Intracellular delivery of nanoparticles fabricated from monoclonal and polyclonal antibodies against influenza A and hepatitis B virus antigens

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Abstract. Specific antibodies (AB) may serve as molecular tools for both targeted, etiotropic and immunomodulation therapy of the infectious and oncological diseases. Despite their ability to bind with extracellular virions and isolated viral antigens AB may not pass through host cell membranes to inhibit intracellular infectious agents. Nanoparticles (NP) were fabricated from polyclonal and monoclonal AB against the influenza A structural and non-structural proteins as well as against the hepatitis B virus surface antigen (HBsAg). ELISA and immunofluorescent analysis revealed the ligand-binding activity of the AB NP comparable with the properties of the original AB. Fluorescent microscopy and quantitation showed efficient cellular uptake of the labeled AB NP and their degradation in 5 days posttreatment. Intracellular AB NP induced interferon (IFN) gene expression but not interleukin (IL) 4 and IL10 transcription. The cytokines mediated immunomodulation potential of the protein NP.

1. Introduction
Specific AB are used for targeted immunotherapy. Among 75 approved and commercially available drugs on the base of AB more than half are checkpoint inhibitors against surface receptors. In spite of evident advantages of AB such as their high specificity, affinity and prolonged action the remaining problem is their lacking penetration in all eukaryotic cells excepting B lymphocytes, follicular dendritic cells, natural killer cells, macrophages, neutrophils, eosinophils, basophils, human platelets, and mast cells with receptors to Fc fragments of heavy chains of AB [1]. Since AB cannot entry into host cells and inhibit or neutralize intracellular viruses there then the AB-mediated treatment may not prevent a persistent infection inside cells. To eliminate viruses the specific and active AB must be delivered inside infected cells near virus replication sites. Targeted drug delivery may be mediated by AB on NP surface [2]. Since 1990 US Food and Drug Administration approved 51 nanomedicines [3]. The number of nanotherapies remains low partly due to acute and chronic toxicity. Various combination of immunomodulating cytokines, chemokines and Toll-like receptor agonists targeting various immune cells as well as immunocytokines that combine AB with cytokines have been
designed, developed and evaluated in many preclinical trials [4]. Moreover, beyond providing delivery platforms different nanosystems possess intrinsic immunomodulatory properties causing pleiotropic and possible side effects [4]. Despite clinical implementation of protein NP little is currently known about their safety, cellular uptake, intracellular distribution patterns, and the clearance mechanisms [2-4]. Our goal was the fabrication of NP from polyclonal and monoclonal AB against the influenza A structural and non-structural proteins as well as against HBsAg, comparative immunoassays of their binding with specific antigens and analysis of intracellular delivery of AB NP.

2. Methods

2.1. Tissue cultures.
Human larynx carcinoma HEP-2 cells were obtained from the National Research Center of Epidemiology and Microbiology of N.F. Gamaleya of the Russian Ministry of Health, Moscow, Russia and grown in culture medium 199 with 8% fetal bovine serum in the presence of 100 U/ml penicillin and 100 U/ml streptomycin until monolayer formation.

2.2. Fluorescent labeling of AB.
The immunoglobulins (Ig) were labeled with rhodamine B dye (Rho B) in 0.1 M Na$_2$CO$_3$ solution at pH 9.3 for 1 h at room temperature or overnight in refrigerator and then purified by Sephadex G25 gel-chromatography with centrifugation at 700 g.

2.3. AB NP fabrication.
NP were constructed from AB solutions in hexafluoro-propan-2-ol (HFIP) by nanoprecipitation as previously described [5].

2.4. Circular dichroism (CD) spectroscopy.
CD spectra were obtained by Chirascan spectrophotometer (Applied Photophysics, UK).

2.5. Fluorescent microscopy and quantitation.
The HEP-2 cells with fluorescent AB NP were imaged using the fluorescent microscope Eclipse Ti (Nikon, Japan) with a Plan Fluor 40x/0.6 objective and filters providing excitation/emission for RhoB (528-553 nm/590-650 nm) and Hoechst 33342 (340-380 nm/435-485 nm). The images were captured with the camera ORCA-Flash4.0 (Hamamatsu, Japan) with the exposure time of 3-5 s. Fluorescence intensity at 580 nm of cell lysates at different time points was measured using spectrofluorometer “Fluoromax+” (Horiba Scientific, Japan).

2.6. Cytotoxicity assay.
MTT assay was performed to evaluate the in vitro cytotoxicity of RhoB, fluorescent AB and corresponding NP. The absorbance was measured at 570 nm.

2.7. Cytokine gene expression.
Cytokine RNA including IFN α, β, γ, λ, IL 2, 4, 6, 10, 12, 23 were detected by reverse transcription with quantitative real time PCR (RT$^2$-qPCR) as previously described [6].

3. Results and Discussion
Nanoprecipitation of polyclonal and monoclonal AB of different origin, classes and specificity from their solutions in fluoroalcohol HFIP permitted to fabricate water-insoluble and stable NP without cross-linking. Mixed protein NP could be constructed by the nanoprecipitation of core albumin and its subsequent cross-linking with surface AB shells using 0.1% glutaraldehyde. The AB NP might be also constructed from blood sera containing the specific AB diluted with HFIP (ratio 1:3). Similar CD spectra of Ig in water solutions and in NP revealed AB stable secondary structures after nanoprecipitation. Noteworthy that the protein NP including those from different AB are very stable up to 1 year of storage in water at +4°C [5], in culture media and in biological fluids (blood sera,
saliva – for 5 days at 37°C). Binding of the AB NP with specific influenza A virus antigens and HBsAg was shown by ELISA, immunofluorescent analysis (IFA), hemagglutination inhibition (HI) and neutralization tests. According to MTT assay both AB and the AB NP with concentrations up to 2 mg/ml were not toxic for human cell lines (Fig. 1). Main part (50-80%) of the fluorescent AB NP appeared to be associated with cellular monolayers. According to our estimations each AB NP of average diameter near 100 nm contained approximately \(10^4\) Ig molecules in their native conformations with specific ligand-binding properties and up to \(10^5\) NP can be found per cell. The average size of one eukaryotic cell is known to be 10 \(\mu\)m, consequently, the cellular surface area \(S=4\pi R^2\) is \(\approx 314\) \(\mu\)m\(^2=3.14*10^8\) nm\(^2\). Thus, \(10^5\) NP of average diameter 100 nm may cover up to 10% of the cellular surface. However, cellular uptake of the AB NP was not simultaneous. The AB NP could be found in cells in 2 hours posttreatment with maximum after 2 days and subsequent gradual decline until background values (Fig. 2). The slow enhancement of the cell-associated fluorescent Ig NP amounts could be resulted from gradual entry of the NP into cells and further stepwise degradation.

![AB anti-influenza A-HA-RhoB NP and AB anti-HBsAg-RhoB NP](image)

**Figure 1.** Cellular uptake of AB NP in 2 days posttreatment. Nuclei were stained with Hoechst 33342 (blue color), AB NP were labelled with Rho B (red color).

Cellular uptake relies on 4 known mechanisms: clathrin- and caveolar-mediated endocytosis, phagocytosis, macropinocytosis, and pinocytosis [2]. Specific clathrin- and caveolin-mediated endocytosis pathways can be excluded from consideration because of the absence of specific receptors for AB on HEP-2 cells [1]. Therefore, the fluorescent AB attachment to human cell monolayers was not essential (Fig. 2). Phagocytosis is used by macrophages, dendritic cells and neutrophils engulfing particles larger than 0.5 \(\mu\)m only [2]. Consequently, macropinocytosis remains the most probable pathway by which cells internalize AB NP, and large vesicles (0.2–5 \(\mu\)m) are formed inside cells [2]. For Rho B dye the pinocytosis seems to be a possible endocytic process by which the eukaryotic cells absorb extracellular fluids and small molecules (Fig. 2). Because of the AB NP degradation in the eukaryotic cells (Fig. 2) their binding with intracellular infectious agents could be possible near outer cellular membranes and in endosomes during the first two days postinfections. The step-by-step cleavage of Ig on the NP surface did not exclude the antigen-binding properties of the AB from underlying, deeper and core layers. The evident advantages of the AB NP disintegration include their low toxicity and the cytokine gene expression induction. The slow gradual increase in intracellular fluorescent NP accumulation from 30 minutes to 48 hours posttreatment and subsequent decrease for 3 days until background values suggested that the AB NP entry in the cells was not synchronous.
Figure 2. Comparison of cellular uptake of RhoB dye, AB and corresponding NP during 3 days after addition.

Despite immunological tolerance of cells to Ig from growth culture media and degradation of fluorescent protein NP during 3 days (Fig. 2), the AB NP induced transcription of IFN genes of types I and III in 1-2 days posttreatment. IL 4 and IL10 RNA were not detected using RT²-PCR. The cytokine spectra and the polarization indexes (PI) calculated as ratio Th2:Th1 cytokines were similar for NP constructed from various AB. PI near 0 with the absence of Th2 cytokines suggested Th1 polarization of innate immunity induced by the AB NP. The protein NP remain non-immunogenic [7]. The absence of detectable antibodies after the protein NP administration could be caused by Th1 polarized mainly cellular immune response. The Th1 innate immunity may protect cells from invasions. Degradation of the fluorescent AB NP in 3-5 days posttreatment together with the IFN α, β and λ gene expression could be resulted from the fluorescent AB proteolitic hydrolysis in lysosomes but not in intracellular vesicles without proteases. So, the macropinocytosis was not the only possible way of the AB NP endocytosis [2]. Exocytosis of the RhoB-AB NP and their gradual accumulation in the culture medium after 2 days posttreatment were not observed. Taken together, the stable AB NP with the antigen-binding activity can be used for targeted delivery and immunimodulation towards Th1 mainly cellular immune response. Their intracellular binding with the virions may be limited because of proteolytic degradation after 2 days.

4. Summary
AB NP retained the ability of the original AB to bind with specific antigens, were not toxic for human cells, gradually accumulated in cells and induced Th1 polarized cytokine gene expression.

5. References
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