibody. The Bio-Plex assay was performed on MAGPIX Multiplex Reader 60 in collaboration with Dr. Vrzalová (Laboratory of immunochemical diagnosis, University Hospital in Pilsen).

RESULTS

Preparation of IgY samples

The prepared IgY (300 mg) was in part enzymatically digested with papain to the Fab (44 kDa) and Fc (55 kDa) fragments. The digest was separated on DEAE-ion exchange chromatography. The elution profile is shown in Figure 1. The fractions with a high protein content were subjected to electrophoretic analysis. Figure 2 indicates that ion exchange chromatography successfully separated Fab from Fc fragments. The

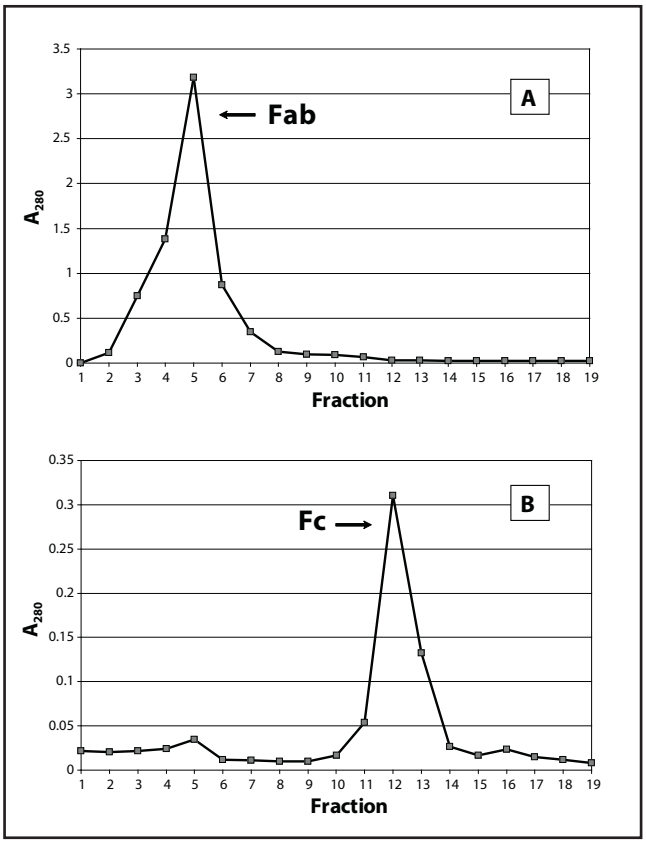

Fig. 1. Separation of IgY papain digest on DEAE-Sephacel. The column was eluted with 100 mM TRIS-HCl, pH 8.0 to collect the Fab fraction (panel **A**). The Fc fragment was eluted with 100 mM TRIS-HCl, pH 8.0, containing 200 mM NaCl (panel **B**).

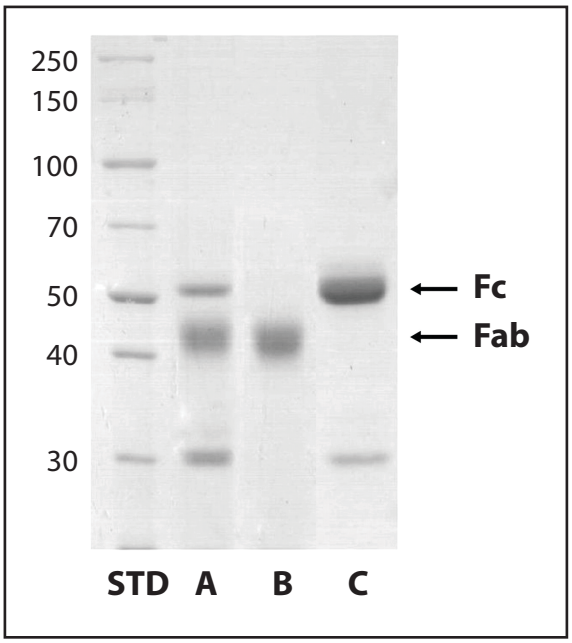

Figure 2. SDS-electrophoresis on 12% polyacrylamide separation gel under reduced conditions. Lines labeled as STD, A, B, and C were loaded with molecular weight standards (Page Ruler Broad Range), IgY papain digest, Fab fraction, and Fc fraction, respectively. Arrows show relative mobility of Fc and Fab fragments. The gel was stained with Coomassie Brilliant Blue R-250 for proteins.

Fab fragment was also identified with a specific antibody on Western blots. The presence of carbohydrate components of the Fc fragment was detected with concanavalin A, recognizing its mannosylation site (data not shown).

Exposure tests and cytokine assays

The influence of chicken IgY, its Fab fragment and mammalian immunoglobulins, human and goat IgG, on the production of inflammatory cytokines was examined with A549, CuFi and NuLi cells lines. Other compounds, OVA (possible contaminant of IgY fraction) and bacterial LPS (known inducer of cytokines) (Hansen, L. *et al.* 1999) were also included in the tests. Determination of cytokines TNF-α, IL-1β, IL-6 and GM-CSF in the cell medium was performed using the Bio-Plex method. Data are presented in Figure 3 for each cytokine separately.

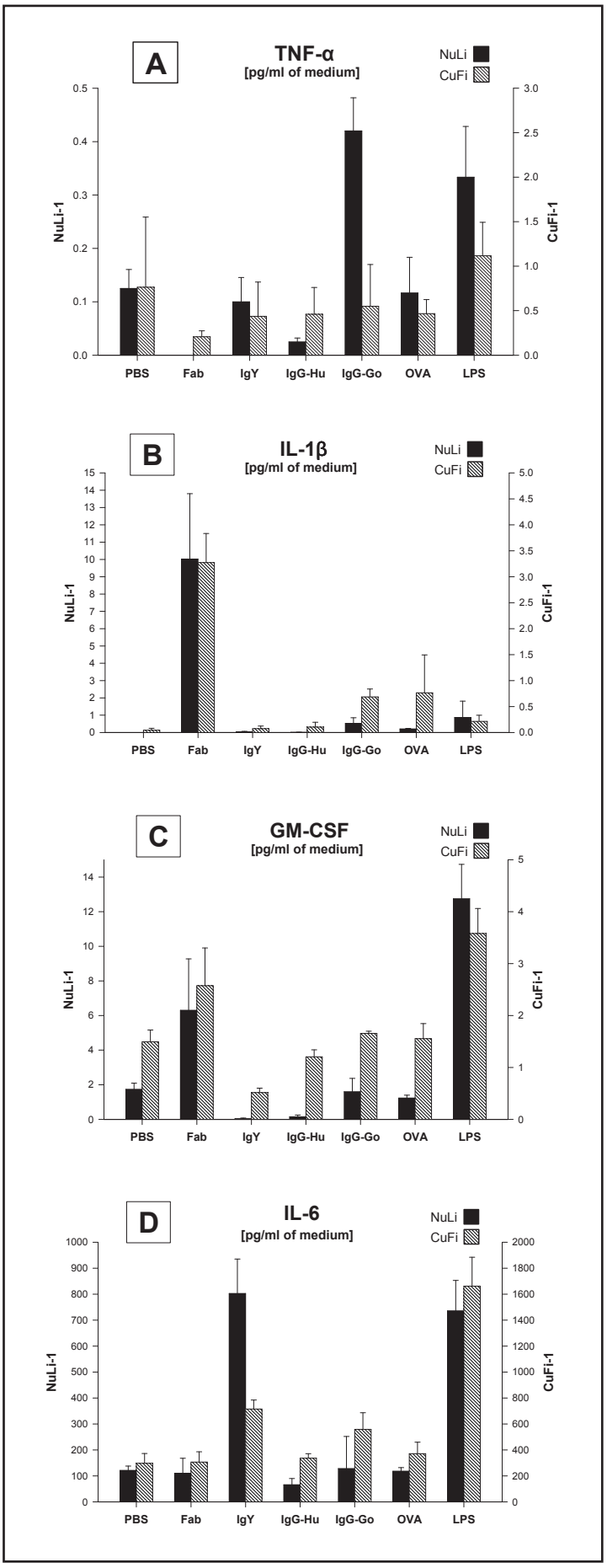

Figure 3. Cytokine concentrations in CuFi and NuLi cell cultures. Cytokines TNF-α (panel **A**), IL-1β (panel **B**), GM-CSF (panel **C**) and IL-6 (panel **D**) were determined by Bioplex assay. CuFi (hatched bars) and NuLi (black bars) cells were treated with PBS, the Fab fragment of IgY (Fab), chicken yolk immunoglobulin (IgY), human IgG (IgG-Hu), goat IgG (IgG-Go), egg ovalbumin (OVA) in the final concentration of 1 mg/ml or 80 μg/ml bacterial lipopolysaccharide (LPS). Plotted data are means ± S.D. of three independent measurements.

Treatment of A549 cells

The tumor cell line A549 produced cytokines in trace concentrations, which did not allow their precise detection. Cytokine IL-6 was the only detectable analyte. In response to the Fab fragment, this cytokine was induced to levels comparable to LPS. Other samples induced IL-6 to much lower extent. It is possible to conclude that goat IgG stimulated the production to higher extent than human IgG (data not shown).

Treatment of NuLi cells

The immortalized cell line NuLi of healthy individual secreted detectable amounts of cytokines IL-1β, IL-6 and GM-CSF. Whereas concentrations of TNF-α were close to detection limit (0.6 pg/ml), some increase of this cytokine was detected for gIgG exposure. The production of IL-1β (compared to PBS control) was not induced by any immunoglobulin used. From all other compounds studied, only the Fab fragment elevated levels of IL-1β. Similarly, the Fab fragment markedly stimulated production of GM-CSF. Contrary to the impact on IL-1β, LPS induced GM-CSF more than the Fab fragment did. IgY did not caused any induction of GM-CSF. Formation of cytokine IL-6 shows the opposite trend in respect to Fab and IgY. The presence of IgY increased the IL-6 to the level that is comparable to the effect of LPS. In NuLi cells LPS proved to be the most effective stimulant of selected cytokines with the exception of IL-1β induction by the Fab fragment.

Treatment of CuFi cells

All four types of inflammatory cytokines were measured in immortalized CuFi cell line originating from CF patient airways. Similarly to NuLi cell, TNF-α formation was close to the detection limits. Thus, none of the tested compounds caused significant elevation of this cytokine. In line with results from NuLi cells the production of IL-1β (compared to PBS control) was highly induced by the Fab fragment to levels exceeding the effect of LPS. Some, but much lower effect of Fab fragment was detected on the GM-CSF production. Chicken IgY did not induce this cytokine. In contrast, cytokine IL-6 was slightly induced by IgY, whereas the Fab fragment caused only a low impact on IL-6 induction.

DISCUSSION

Chicken eggs are becoming a widely respected source of unique antibodies. The preparation of IgYs from egg yolks is, in comparison to the procedure utilizing blood of mammals, more acceptable from the point of view of animal welfare. Moreover, the possibility of a large scale production of IgY, makes chicken immunoglobulins an excellent tool for passive immunization. Thus, egg yolk immunoglobulins might be considered for prevention of infections with opportunistic pathogens affecting e.g. lungs of CF patients.

The aim of this study was to determine the effect of IgY on epithelial cells in the respect to inflammation development. The secretion of selected cytokines in cell media was determined to trace initial signs of a possible inflammatory reaction caused by IgY. Beside the IgY, the Fab fragment was included in exposure tests, too, as it is assumed to be less immunogenic than IgY due to the absence of a glycosylation site and a reduced molecular weight (Sarnesto *et al.* 1983; Cheung *et al.* 2003). It was confirmed by SDS-electrophoresis that the most of Fab fragment fractions contained only insignificant amounts of additional protein of a similar molecular weight as the Fc fragment. Experiments with OVA were carried out to examine the impact of this protein, which might be a possible contaminant of IgY fractions.

The data of Bioplex assay show that all epithelial cells responded effectively to LPS *via* secretion of three of pro-inflammatory cytokines studied. This observation confirms that under conditions used, the *ex vivo* cell cultivation allows to evaluate the effect of tested compounds on the induction of inflammatory reactions. Of the all immunoglobulins, only the Fab fragment of IgY highly stimulated levels of IL-1β and GM-CSF in CuFi and Nuli cell lines. Although A549 cells mostly did not produced detectable amounts of cytokines, the Fab fragment induced cytokine IL-6 to levels comparable to LPS. By analogy to human IgG, the Fab fragment of IgY should be less immunogenic than the Fc fragment or the whole immunoglobulin molecule (Sarnesto *et al.* 1983), however, our data contradict this assumption. In our previous experiments with rats, we observed that Fab, when inhaled, induced the production of IL-1β in serum. Likewise, although to a lesser extent, the treatment with Fab increased serum concentrations of IL-18 and GM-CSF (Hadrabova *et al.* 2014). The reason why Fab triggered the induction of cytokines in epithelial cell lines is unclear.

When comparing cytokine inducing effects of immunoglobulins tested, IgY increased solely levels of IL-6 in both cell lines comparably. In CuFi cells, the IgY-mediated stimulation of IL-6 formation was similar to that produced by the other immunoglobulin of foreign origin, goat IgG. On the other hand, OVA at a concentration of 1 mg/ml was not demonstrated to provide any marked inflammatory response. Hence, the possible presence of OVA in the IgY sample does not play a significant role.

It is interesting to compare concentrations of determined cytokines in a cell medium with their physiological values in human serum. Concentration of TNF-α, IL-1β and GM-CSF in serum are 3.21 pg/ml, 2.04 pg/ml and 40.9 pg/ml, respectively (Kim *et al.* 2011). Although the direct comparison of cytokine concentrations in serum and cell media might be misleading, the serum values provide a basis for our result interpretation. Given of these data we can state that serum physiological levels were exceeded only in two cases when CuFi and NuLi cells were exposed to the Fab fragment. The

concentration of cytokine IL-1β secreted to media by NuLi cells was ~5 folds higher than the serum level, while the only slight increase of IL-1β was found with CuFi cells.

Present data confirmed our previous results from animal studies (Hadrabova *et al.* 2014) that IgY is not inflammatory and therefore might be considered as a tool of passive immunization. Since the Fab fragment of IgY was shown to be inflammatory for cells that should be taken under consideration when possible used *in vivo*.

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