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Expression of the endoplasmic TLRs, NLRs and RLRs in human dendritic cells exposed to inactivated and live virus vaccines in combination with chitosan

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ABSTRACT

We assessed the effect of live and inactivated influenza vaccines and live polio vaccine admixed with adjuvant (chitosan glutamate) for cytoplasmic expression of several pattern recognition receptors (PRRs) in human dendritic cells. The receptors studied included endosomal TLRs (TLR3, TLR7, TLR8, TLR9), NLRs (NOD1, NOD2) and RLRs (Rig-1, Rig-2). Our data suggest that one of the possible mechanisms of immune response polarization to Th-1 type after immunization with live and inactivated viral vaccines in combination with chitosan glutamate as adjuvant is increased activation of pattern recognition receptors localized in dendritic cells.

KEYWORDS: cytoplasmic receptor, TLR, NLR, RLR, adjuvant, chitosan, dendritic cells, immune response polarization

INTRODUCTION

Immunization with live and inactivated viral vaccines activates innate immunity through various pattern recognition receptors, the most common of which are the Toll-like receptors (TLR), Rig-like receptors (RLR) and NOD-like receptors (NLR). Interaction of pathogens with the receptors result in different signaling pathways, which eventually lead to activation of antigen-presenting cells (APCs), with the subsequent activation of adaptive immune response.

Interaction of live viral vaccine antigens with immunocompetent cells is determined by a set of receptors that recognize ligands of microorganisms, and activate signaling pathways that induce further development of innate and adaptive immunity [1, 2, 3].

It is known that some inactivated virus vaccines are characterized by low immunogenicity and, therefore, require the use of adjuvants. However, the mechanism that is responsible for adjuvant-induced stimulation of immune response remains poorly known. Understanding these mechanisms can help in increasing efficiency of viral vaccines.

We have shown previously that immunization with inactivated poliovirus and influenza vaccines in combination with derivatives of chitosan as an adjuvant leads to a polarization of the immune response to Th-1 type [4, 5]. One of the possible mechanisms of this polarization could be increased activation of cytoplasmic PRRs promoted by viral antigens in combination with chitosan. In this respect, in the present study we attempted to investigate the impact of the live and inactivated viral vaccines admixed with chitosan glutamate as an adjuvant on cytoplasmic expression of the TLRs, RLRs and NLRs in human dendritic cells.

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MATERIALS AND METHODS

Chitosan

In the experiments we used a 1% solution of the chitosan glutamate (MW 300 kD, degree of deacetylation 85%) produced by Georgy Krivtsov at the Institute for Vaccines and Sera, Moscow, Russia. An equal volume of 1% solution of chitosan glutamate in 0.2 M buffer (pH 6.2) was added to the vaccine (final concentration of chitosan 0.5%) (Russian patent number 2323742).

Vaccines

We used the following inactivated and live influenza vaccines, and polio vaccine:

1) Purified inactivated trivalent subunit vaccine "Agripal S1" (Novartis vaccines and diagnostics, Italy) for 2010/2011 season. The vaccine contained the following strains: A/California/07/2009 NYMC X-181 (H1N1), A/Victoria/210/2009 NIMC X-187 (H3N2), B/Brisbane/60/2008.
2) Cold-adapted (*ca*) influenza strain A/Krasnodar/101/35/59 (H2N2), designed to be an attenuation donor for live influenza vaccine production (Institute for Vaccines and Sera, Moscow, Russia, Russian patent number 2354695).
3) Inactivated "Imovax Polio" vaccine (Sanofi-Pasteur, France), containing poliovirus type 1, 2, and 3. One vaccine dose (0.5 ml) contained 40 units of the D antigen type 1; 8 units of the D antigen of the second type, and 32 units of the D antigen type 3.
4) Live oral poliovaccine, containing poliovirus type 1, 2, and 3 (Chumakov Institute of Poliomyelitis and Viral Encephalitides, Moscow, Russia).

Preparation of dendritic cells (DC) from peripheral blood mononuclear leukocytes (PBMC)

Monocytes were isolated from buffy coats by Ficoll density gradient (ρ = 1.077) (BioShrom) and Percoll (PercollTM GE Healthcare, UK) [6]. The corresponding fraction was collected and washed 3 times with PBS.

Monocytes (5 x 10^5) derived from PBMCs were cultured in growth medium RPMI-1640 supplemented with Hepes (containing 0.32 mg/ml glutamine, 10% foetal calf serum (PanEco, Russia) and 0.1 mg/ml gentamicin sulfate (Sigma) in the presence of 20 ng/ml of recombinant granulocyte - macrophage colony stimulating factor (GM-CSF) and 20 ng/ml interleukin-4 (IL-4) (BioSource International Inc, Belgium). On the sixth day of incubation, the growth medium was replaced, and vaccines (50 µl/ml), derivatives of chitosan (1% solution of chitosonia glutamate, 50 µl/ml) or vaccines with chitosan glutamate solution (50 µl/ml + 50 µl/ml) were added to induce expression of TLRs. Two days later, DCs were washed from the culture medium and used in further experiments.

Evaluation of the TLRs expression

Expression of TLRs in DCs was monitored by flow cytometry using monoclonal antibodies against TLR3, TLR7, TLR8, or TLR9. Cells were washed with cold phosphate-saline (PBS) with 1% fetal calf serum and incubated with Permeabilization Buffer (10^6 cells in 40 µl Permeabilization Wash buffer, BioLegend, USA) for 20 min at 20 °C. DCs were washed with PBS and stained with FITC-and PE-labeled antibodies to TLR3, TLR7, TLR8, and TLR9, according to manufacturer's instructions. Then DCs were washed 2 times with cold PBS and resuspended in 400 µl of 1% formaldehyde. Fluorescent-labelled cells were analyzed on a flow cytometer FC-500 (Beckman Culter, USA).

Isolation of cellular RNA

To isolate total RNA from the samples, we used a set of RNeasy Mini Kit, «Qiagen», cat # 74104 (Germany) according to the standard protocol. The samples were stored at –70 °C.

Reverse transcription reaction

To obtain cDNA libraries, we used a set of reagents GoScript Reverse Transcription System, «Promega», cat # A5001, (USA). Synthesis was performed by using both OligodT, and Random-Hex primers.

Polymerase chain reaction in real time (qPCR)

Primers and probes for qPCR were selected in the program Vector NTI 8.0 and manufactured in the form of ready-to-use TaqMan sets ("DNA synthesis", Russia).

Design of primers was carried out in accordance with mRNA sequences of genes under study:

1) hNOD1, nucleotide-binding oligomerization domain 1, Gen ID: 10392
2) hNOD2, nucleotide-binding oligomerization domain 2, Gen ID: 64127
3) hDDX58 (RIG-I), box peptide 58, Gen ID: 23568
4) hIFIH1 (RLR-2), interferon-induced with helicase C domain 1, Gen ID: 64145.

As representative housekeeping genes (HS genes), we selected:

1) hACTB, actin cytoplasmic, β-actin, Gen ID: 60
2) hGAPDH, glyceraldehyde-3-phosphate-dehydrogenase, Gen ID: 2597.

The reaction was performed in accordance with manufacturer's recommendations (CFX-96 (Bio-Rad)).

Fluorescence measurement was performed in the channel «FAM, λmax = 490 nm». Expression was analyzed by using relative valuation ΔΔCt.

Calculation of the relative gene expression was performed by ΔΔCt with regard to the efficiency of PCR as determined by a standard curve [7].

Statistical analysis was carried out using a software package WINMDI 2.8. Significant differences between the values of the DC control group were evaluated using the Wilcoxon test and Student t-test.

RESULTS

In order to access the activation of PRRs promoted by influenza vaccines with and without adjuvant, we incubated dendritic cells (DCs) isolated from human blood with live or inactivated influenza vaccine with or without chitosan. On the eighth day after treatment we determined the level of expression of endosomal TLRs by flow cytometry (Table 1).

When any of the investigated vaccines alone were added to the culture of immature DCs, the level of TLR3 did not significantly change (it varied between 14.5 and 17.2%). As for the chitosan-adjuvanted vaccines, chitosan in combination with a live cold-adapted (*ca*) influenza vaccine strain and with live poliovirus vaccine increased the number of TLR3 cells (27.3% and 25.4%, respectively). A moderate elevation of TLR3+ cells was also observed with Immovax polio vaccine admixed with chitosan (18.3%).

Table 1. The effect of inactivated and live virus vaccines in combination with chitosan glutamate on the expression of the endosomal TLRs in human dendritic cells (DC).

No.		% of the total number of analyzed cells (M ± SD)			
		TLR3	TLR7	TLR8	TLR9
1	DC + 0.9% NaCl	14.3 ± 0.73	27.9 ± 0.5	22.4 ± 0.83	8.2 ± 0.7
2	DC + CG	11.73 ± 1.6	24.1 ± 0.5	26.3 ± 1.01	13.5 ± 1.1
3	DC + Agripal S1	14.5 ± 1.0	33 ± 2.7	29.1 ± 7.2	15 ± 1.6*
4	DC + AgripalS1 + CG	17.5 ± 1.0	**41.5 ± 1.5****#	**42 ± 1.4****#	**31.5 ± 1.5****#
5	DC + Live CAIV	17.2 ± 1.2	46.6 ± 2.3**	70.5 ± 4.4**	8.1 ± 1.5
6	DC + Live CAIV + CG	**27.3 ± 1.57*#**	**77.3 ± 3.4****#	74.8 ± 3.6**	**33.1 ± 2.5****#
7	DC + Imovax-Polio	13.3 ± 1.2	34.1 ± 2.55*	36.3 ± 1.65*	11.6 ± 1.1
8	DC + Imovax-Polio + CG	**18.3 ± 1.85*#**	38.4 ± 2.5*	**47.5 ± 4.7****#	**13.3 ± 1.3*#**
9	DC + LPV	16.4 ± 1.6	50.3 ± 3.17**	61.5 ± 4**	10.6 ± 1.25
10	DC + LPV + CG	**25.4 ± 1.9*#**	**60.3 ± 4.2****#	**73.8 ± 3.7****#	**30.1 ± 2.54****#

Note. The significance of differences compared with the control (Group 1): *-p < 0.05; **-p < 0.01, #-p < 0.05 between Groups 3 and 4, 5 and 6, 7 and 8. DC - dendritic cells, CAIV - cold-adapted influenza vaccine donor strain, LPV - live polio vaccine, CG - chitosan glutamate.

The content of the TLR7-expressing cells increased ($p < 0.05$) when DCs were treated with live vaccines (46.6% for live influenza *ca* vaccine, and 50.3% for live poliovaccine), and a slight increase was observed in the case of Imovax-Polio vaccine (34.1%). Imovax-Polio vaccine in combination with chitosan did not significantly increase the level of TLR7 (38.4% ($p > 0.05$)). Chitosan significantly enhanced the effect of all the other vaccines tested (41.5% for Agripal S1 vaccine, 7.3% for live influenza *ca* vaccine strain, 60.3% for live polio vaccine ($p < 0.05$ in all the cases).

TLR8 expression in DCs was elevated after treatment of the cells with cell culture vaccines (except Agripal S1, 29.1%), but a combination of viral vaccines with chitosan greatly enhanced the number of TLR8+ cells (70.5% for live influenza *ca* vaccine strain, 36.3% for Imovax-Polio vaccine, 61.5% for live polio vaccine). However, chitosan in combination with the live influenza *ca* vaccine strain virtually did not increase the number of TLR8+ DC cells (74.8%), which is probably due to the immunoregulatory capacity of chitosan.

The level of the TLR9-positive DCs increased only when the cells were exposed to Agripal S1 vaccine (15%). Other vaccines by themselves did not have any influence on TLR9 expression. However, addition of chitosan to any of the vaccines induced an increase in the number of TLR9+ cells (31.5% for Agripal S1, 33.1% for live influenza *ca* vaccine strain, 30.13% for live poliovaccine). The level of TLR9-expressing DCs was enhanced to a lesser extent (13.3%) by Imovax-Polio vaccine admixed with chitosan.

After that we studied the effects of live and inactivated virus vaccines in combination with the chitosan glutamate on the cytoplasmic expression of NLRs (NOD1, NOD2) and RLRs (Rig-1, Rig-2) in human dendritic cells. As can be seen from Fig. 1, in pure form chitosan glutamate had virtually no effect on the NLR gene expression, but it raised the NLR gene expression in DC, to varying degrees, when combined with live and inactivated viral vaccines. Expression level of NOD1 did not change under the influence of inactivated influenza vaccine (Agripal S1) alone, but increased twice after incubation of the cells with the same vaccine with chitosan glutamate. In the case of the inactivated influenza vaccine (IIV), addition of the adjuvant had also a minor effect

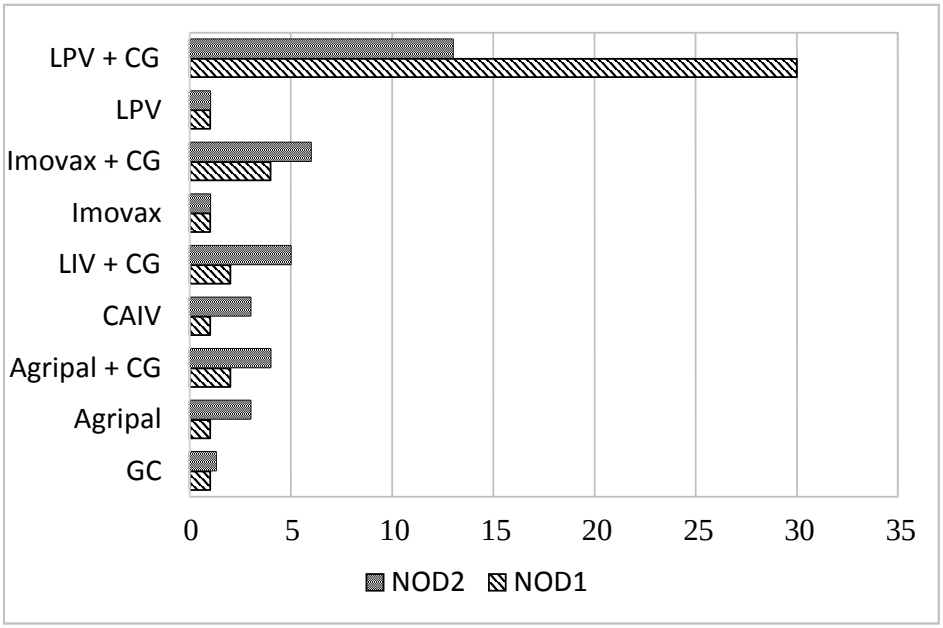

Fig. 1. Normalized NLRs gene expression under the influence of inactivated and live virus vaccines in combination with chitosan glutamate. CAIV - cold-adapted influenza vaccine donor strain, LPV - live polio vaccine, CG - chitosan glutamate.

compared to the impact of the vaccine itself. Adjuvant addition to live influenza vaccine caused a moderate expression of NOD1, and NOD2 genes. More interesting results were observed in experiments with poliomyelitis vaccines: addition of chitosan to live poliovirus vaccine enhanced the expression of the receptor 30 times for NOD1 and 13 times for NOD2, compared to unadjuvanted control. Addition of chitosan to inactivated polio vaccine increased 4 times the expression of NOD1, and 6 times the level of NOD2.

The inactivated influenza vaccine Agripal S1 and the Imovax-Polio vaccine have almost no influence on the receptor Rig-1 and Rig-2 expression (Fig. 2). However, adjuvanted inactivated poliovirus vaccine caused increased expression of the Rig-1 and Rig-2 (8 and 7 times, respectively), and the inactivated influenza vaccine resulted in an 8-fold increase in the expression of Rig-2. Live virus vaccines did not induce Rig-1 expression. However, addition of chitosan glutamate to the live influenza vaccine increased 12 times the expression of Rig-2, and the addition of the adjuvant to the live polio vaccine increased 4 times the expression of Rig-2. However, the highest raise in RLR-expression was observed in the DCs exposed to live poliovaccine in combination with the adjuvant: a 15-fold increase in the level of the Rig-2, and a 30-fold increase in the level of Rig-1.

DISCUSSIONS

Our data shows that inactivated virus vaccines induce a weak expression of cytoplasmic PRRs. Agripal S1 had no effect on the NOD1, Rig-1 and TLR3 receptors and very moderately induced the expression of the other receptors studied. A similar pattern was observed with respect to inactivated Imovax-Polio vaccine. This vaccine was absolutely unable to stimulate NLRs and RLRs (Fig. 1, 2) as well as TLR3, and only very moderately increased the expression of TLR7, TLR8 and TLR9. This explains to a certain extent the weak immunogenicity of the inactivated polio vaccine, and the need for multiple immunizations to produce an acceptable immune response.

However, addition of chitosan glutamate to inactivated viral vaccines led to increased activation of most of the cytoplasmic PRRs in DCs. As seen from Table 1, chitosan-adjuvanted

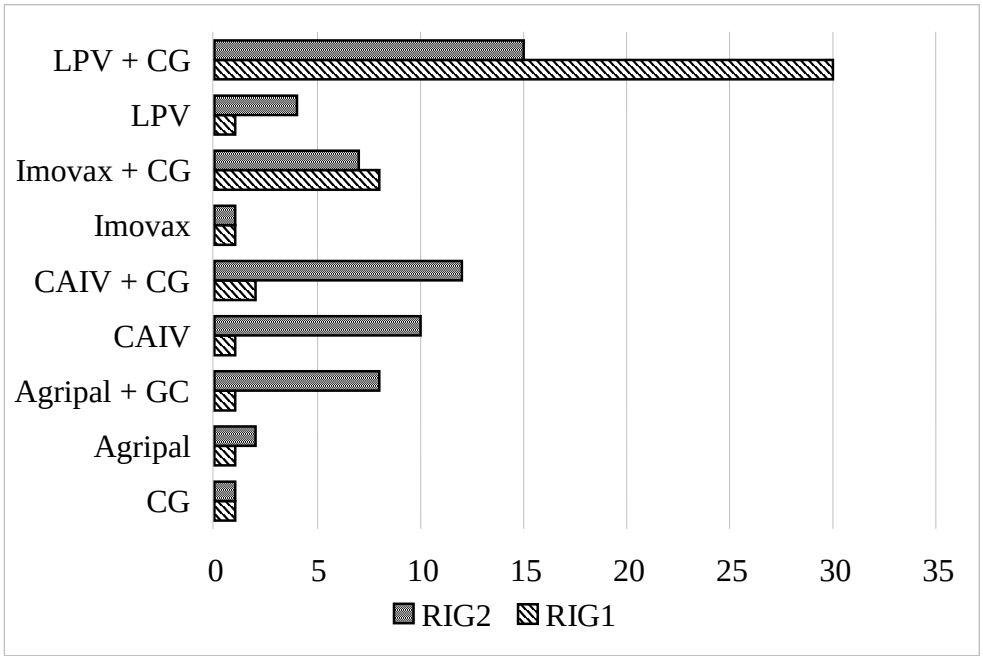

Fig. 2. Normalized Rig-like gene expression under the influence of inactivated and live virus vaccines in combination with chitosan glutamate. CAIV - cold-adapted influenza vaccine donor strain, LPV - live polio vaccine, CG - chitosan glutamate.

vaccine Agripal S1 induced an increase in the number of TLR9-expressing cells (31.5% TLR9+ cells for vaccine with chitosan compared to 15% for vaccine alone), and enhanced the expression of TLR7 (41.5% compared to 33% for vaccine alone). The level of NOD1 expression after incubation of cells with inactivated influenza vaccine was not changed, but upon addition of chitosan glutamate it increased 2-fold, and the expression of NOD2 increased 3 and 4 times respectively in comparison with untreated DCs. Adjuvanted inactivated influenza vaccine increased the level of Rig-2-expressing cells 8 times.

We observed an increased level of the Rig-2 and Rig-1 expression only when vaccines were admixed with chitosan. NOD2-expression was also promoted by chitosan addition to the inactivated polio vaccine. All these data indicate that one of the possible mechanisms of immune response polarization to Th-1 type upon immunization with chitosan-adjuvanted virus vaccines may be an increase in the expression of cytoplasmic PRRs under influence of viral antigens with chitosan.

Interestingly, addition of chitosan glutamate to any of the virus vaccines resulted in increased expression of at least some cytoplasmic receptors. For instance, adjuvanted live polio vaccine induced an increase in the expression of all the studied NLRs and RLRs, and TLR7 and TLR8. At the same time, adjuvanted inactivated influenza vaccine activated mainly NOD2, Rig-2, TLR7, and TLR9.

The most dramatic effect on the expression of TLR7 was observed upon addition of live vaccines, while the activity of inactivated vaccines was significantly lower. Chitosan glutamate enhanced the effect of live vaccines, but a higher adjuvant effect of chitosan was manifested when administered with the live influenza vaccine compared with the live polio vaccine (a 1.7 and 1.2-fold increase in TLR7 expression). In previous studies we have shown that mice immunization with inactivated poliovirus and influenza vaccines in combination with the derivatives of chitosan as adjuvants leads to a polarization of the immune response to the Th-1 type [8, 9, 10]. Our data suggest that one of the possible mechanisms of immune response polarization to Th-1 type in the immunization with the live and inactivated viral vaccines in combination with chitosan glutamate as adjuvant is the increased cytoplasmic activation of pathogen recognition receptors localized in dendritic cells.

A distinctive feature of the live vaccines was a powerful expression of TLR8 after treatment of DCs, but only the effect promoted by live polio vaccine was enhanced by addition of chitosan glutamate (1.2 times). Lack of response to the addition of chitosan to the live influenza vaccine strain can be attributed to various causes. Perhaps this phenomenon is associated with the immunomodulatory activity of the chitosan, which prevents excessive cell proliferation. On the other hand, this phenomenon can be explained by limited efficiency of signaling pathways related to this type of receptor.

The study of the mechanisms of action of adjuvants on the various branches of innate immunity will contribute to the development of new approaches to improve effectiveness of live and inactivated viral vaccines.

CONCLUSIONS

1. Inactivated virus vaccines (Agripal and Imovax-Polio) resulted in only a weak induction of expression of cytoplasmic PRRs compared to the live vaccines (live influenza virus and live polio vaccines). This fact explains to a certain extent the weak immunogenicity of inactivated poliovaccine, and the need for multiple immunizations to get an acceptable immune response.
2. The addition of the chitosan glutamate to the inactivated and live virus vaccines has led to increased activation of cytoplasmic PRRs (TLR7, TLR8, TLR9, NLRs and RLRs).

CONFLICT OF INTEREST STATEMENT

In our article, we have no conflicts of interest.

REFERENCES

1. Medzhitov, R. and Dzhanevey, C. 2005, Kazan Med. J., LXXXV(3), 161-167.
2. Culter, C. W. and Jotwani, R. 2006, J. Dent. Res., 85(8), 678-689.
3. Romange, F. 2007, Drug Dis. Today, 12(1/2), 80-87.

4. Akhmatova, N. K., Pereversev, A. D., Markushin, S. G., Lebedynskaya, O. V. and Ahmatov, E. A. 2011, Med. Immunol., 134(4-5), 516.
5. Markushin, S. G., Pereversev, A. D., Akhmatova, N. K. and Kryvtsov, G. G. 2011, Russian J. Immunol., 5(14), 233-243.
6. Grachev, A. N., Karagodin, V. P., Myasoedova, V. A., Kirichenko, T. V., Rudimov, E. H., Orekhova, E. A., Khrenov, M. O., Avhacheva, N. V., Mubarakshina, E. C., Kzhyshkovskaya, U. G. and Orekhov, A. N. 2009, Bull. Moscow Society of Naturalists, 114(3), 291-296.
7. Livak, K. J. and Schmittgen, T. D. 2001, Methods, 25(4), 402-8.
8. Markushin, S. G. and Akhmatova, N. K. 2012, Palmarium (Ed.), 180.
9. Akhmatova, N. K., Markushin, S. G., Akhmatov, E. A., Sorokina, E. V., Khomenkov, V. G., Sukhno, A. S., Pereversev, A. D. and Zverev, V. V. 2012, Russian J. Immun., 6(15), 124-131.
10. Akhmatova, N. K., Markushin, S. G., Pereversev, A. D., Kryvtsov, G. G. and Akhmatov, E. A. 2011, Immunol., 6, 292-296.