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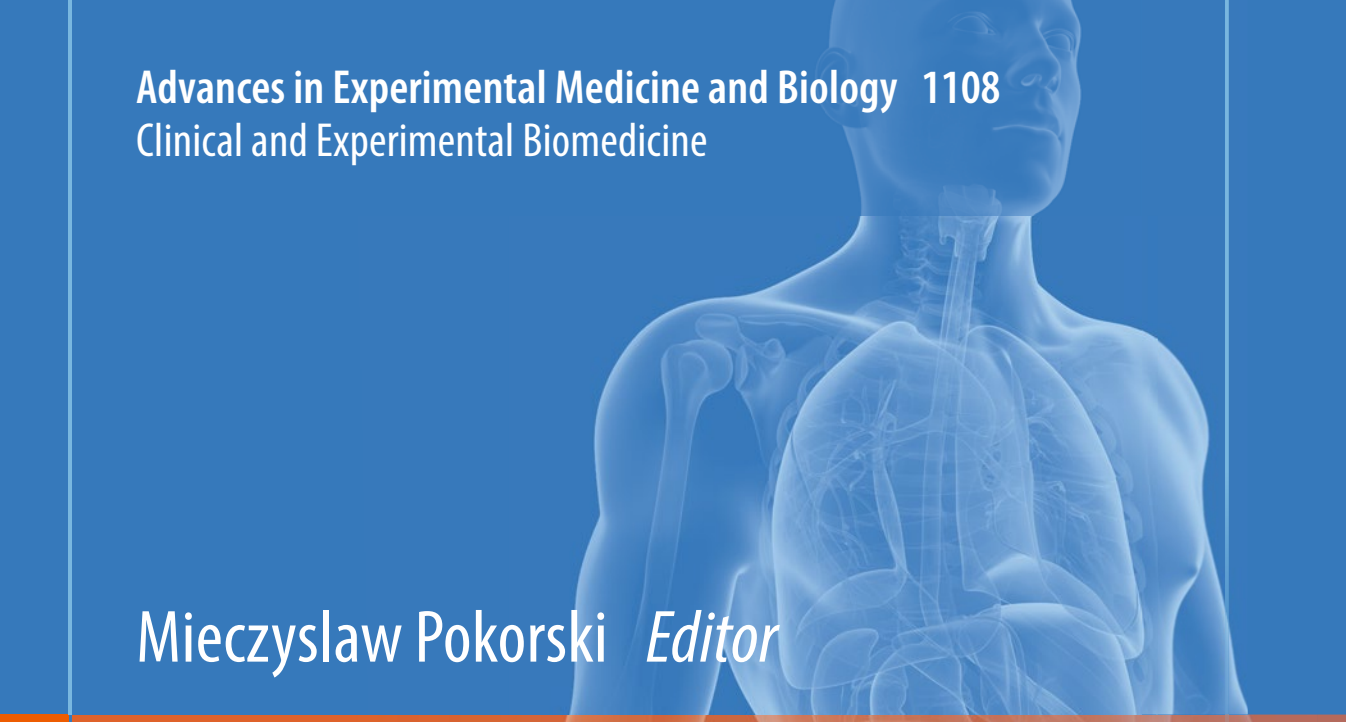

Mieczyslaw Pokorski *Editor*

Current Trends in Immunity and Respiratory Infections

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Influence of Different Bacteria Strains Isolated from Septic Children on Release and Degradation of Extracellular Traps by Neutrophils from Healthy Adults

Weronika Bystrzycka, Sandra Sieczkowska, Aneta Manda-Handzlik, Joanna Rogulska, Aneta Moskalik, Urszula Demkow, and Olga Ciepiela

Abstract

Neutrophils are the first line of immune defense against pathogens. They use three major antimicrobial mechanisms: phagocytosis, degranulation, and release of neutrophil extracellular traps (NETs). NETs are structures which consist of nuclear DNA conjugated with antibacterial proteins. They are formed to entrap and kill pathogens. The aim of the study was to evaluate the influence of *Escherichia coli* (*E. coli*), *Streptococcus pneumoniae* (*S. pneumoniae*), *Stenotrophomonas maltophilia* (*S. maltophilia*), and *Pseudomonas aeruginosa* (*P. aeruginosa*), isolated from the peripheral blood of children with sepsis, on the release and degradation of NETs by neutrophils isolated from blood healthy adult subjects. Neutrophils were stimulated with the bacterial strains outlined above. The quantitative and qualitative analyses of NETs release were performed by fluorometric measurement and immunofluorescence, respectively. The ability of bacteria to degrade NETs was studied qualitatively. Oxidative burst was assessed by flow cytometry. Histone H3 citrullination was evaluated by Western blot. We found that NETs were formed only when neutrophils were incubated with *S. pneumoniae*. *E. coli*, *P. aeruginosa*, and *S. maltophilia* did not induce the release of the NETs. *P. aeruginosa*, *S. pneumoniae*, and *E. coli* induced the production of reactive oxygen species (ROS) by neutrophils. Two studied

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bacterial strains (*S. pneumoniae* and *E. coli*) were able to degrade NETs. However, none of the strains induced the citrullination of histone H3. We conclude that the ability of bacteria to induce and degrade NETs depends on the specific bacterial strain.

Keywords
Bacterial strain · Neutrophil extracellular traps · Oxidative stress · Sepsis · Septic children · *Streptococcus pneumoniae*

1 Introduction

Neutrophils play a crucial role in innate immunity against bacterial infections. They act by phagocytosis, degranulation, and the formation of neutrophil extracellular traps, called NETosis, to catch, immobilize, kill, and remove pathogens from the body (Pruchniak et al. 2013). Bacteria employ a wide range of mechanisms which protect them against the immunological challenge. These mechanisms include the formation of a polysaccharide capsule that protects against being trapped in the neutrophil extracellular traps (NETs), the alteration of cell membrane potential to avoid DNA binding, the creation of a biofilm, or the production of enzymes that degrade or inhibit the NETs release such as nucleases and catalases (Arazna et al. 2013). Phagocytosis and degranulation are the well-known bactericidal mechanisms, while NETosis, based on the release of NETs from cells in response to stimulation, remains a largely unknown way of neutrophilic action (Brinkmann et al. 2004). During NETosis, elastase is translocated to the neutrophilic nucleus, histones undergo modifications such as citrullination and degradation, chromatin undergoes decondensation, nuclear and cytoplasmic granules mix, and nuclear DNA furnished with cytoplasmic antimicrobial enzymes is released into the extracellular space (Papayannopoulos et al. 2010). The release of NETs is called a "double-edge sword of innate immunity". On the one hand, NETs present antiviral, antibacterial, and antiparasitic activity by immobilization and killing of pathogens, preventing their spread within the body, but on the other hand, NETs may constitute a source of antigens against which autoantibodies are produced, which can trigger disorders (Manda et al. 2014; Kaplan and Radic 2012). A variety of mechanisms have been proposed as the inducers of NETs release by neutrophils, and the list still goes on (Hahn et al. 2013). Several bacterial species have been identified as stimulants that cause neutrophils to release NETs. Interestingly, the ability to induce or degrade NETs may differ between bacterial strains among one species, which depends on the virulence factors expressed by a specific strain (Arazna et al. 2013; Hahn et al. 2013).

Bacteria that induce sepsis can be either pathogenic or opportunistic. The development of sepsis involves both innate, including the release of NETs, and adaptive immunity (Araujo et al. 2016; Slotwinski et al. 2015). Studies have shown that neutrophils collected from septic patients release fewer NETs compared to the cells isolated from healthy individuals. The septic neutrophils' ability to neutralize bacteria also is weaker than that of neutrophils isolated from healthy subjects (Hashiba et al. 2015). Bacterial products, such as cytokines and complement proteins, released by the immune system in the course of sepsis impair margination, migration, and bactericidal function of neutrophils consisting of phagocytosis, reactive oxygen species (ROS) formation, and NETosis (Shen et al. 2017). Neutrophilic activation during sepsis could be driven by the mechanisms other than those acting during local infection (O'Brien et al. 2017; Park et al. 2017). Importantly, pro-inflammatory and pro-coagulating effects of NETosis may promote a multi-organ dysfunction in the course of sepsis. Disseminated intravascular coagulation, a septic symptom being a death risk factor, develops on the basis of NETs formation (McDonald et al. 2017; Yang et al. 2017; Tanaka et al. 2014). The release of NETs by neutrophils in the course of bacterial infection

depends on the bacteria type (Branzk et al. 2014). The knowledge of an interaction between neutrophils and bacteria strain would enable the control of excessive innate immune response in the course of disseminated infection. The present study seeks to assess the ability of four different bacteria strains isolated from peripheral blood of septic patients such as *Escherichia coli* (*E. coli*), *Streptococcus pneumoniae* (*S. pneumoniae*), *Stenotrophomonas maltophilia* (*S. maltophilia*), and *Pseudomonas aeruginosa* (*P. aeruginosa*) to induce NETosis in neutrophils isolated from healthy individuals and the ability of neutrophils to degrade NETs.

2 Methods

2.1 Neutrophils Isolation

The study was accepted by the Ethics Committee of Warsaw Medical University in Warsaw, Poland, and a written informed consent was obtained from each subject. Peripheral venous blood was collected on sodium citrate from healthy adult volunteers, age range of 22–35 years. Platelet-rich plasma was discarded by centrifugation at 1000 rpm for 10 min. The pellet was half-diluted with 0.9% NaCl, and neutrophils were isolated by density centrifugation; the diluted suspension of cells was layered onto Histopaque 1077 (Merck; Darmstadt, Germany) and centrifuged for 30 min at 400 × *g*. Next, mononuclear cells and Histopaque were removed, and the pellet containing red blood cells and neutrophils was gently mixed with 1% polyvinyl alcohol (Avantor Performance Materials; Gliwice, Poland) and left for 20 min at room temperature for erythrocytes sedimentation. Residual red blood cells were lysed with water for 40 s; the lysis was stopped by the addition of twice concentrated phosphate-buffered saline. Neutrophils were washed twice and suspended in no-phenol red protein-free Roswell Park Memorial Institute (RPMI) medium (Thermo Fisher Scientific; Waltham, MA).

2.2 Bacteria Isolation and Culture

Peripheral venous blood of septic children of up to 14 years of age was collected to BacT/Alert PF culture bottles which were incubated in a BacT/Alert 3D analyzer (bioMérieux, Inc.; Durham, NC) for up to 18–24 h, until the positivity of culture was detected consisting of a change in pH due to CO_2 released from bacteria. The medium containing proliferating bacteria was aseptically collected to a 1 ml syringe. A bacterium strain was identified from smears for Gram staining and cultures in the differentiating media (blood agar, MacConkey agar, chocolate agar, Sabouraud agar). All of the bacteria were identified according to the criteria set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2015). The identification of bacteria was made using a Vitek 2 compact analyzer (bioMérieux, Inc.; Durham, NC), antibiotic sensitivity test using a disk diffusion method, or E-test. After the identification, bacteria strains were preserved at −70 °C until further use. A day before the experiment, bacteria were thawed and cultured on MacConkey agar (Gram negative) or blood agar (Gram positive) for 24 h.

The bacteria used for neutrophil stimulation were identified as *E. coli* ESBL+ (extended spectrum beta-lactamase positive), *S. pneumoniae* PSSP (penicillin-susceptible *S. pneumoniae*), *S. maltophilia* sensitive to trimethoprim and sulfamethoxazole, and *P. aeruginosa* ESBL– (extended spectrum beta-lactamase negative).

2.3 Respiratory Burst

To analyze whether the bacteria studied induce the respiratory burst in neutrophils, dihydrorhodamine 123 (DHR 123) (Thermo Fisher Scientific; Waltham, MA) was used as a fluorescent marker of the intracellular production of reactive oxygen species (ROS). Briefly, 2.5×10^5/ml of neutrophils were incubated with 4 μg/ml DHR123 in 5% CO_2 atmosphere for 30 min at 37 °C. Subsequently, the cells were incubated for 1 h with 30 μl of the bacterial suspension at optical density (OD) 600 = 0.5. A sample with

100 nM phorbol myristate acetate (PMA) as a stimulator of ROS production was used as positive control, while the negative control contained cells resuspended in RPMI 1640 medium. The analysis of fluorescence intensity of DHR123, formed due to reactivity of ROS, was measured at the first fluorescence channel with flow cytometry (Cytomics FC500, Beckman Coulter; Brea, CA).

2.4 NETs Quantification, Visualization, and Degradation

Isolated cells were seeded into 96-well black plates at density of 1×10^5 cells/well. Samples were divided into two groups: neutrophils incubated in RPMI 1640 medium for 4 h as negative control and neutrophils incubated with 80 μl of bacterial suspension at OD 600 = 0.5 for 4 h. The plates were incubated in 5% CO_2 atmosphere at 37 °C. After incubation, 100 nM Sytox Green fluorescent dye (Thermo Fisher Scientific; Waltham, MA) was added to each well to measure the amount of extracellular DNA by fluorometry.

To visualize the formation of NETs, a qualitative method with the use of a fluorescent microscope was applied. Neutrophils (2.5×10^4) were seeded into 8-well Lab-Tek chamber slides (Thermo Fisher Scientific, Waltham, MA). The samples were incubated in 5% CO_2 atmosphere for 4 h at 37 °C, with or without the bacteria as described in the NETs quantification procedure outlined above and with 100 nM PMA as positive control. Then, samples were fixed with 4% paraformaldehyde and washed three times with PBS. Permeabilization of cells was investigated with 0.1% Triton X (Sigma-Aldrich, St. Louis, MA), and the slides were again washed with PBS. After overnight staining with anti-myeloperoxidase (MPO)-fluorescein isothiocyanate (FITC)-labeled monoclonal antibody (ab11729; 0.1 mg/ml) (Abcam; Cambridge, UK), the chamber was washed with 0.9% NaCl. DNA was counterstained with the nucleic acid dye Sytox Orange (Thermo Fisher Scientific; Waltham, MA), and the samples were examined under the fluorescent Leica DMi8 microscope (Wetzlar, Germany).

Studies on the ability of bacteria to degrade NETs were performed in 24-well plates, where 5×10^4 neutrophils were seeded in RPMI 1640 medium. Then, 100 nM PMA was added to selected wells to induce NETosis or RPMI 1640 to the unstimulated control. The plates were incubated in 5% CO_2 atmosphere for 3 h at 37 °C. Subsequently, 40 μl of each bacterium suspension at OD 600 = 0.5, or 50 units of micrococcal nuclease (Thermo Fisher Scientific; Waltham, MA) to positive controls or RPMI 1640 to negative controls, was added, and the wells were examined after 1, 2, 3, and 4 h of incubation (separate wells were prepared for each time point). At each time point, Sytox Green fluorescent dye at a final concentration of 100 nM was added to the wells, and the presence of NETs and their degradation was assessed by fluorescent microscopy.

2.5 Western Blot Analysis of Histone 3 Citrullination

Neutrophils were incubated with RPMI 1640 alone, PMA (100 nM), or calcium ionophore A23187 (4 μM) for 2 h. Subsequently, bacterial suspensions were added to the samples, and neutrophils were further incubated for 2 h, after which the tubes containing neutrophils were centrifuged and washed with ice-cold PBS. The cell pellets were lysed with radioimmunoprecipitation assay (RIPA) buffer supplemented with a protease inhibitor cocktail. The lysates were sonicated, boiled for 5 min at 95 °C with 5 × Laemmli buffer, and loaded onto 15% polyacrylamide gel, and the proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% milk for 1 h at room temperature and incubated with the primary anti-citH3 antibody (ab5103, 1:1000) in 5% milk, overnight at 4 °C. That was followed by further incubation with a secondary antibody conjugated with horseradish peroxidase (HRP) (ab97051; 1:50000) (Abcam; Cambridge, UK) in 5% milk for 1 h at room

temperature. The incubation in like manner with anti-actin antibody conjugated with HRP (A3854, 1:50000) (Sigma-Aldrich; St. Louis, MA) was used as a loading control. The results of Western blotting were visualized on X-ray film after the HRP reaction with the luminol substrate (Amersham ECL Prime Western Blotting Detection Reagent; GE Medical Systems, Poland).

2.6 Statistical Analysis

Data were presented as means ±SE. Data distribution was assessed with the Shapiro-Wilk test. Differences were compared with a *t*-test or Wilcoxon matched-pair test. A p-value <0.05 defined the statistically significant differences. The evaluation was performed using GraphPad Prism v6.0 (GraphPad Software; La Jolla, CA).

3 Results

3.1 Qualitative and Quantitative Analysis of NETs Formation

P. aeruginosa, *E. coli*, and *S. maltophilia* did not induce the release of NETs from neutrophils isolated from healthy blood donors. The ability to induce NETs, compared with spontaneous NETs release, was shown only for *S. pneumoniae*. Fluorescence of the extracellular DNA released from the unstimulated neutrophils amounted to 18,197 ± 5,511 RFU (relative fluorescence units), while the cells incubated with *S. pneumoniae* showed 31,831 ± 5,577 RFU ($p = 0.003$) (Fig. 1a). Fluorescent microscopy confirmed these observations (Fig. 2).

3.2 Reactive Oxygen Species Production

There were noticeable increases in the mean fluorescence intensity peak (MFI) of DHR123 in neutrophils incubated with *E. coli*, *P. aeruginosa*, and *S. pneumoniae* compared with those present in the resting neutrophils. The levels of MFI in bacteria stimulated *versus* resting neutrophils were as follows: 23.9 ± 1.3 *vs*. 18.1 ± 1.8 for *E. coli*, 26.8 ± 10.8 *vs*. 14.2 ± 4.8 for *P. aeruginosa*, and 19.3 ± 4.1 *vs*. 12.2 ± 3.3 MFI for *S. pneumoniae*, respectively; $p = 0.031$ for all comparisons. *S. maltophilia* did not induce ROS production; 24.0 ± 13.7 *vs*. 22.6 ± 9.0 MFI, respectively; $p > 0.05$) (Fig. 1b).

3.3 Effect of Bacteria on NETs Degradation and Histone Citrullination

NETs structures in unstimulated and PMS-stimulated neutrophils, as well as in positive controls treated with micrococcal nuclease (MNAse), are shown in Fig. 3a. *E. coli* effectively degraded NETs. The structure of NETs changed, with the signal resembling the nuclear area of neutrophils and no visible fine DNA threads after a 2-h incubation of *E. coli* with formed NETs. After further 2 h, there were no NETs in a sample, which was akin to the positive control treated with MNAse (Fig. 3b). *S. pneumoniae* also degraded the NET structure, but less efficiently than *E. coli* (Fig. 3c). *P. aeruginosa* failed to degrade NETs, as the fluorescent pattern of their DNA backbone, stained with Sytox Green, was similar at the beginning and end of 4-h incubation, although the NET structure assumed a somehow riddled-like appearance (Fig. 3d). Likewise, *S. maltophilia* failed to appreciably affect the structure of NETs formed (Fig. 3e).

None of the bacteria investigated induced citrullination of histones, as shown in Western blot analysis. Neutrophils stimulated with calcium ionophore were used as positive control (Fig. 1c).

4 Discussion

In this study we investigated four different bacteria strains with regard to their ability to induce and degrade NETs. The findings were that the bacterial ability to trigger NETs release differed

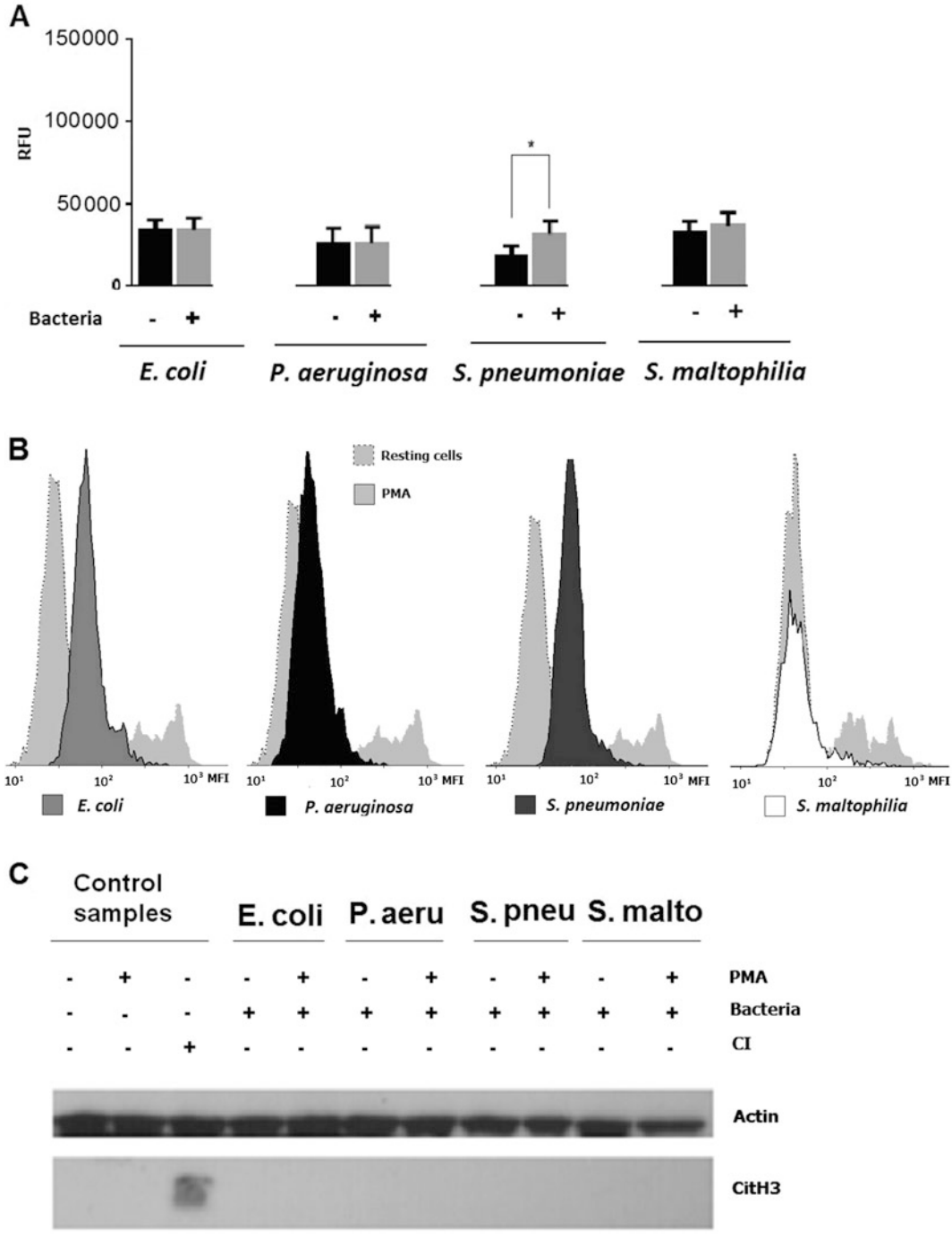

Fig. 1 **(a)** Quantitative analysis of NETs release from neutrophils stimulated with the bacteria investigated. NETs release was measured as fluorescence of extracellular DNA stained with Sytox Green in suspension of cells after 3-h stimulation. Neutrophils were isolated from six different blood donors (n = 6). **(b)** Analysis of ROS induction in neutrophils incubated with the bacteria investigated. The presence of ROS was assessed from the intensity of DHR fluorescence measured by flow cytometry. Neutrophils were isolated from six different blood donors (n = 6). **(c)** Western blot detection of citrullinated histone H3. Actin was used as a loading control; expression of citH3 was analyzed in cellular lysates after incubation of neutrophils with phorbol myristate acetate (PMA), selected bacteria, and calcium ionophore (CI), whenever applicable. None of the bacteria investigated induced histone citrullination. Neutrophils were isolated from three different blood donors (n = 3)

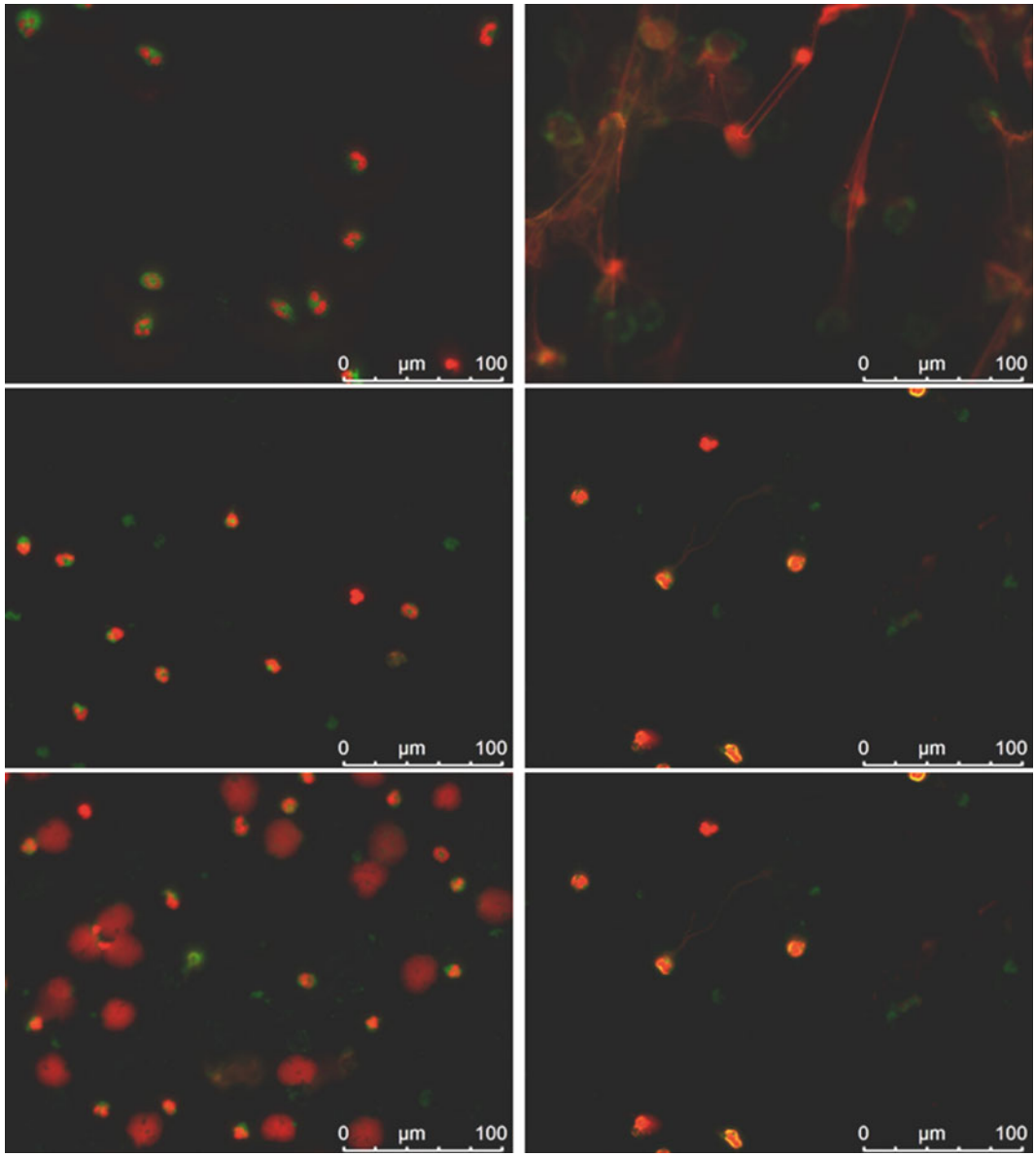

Fig. 2 Immunofluorescent analysis of NETs release from neutrophils incubated with different bacteria strains. Myeloperoxidase (MPO) was labeled with monoclonal antibody conjugated with fluorescein isothiocyanate (*FITC)* (green fluorescence); DNA was stained with Sytox Orange (red fluorescence)

significantly between species, despite that all of them are causative organisms associated with sepsis. *E. coli* failed to induce NETs release from neutrophils obtained from healthy donors. It is worth mentioning that neutrophils incubated with bacteria were not pre-activated. Pre-activation may better correspond with the in vivo environment, where a variety of cytokines keep neutrophils ready for bactericidal action. Specific virulence factors, presented by different bacterial strains within the same species, determine the bacterial ability to interact with the

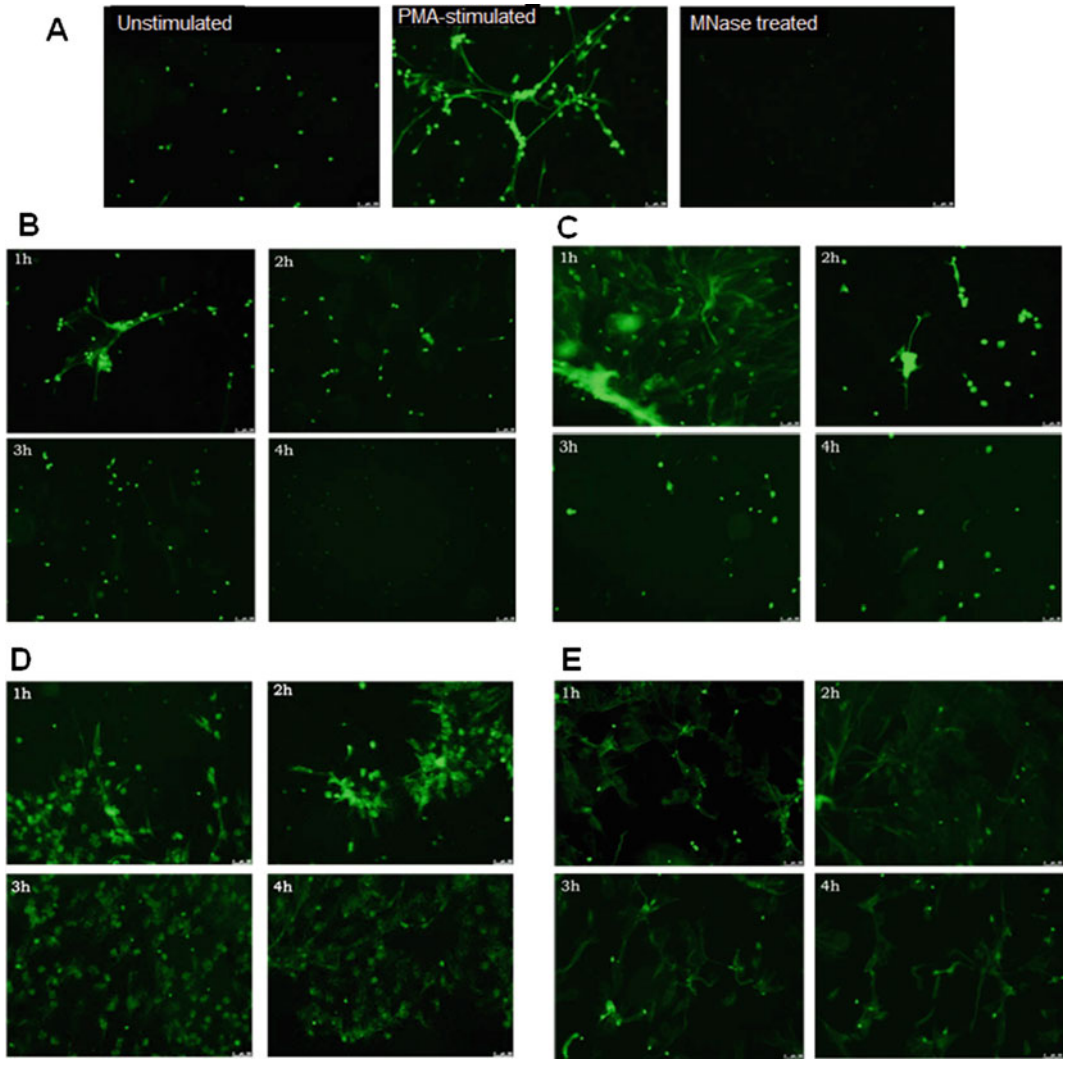

Fig. 3 Degradation of NETs by bacteria strains isolated from peripheral blood of septic patients. (**a**), unstimulated cells; (**b**), *E. coli*; (**c**), *S. pneumoniae*; (**d**), *P. aeruginosa*; and (**e**), *S. maltophilia*; 1, 2, 3, and 4 h of incubations are marked in panels B, C, and D. All experiments were performed in neutrophils isolated from four different blood donors (n = 4)

human immune system. Grinberg et al. (2008) have found that mammary pathogenic *E. coli* may stimulate the release of NETs from bovine neutrophils. Interestingly, Marin-Esteban et al. (2012) have used different strains of *E. coli* to induce NETs and found that these bacteria induce NETs in different manner depending on the strain, which might explain why we were unable to observe *E. coli*-induced NETs release. One of the *E. coli* virulence factors that stimulates human innate immunity is flagellin, a chemoattractant for murine neutrophils (Zgair 2012). The ability of *E. coli* to induce the innate immune response may result from the presence of virulence factors, which inhibit migration, adhesion, and pro-inflammatory function of neutrophils. These factors were detected in uro-pathogenic strains of *E. coli*. Such strains are able to produce cytotoxic

necrotizing factor 1 (CNF1), which inhibits chemotaxis, phagocytic activity, and the oxidative burst of peripheral blood neutrophils (Olson and Hunstad 2016). An indirect evidence for neutrophilic infiltration in response to *E. coli* has been presented by Strus et al. (2015) who show increased MPO activity in the area of infection after retroperitoneal injection into the mouse of *E. coli* isolated from human microbiota. The increased MPO activity in the area of infection could result not only from cell degranulation but also from release of NETs. In the present study, we showed that *E. coli* was able to degrade NETs, which confirms that these bacteria produce nucleases (Connelly and Leach 1996). Nonetheless, it cannot be determined whether the nucleases act against single- or double-stranded DNA.

The studied strain of *P. aeruginosa* did not induce the release of NETs either. This finding is at variance with that of Yuen et al. (2016) who have found that *P. aeruginosa* induces the release of NETs. This divergence lends support to the notion that a different level of activation of the immune system may result from distinct virulence factors presented by bacteria. That also was confirmed by Shan et al. (2014) who compared seven cytotoxic and invasive strains of *P. aeruginosa* and showed a different level of the immune system activation in response to different strains. Moreover, those authors have shown that the levels of induction of neutrophils by *P. aeruginosa* in *in vitro* and *in vivo* models significantly differ from each other. Thus, immune cells need pre-activation and a specific microenvironment to fully present their function. *P. aeruginosa* isolated from airways of cystic fibrosis patients, akin to its standard laboratory strains, may induce the release of NETs, but it does so in a distinct manner. Interestingly, early *Pseudomonas* isolates enhance the release of NETs more than late isolates obtained from the same cystic fibrosis patient, which shows that a longer bacteria adjustment to the condition of cystic fibrosis airways is needed to decrease the induction of NETs (Yoo et al. 2014a). In line with those results, Floyd et al. (2016) have found that *Pseudomonas* induces NETs most potently at an early stage of growth, which is explicable by its virulence factors and motility typical of the early growth. Those authors, using flagellated and non-flagellated *P. aeruginosa*, have confirmed that the flagellum is a major factor inducing the release of NETs from neutrophils.

In the present study, we failed to notice citrullination of histone 3 in neutrophils incubated with *P. aeruginosa*, which opposes the findings of other authors (Yoo et al. 2014b; Douda et al. 2011). This divergence might be associated with different strains and conditions, under which neutrophil granulocytes were stimulated. Pieterse et al. (2016) have found that under platelet-free and serum-free conditions in vitro, neutrophils release NETs in the presence of only some bacterial strains. More precisely, NETs are released after treatment with lipopolysaccharide (LPS) isolated from a single *E. coli* strain and a single *P. aeruginosa* strain among several strains tested. The importance of platelets and serum for this process has been underlain as in their presence NETs are released from neutrophils incubated with all strains tested.

In the present study, ROS were formed by neutrophils incubated with *P. aeruginosa*. Floyd et al. (2016) have found that ROS production is dependent on bacterial motility associated with the presence of a functional flagellum. The present finding that *Pseudomonas* did not entirely degrade the NET structure suggests that the strain investigated did not produce nucleases. Nonetheless, we found a degraded change in the NET structures, resembling a mesh strainer, when neutrophils were incubated with *Pseudomonas*, which might stem from the activity of bacterial proteases.

A strain of *S. pneumoniae*, which was used in this study, induced the release of NETs from non-activated neutrophils. The finding corresponds with the observations made by other authors (Ullah et al. 2017; Narayana Moorthy et al. 2013). Mori et al. (2012) who have argued that α-enolase expressed by *S. pneumoniae* is responsible for the immune system stimulation. They have showed that α-enolase induces the release of NETs using a pathway different than PMA, IL-8, or LPS.

G Nel et al. (2016) have shown that the release of NETs from cells incubated with *S. pneumoniae* is dependent on pneumolysin; the mechanism is independent from Toll-like receptors 4 (TLR4) or ROS. Contrary results have been obtained by Ullah et al. (2017) who show that the release of NETs upon stimulation with *S. pneumoniae* is not associated with pneumolysin activity, but rather with autophagy. Hence, direct mechanisms of NET induction by *S. pneumoniae* remain debatable. In contrast, the process of NET degradation leaves no doubts. One of the enzymes responsible for the degradation process is endonuclease EndA (Narayana Moorthy et al. 2013; Zhu et al. 2013; Beiter et al. 2006). In this study we also noticed a NET degradation by *S. pneumoniae*. We show that *S. pneumoniae* induced oxidative burst *in vitro*; the finding is in line with *in vivo* observations, where the bacterium was able to induce ROS formation in the lungs of infected mice (Narayana Moorthy et al. 2013).

The present study is the first to analyze the effect of *S. maltophilia* on the release of NETs. These environmental Gram-negative bacteria rarely cause opportunistic infections. A strain isolated from a septic individual did not induce NET release. Zgair and Al-Adressi (2013) have found that this bacterium present in the urine bladder stimulates the innate immune response, resulting in the formation of pro-inflammatory cytokines, chemokines, and nitric oxide (NO). Those authors have also shown the increased MPO activity in tissues, reflecting the neutrophil infiltration, degradation, or even the NETosis-inducing action. Akin to *Pseudomonas*, *S. maltophilia*'s flagellum is a key factor stimulating the release of NETs from neutrophils (Rada 2017; Zgair and Chhibber 2012a; Zgair and Chhibber 2012b). Similarly to other studied bacteria, *S. maltophilia*, like other currently investigated bacteria, failed to cause citrullination of histone 3. Interestingly, the strain did not stimulate neutrophils to release ROS. A failure to notice the degradation of NETs in the presence of *S. maltophilia* is suggestive of the lack of nuclease formation by the isolated strain.

In the present study, we assessed the effects of bacteria isolated from septic pediatric patients on neutrophil granulocytes isolated from peripheral blood of healthy individuals. It is possible the results would be different had we had neutrophils isolated from septic children. Hashiba et al. (2015) have shown that the generation of NETs *ex vivo* by neutrophils isolated from septic patients is appreciably less than that from healthy subjects. At any rate, those authors have demonstrated a high concentration of free plasma DNA accompanied by histones in healthy subjects as well, which is indicative of NET release in vivo.

In conclusion, this study confirmed that stimulation of the innate immune system by bacteria highly depends on the type of bacteria. Since most of the pathogens studied were opportunistic, the effects of bacteria *in vivo* strictly depended on the patients' immunity. Apparently, *in vivo* experimental models enhance and update the knowledge on the effects of bacteria on the release of NETs.

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Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Immunomodulatory Role of Vitamin D: A Review

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Abstract

Vitamin D is well known for its classical hormonal action related to the maintenance of mineral and skeletal homeostasis. However, the discovery that vitamin D receptor (VDR) is expressed in most non-skeletal tissues points to its broad role in the human organism. Current literature emphasizes a multidirectional role of vitamin D, with a special focus on its immunomodulatory properties. As VDR and the enzyme 1-α-hydroxylase are expressed in most immune cells, vitamin D modulates the phagocytic activity of macrophages and natural killer cells. In addition, it induces the microbicidal activity of phagocytes. In contrast, vitamin D suppresses differentiation and maturation of antigen-presenting dendritic cells and B lymphocytes, and it inhibits proliferation of Th1 and Th17 cells. In this review we aimed to describe the current scientific discoveries on the role of vitamin D as immunomodulator.

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Keywords

Immune cells · Immunity · Immunomodulation · Phagocytic activity · Vitamin D · Vitamin D receptor

1 Introduction

During the past decades, our knowledge about the vitamin D has radically changed. Since the discoveries that the vitamin D receptor (VDR) and the vitamin D-activating enzyme 1-α-hydroxylase, member of the cytochrome P450 (CYP), are expressed in most tissues, vitamin D became a central focus for a number of scientists. A growing body of evidence shows the multiple biologic roles of vitamin D and its much broader influence on human health than initially accepted (Bikle 2014; Prietl et al. 2013; Baeke et al. 2010b). It is well known that vitamin D is essential for the development and growth of the organism and for the maintenance of mineral and bone homeostasis (Białek-Gosk et al. 2018; Kitay and Geibel 2017). The classical, hormonal activity of vitamin D is related to bones, kidneys, and intestines, with the main role to sustain the body's adequate calcium level (Christakos et al. 2017; Prietl et al. 2013; Holick 2003). It is currently accepted that the action of vitamin D is also associated with various other non-classical tissues and cells in the immune system (T and B cells, macrophages, and monocytes), the reproductive

system (uterus, testis, ovary, prostate, placenta, and mammary glands), the endocrine system (pancreas, pituitary, thyroid, and adrenal cortex), muscles (skeletal, smooth, and heart muscles), and the brain, skin, and liver (Verstuyf et al. 2010).

2 Vitamin D Metabolism

In humans, vitamin D can be obtained from either dietary products (cod-liver oil, oily fish, and eggs) or be synthesized in the skin in a process mediated by the ultraviolet (UV) sun radiation (Mohania et al. 2017; Lamberg-Allardt 2006) (Fig. 1). In the epidermal layer of the skin, UV rays (280–315 nm) lead to photolytic cleavage of 7-dehydrocholesterol (7-DHC) into pre-vitamin D that undergoes thermal isomerization into vitamin D3 (Holick 2017; Holick 2003). This metabolite of vitamin D undergoes further modifications to acquire biological activity. Firstly, vitamin D3 is converted to 25-hydroxyvitamin D3 (25(OH)D3) by hydroxylation of carbon 25 by 25-hydroxylase. This step of hydroxylation occurs in the liver and is catalyzed by various mitochondrial and microsomal isoforms of cytochrome P450 (CYP) (Prosser and Jones 2004). Since the 25(OH)D3 is the main metabolite of vitamin D present in the human blood, with a half-life of 2 weeks and a proportional increase along with vitamin D intake, plasma levels of this metabolite are widely used as an indicator of vitamin D status. The concentration of 25(OH)D3 in the serum of a healthy human should be in a range of 30–50 ng/ml. Second hydroxylation of vitamin D takes place in the kidney and leads to the generation of the bioactive metabolite, 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) (Dusso et al. 2005). The key enzyme responsible for this modification is a 1-α-hydroxylase (CYP27B1), which is expressed mainly in proximal renal tubules, but may be also found in other

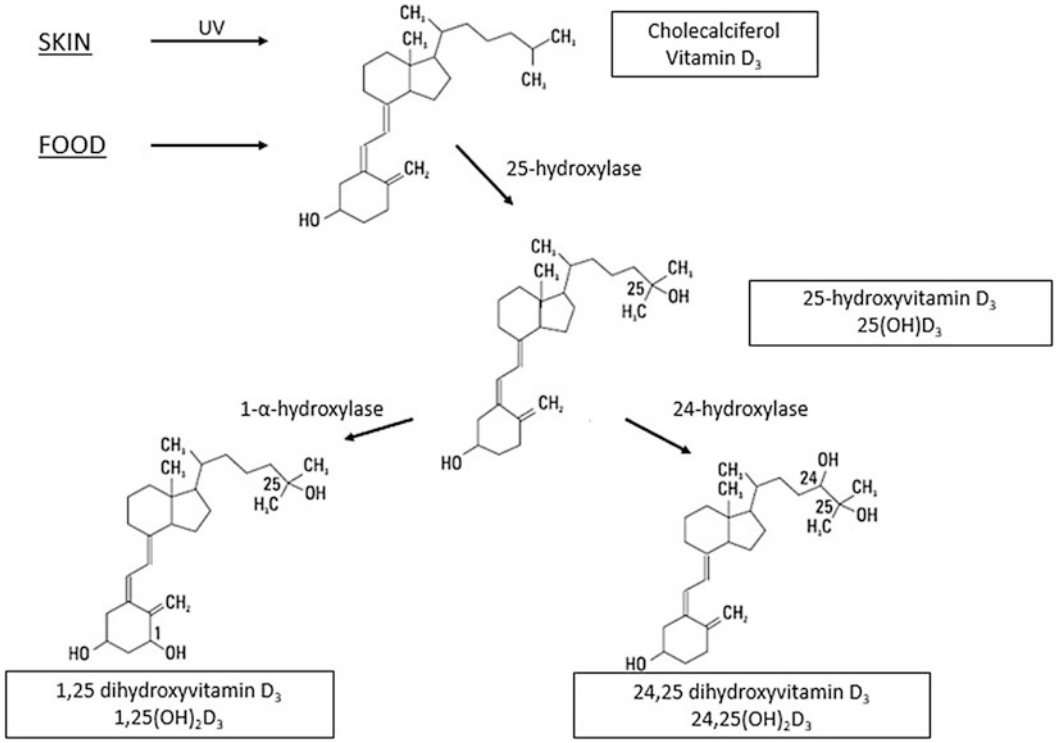

Fig. 1 Metabolism of vitamin D

tissues. Renal 1-α-hydroxylase activity is tightly regulated by the parathyroid hormone (PTH) and a phosphaturic hormone, fibroblast growth factor 23 (FGF-23).

Importantly, concentration of 1,25(OH)2D3 is strictly controlled by a renal negative feedback loop. A high level of 1,25(OH)2D3 inhibits 1-α-hydroxylase and FGF-23 followed by the induction of 24-hydroxylase (CYP24A1). This enzyme is responsible for a conversion of 1,25(OH)2D3 into the inactive form, 24,25-dihydroxyvitamin D3 (24,25(OH)2D3), that is secreted into the bile. Notably, 1-α-hydroxylase may be expressed by various cell types including the immune cells and epithelia of many tissues, bone, or parathyroid glands. Therefore, those cells are capable of metabolizing inactive 25(OH)D3 into the active form in an autocrine or paracrine manner. This phenomenon enables the immune cells, such as macrophages and dendritic cells, to sustain a level of 1,25(OH)2D3 required for the proper functioning of the immune system, even though they lack a feedback mechanism (Prietl et al. 2013).

3 Mechanism of Action

In order to be transported to the tissues, after synthesis in the kidney, 1,25(OH)2D3 forms a complex with a vitamin D-binding protein (DBP) that is essential to sustain the total amount of vitamin D in the body. The mechanism of action of vitamin D is mediated by the interaction with high-affinity VDR. VDR is a transcription factor, a member of the steroid hormone nuclear receptor family, present mainly in the cell nucleus. The formation of vitamin D-VDR complex results in gene expression at the transcriptional level. VDR acts through heterodimerization with the retinoid X receptors and the subsequent binding to vitamin D response elements (VDRE), located in the promoter region of vitamin D-responsive genes. Depending on the target gene, VDR-interacting nuclear proteins are recruited to the complex either to induce or to repress gene transcription (Rachez and Freedman 2000).

Non-classical actions of vitamin D can be categorized into three regulatory effects: hormone secretion, immune function, cellular proliferation and differentiation. In this review we focus on the influence of vitamin D on the immune system's cells.

4 Vitamin D in the Regulation of Immune Cells

The interest in vitamin D as a modulator of the immune response has arisen over three decades ago when the first reports suggested the influence of 1,25(OH)2D3 on immune cells. Extensive studies of recent years have brought us closer to the understanding of the role of vitamin D in immune system function. Currently, it is accepted that vitamin D can boost the first line of defense against invading pathogens and suppress adaptive immune response, protecting from various autoimmune diseases and limiting graft rejection (Bikle 2009). Due to still growing interest in this phenomenon, we herein summarize the current knowledge concerning vitamin D influence on immune cells.

It has been described that VDR is expressed in most immune cells, including T and B cells, monocytes, and antigen-presenting cells (APC) such as macrophages and dendritic cells (Baeke et al. 2010a; Provvedini et al. 1983). Moreover, immune cells also express the 1-α-hydroxylase enzyme that hydroxylates vitamin D into the active 1,25(OH)2D3 form (Fig. 2).

4.1 Vitamin D Influence on Immune Cells: Innate Immunity

It has been reported that the function of 1,25(OH)2D3-VDR is tightly linked to the development of innate and adaptive immunity against various types of infections. An ample body of literature supports the notion that recurring infections are often related to vitamin D deficiency. Innate immunity is considered as the first line of defense against invading pathogens. The cells engaged in the innate response include

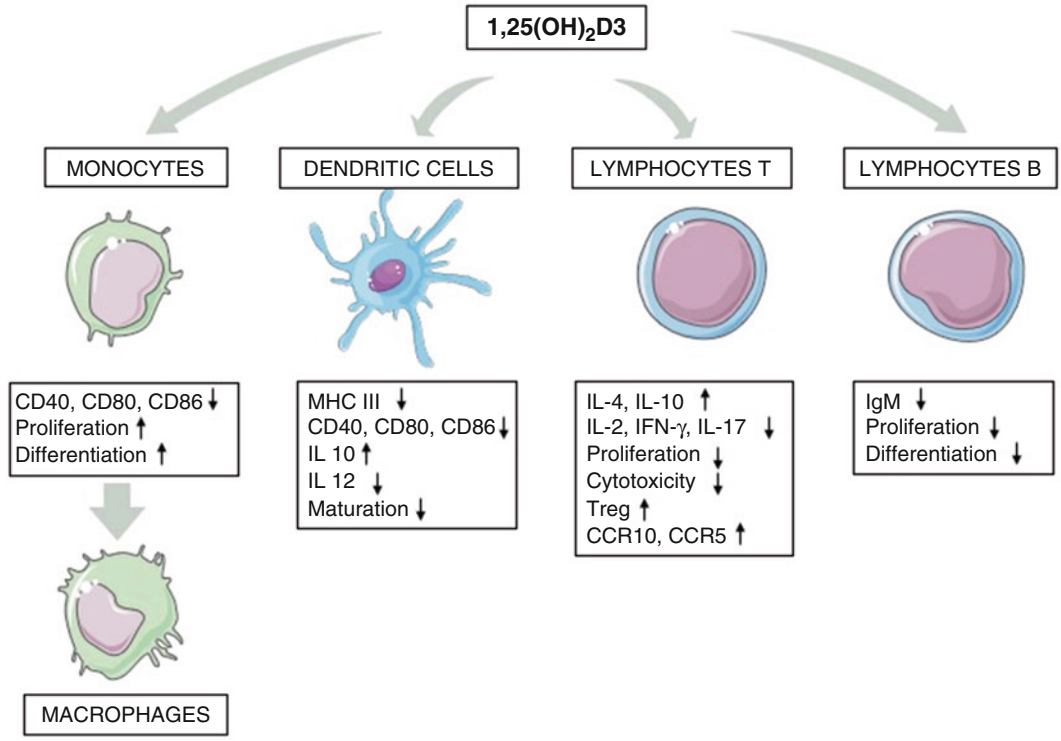

Fig. 2 Effects of 1,25(OH)2D3 on immune cells

phagocytic cells (monocytes, macrophages, and neutrophils), natural killer (NK) cells, and other cells releasing inflammatory mediators (basophils, eosinophils, or mast cells). During the development of an innate immune response, neutrophils and macrophages play an essential role in the elimination of microbes using various mechanisms, including the production of reactive oxygen species (ROS). In addition, this frontline protection is supported by NK cells guarding against infections and tumors (Delves and Roitt 2000).

4.1.1 Monocytes/Macrophages

Monocytes and macrophages are phagocytic cells that comprise the key element of the innate arm of the immune response against pathogens. They act through the recognition of pathogen-associated molecular patterns by specific receptors (Taylor et al. 2005). In the 1980s, Rook et al. (1986) have reported the vital role of vitamin D in the innate immune response. These researchers have shown that 1,25(OH)2D3 is able to inhibit proliferation of the *M. tuberculosis* in human monocytes. Further studies have revealed that 1-α-hydroxylase is induced in human monocytes upon stimulation with the immunogen related to *M. tuberculosis*, whereas Liu et al. (2006) have pointed that the proper function of the innate immune cells is strictly associated with a local synthesis of 1,25(OH)2D3 by monocytes. Interestingly, gene array studies show that the expression of 1-α-hydroxylase and VDR is increased upon activation of Toll-like receptor (TLR) 1 and TLR 2, the pathogen recognition receptors activated by intracellular bacteria. Further, 1,25(OH)2D3 causes changes in the expression of VDR target genes in the TLR-activated macrophages. Under such conditions cathelicidin, an antimicrobial peptide causing intracellular elimination of bacteria, is upregulated. Notably, the elevated cathelicidin level, leading to enhanced *M. tuberculosis* killing, is solely a result of increased levels of 25(OH)D3 (Liu et al. 2006).

These data indicate that changes in vitamin D status have a great influence on the innate immune response to infection of monocytes and that vitamin D deficiency can be a reason for impaired antibacterial activity (Hewison 2012). On the other hand, vitamin D also inhibits expression of TLR2 and TLR4 on monocytes. In most cases this effect is noticeable after 72 h, preventing the excessive TLR activation and progress of inflammation in the late phase of infection (Sadeghi et al. 2006).

As 1,25(OH)2D3 influences the intracellular microbial killing, it has also been suggested that this active metabolite of vitamin D may be involved in the induction of the process of autophagy. Indeed, monocytes treated with 1,25(OH)2D3 reveal a high level of autophagy (Yuk et al. 2009). This membrane-trafficking mechanism is normally responsible for the degradation of cytoplasmic constituents into a lysosome entity in order to sustain cytoplasmic homeostasis (Levine and Yuan 2005). However, double-membrane structures called autophagosomes, formed during autophagy, are also responsible for the isolation of a pathogen. Subsequently, autophagosomes fuse with lysosomes containing digestive enzymes, leading to the elimination of invaders (Maculins et al. 2016).

It is well established that 1,25(OH)2D3 causes monocytic differentiation of leukemic cell lines, peripheral blood monocytes, and bone marrow-derived macrophages (BMDM). This hormone enhances differentiation, maturation, and function of macrophages. Further, macrophages isolated from animals kept under vitamin D3-deficient diet reveal impaired phagocytic ability, reduced chemotaxis and ROS production, decreased ability to release cytokines and to respond to inflammatory stimuli, as well as an abrogated tumoricidal activity. In addition, BMDM from vitamin D-deficient mice reveal lower expression of macrophage-specific antigens, which can be enhanced by an *in vitro* addition of 1,25(OH)2D3, which indicates that 1,25(OH)2D3 is a key player in macrophage maturation (Abu-Amer and Bar-Shavit 1993; Gavison and Bar-Shavit 1989). Interestingly, it has been noticed that macrophages isolated from patients with sarcoidosis or tuberculosis express a high level of 1-α-hydroxylase that is responsible for the transformation of 25(OH)D3 into the 1,25(OH)2D3. Therefore, it has been suggested that hypercalcemia in such patients is caused by the nonrenal production of vitamin D (Adams and Gacad 1985). It is worth mentioning that benzo[a]pyrene (BaP), a main product of cigarette combustion, induces the expression of cytochrome P450, specifically 24-hydroxylase, in human monocyte-derived THP-1 and U937 cells, leading to the generation of inactive 24(OH)2D3. This is a mechanism that may impair the immune response against invading pathogens in patients smoking cigarettes (Matsunawa et al. 2009).

4.1.2 Neutrophils

Neutrophils are the leading cells recruited from the circulation to the site of infection by bacterial-derived stimulators, cytokines, and a chemokine gradient. Neutrophils eliminate microbes through various mechanisms, including phagocytosis, production of ROS, and formation and degranulation of neutrophil extracellular traps (NETs) (Manda-Handzlik and Demkow 2015; Manda et al. 2014). There is evidence that neutrophils, like other immune cells, express functional VDR, at a comparable level to monocytes. It has also been shown that neutrophils isolated from animals fed with vitamin D-deficient chow reveal a reduced migration ability. This observation has been confirmed by pretreatment of HL-60-derived granulocyte-like cells with 100 nM of 1,25(OH)2D3, which leads to diminished migration (Hoe et al. 2016). Thus, it has been suggested that 1,25(OH)2D3 increases the antimicrobial activity of neutrophils. A recent study of Subramanian et al. (2017) highlights a significantly greater power of killing of *S. pneumoniae* by 1,25(OH)2D3-stimulated neutrophils, compared to that in untreated controls. Moreover, 1,25(OH)2D3 can also influence the inflammatory process by modulation of neutrophil gene expression. There are reports showing that 1,25(OH)2D3 can induce the expression of

genes such as cathelicidin and defensin 2 by neutrophils, as well as inhibits the expression of the trappin 2/lafin/SKALP and interleukin-1β in liposaccharide-stimulated neutrophils (Takahashi et al. 2002). The influence of vitamin D on neutrophils has been also studied in the context of NETs formation. NETs are unique structures composed of DNA and antimicrobial proteins released by activated neutrophils. NETs constitute a key mechanism of protection against pathogens, but on the other hand, they can also participate in the pathogenesis of autoimmune diseases, inflammatory disorders, and cancer. Systemic lupus erythematosus (SLE), a complex autoimmune disease, is thought to be associated with the imbalance between the formation and clearance of NETs (Yu and Su 2013). Importantly, the level of 25(OH)D3 in SLE patients, particularly those with active disease and antinuclear antibodies, is significantly lower than that in healthy controls (Chen et al. 2007). Studies with neutrophils subjected to various doses of 1,25(OH)2D3 show that this active metabolite of vitamin D inhibits damage to endothelial cells by reducing the NETs formation in SLE patients (Singgih Wahono et al. 2017).

4.1.3 Natural Killer (NK) Cells

In order to trigger death of a target cell, NK cells use various mechanisms such as granule exocytosis, cytokines, and Fas-mediated apoptosis. Similarly to monocytes and neutrophils, NK cells express VDR and hydroxylases and secrete antimicrobial peptides such as α-defensin and cathelicidin (Hock 2014). This implies that 1,25(OH)2D3 has to be involved in the development of NK cells, as observed in patients with diabetes mellitus (Joshi et al. 2014), and in their maturation, differentiation, and cytotoxicity, as observed in patients on hemodialysis (Quesada et al. 1995). The issue is contentious as there are several reports describing the inhibitory effects of 1,25(OH)2D3 on NK cell activation, cytotoxicity, and IL-8 secretion (Leung 1989). Nonetheless, vitamin D exerts the overall beneficial effects in the control of diabetes (Issa 2017).

4.1.4 Cells Releasing Inflammatory Mediators

Eosinophils/Basophils There are limited data concerning a direct effect of vitamin D on eosinophils, the major effector cell in allergic inflammation. So far, there are reports that eosinophils, similarly to other immune cells, express VDR. The expression of this receptor is upregulated by exposure to 1,25(OH)2D3, as shown in the human eosinophilic cell line EoL-1. Importantly, treatment with 1,25(OH)2D3 prolongs cell survival and upregulates the eosinophil surface expression of CXCR4, an inhibitory chemokine receptor. The 1,25(OH)2D3 reverses the inhibitory effect of IL-5 on the eosinophil migration capacity (Hiraguchi et al. 2012). It also inhibits eosinophil necrosis and release of cytotoxic granules (Ethier et al. 2016). Lu et al. (2017) have shown that eosinophil VDR-deficient mice undergo spontaneous activation in the intestinal mucosa. These authors argue that VDR is responsible for the homeostasis of eosinophils by regulating the gene transcription. Further, there are studies on the association between the serum vitamin D level and the blood absolute eosinophil count. Patients with severe vitamin D deficiency display a significant increase in the eosinophil count compared to the patients with deficient, insufficient, and normal vitamin D levels.

Mast Cells There are reports showing that both mouse and human mast cells can convert 25(OH)D3 to 1,25(OH)2D3 via catalytic activity of 1-α-hydroxylase. Therefore, a direct effect of 1,25(OH)2D3 on mast cells is driven through 1-α-hydroxylase and VDR. Exposure of mast cells to 1,25(OH)2D3 in *in vitro* conditions leads to an increase in VDR expression. To date, experimental data demonstrate that vitamin D is required to maintain stability of mast cells. Deficiency of vitamin D results in mast cell activation, whereas 1,25(OH)2D3 represses IgE-dependent mast cell activation and production of pro-inflammatory and vasodilatory mediators. Vitamin D3 metabolites applied epicutaneously

significantly reduce the magnitude of skin swelling associated with IgE-mediated passive cutaneous anaphylaxis reactions *in vivo* (Liu et al. 2017; Yip et al. 2014).

Dendritic Cells (DCs) DCs are considered the most effectively susceptible to the immunomodulatory effect of vitamin D. These cells reveal a unique ability to induce both innate and adaptive immune responses as well as to provide a balance between immunological tolerance and activation of immune response (Banchereau et al. 2000). Several reports describe the influence of 1,25(OH)2D3 on the function and morphology of DCs. The 1,25(OH)2D3 inhibits maturation and differentiation of DCs from monocytic precursors through the interaction with VDR (Hewison et al. 2003). Further, 1,25(OH)2D3 induces the expression of molecules engaged in the antigen uptake and processing in differentiating monocytes. Interestingly, immature mouse and human DCs subjected to 1,25(OH)2D3 treatment show a tolerogenic phenotype characterized by lower expression of DC-specific surface markers, i.e., histocompatibility complex (MHC) molecules class II, CD1a, CD80, and CD40 co-stimulatory molecules (Canning et al. 2001). The 1,25(OH)2D3 also modulates the expression and secretion of cytokines and chemokines in monocyte-derived DCs. For instance, the secretion of IL-12 and IL-23 as well as TNF-α and IFN-γ (engaged in the differentiation of Th1 and Th17) is inhibited, whereas the production of anti-inflammatory IL-10 is stimulated (Penna et al. 2007; Willheim et al. 1999). Importantly, DCs play a significant role in the development of Th2 immune responses to allergens and Th1 responses involved in the pathogenesis of organ-specific autoimmune disorders. The 1,25(OH)2D3-mediated arrest of DCs in immature state results in retention of the antigen uptake and decreases CD4+ T-cell activation in humans, which is an essential contributor to suppression of allergic and autoimmune reactions, attributed to 1,25(OH)2D3-induced tolerogenic DCs.

5 Vitamin D Influence on Immune Cells: Adaptive Immunity

The adaptive immune response is based on highly specialized T and B lymphocytes able to specifically recognize antigens. B lymphocytes compose humoral immunity responsible for the production of antibodies leading to the elimination of extracellular microorganisms. T lymphocytes are components of the cell-mediated immune response responsible for helping B cells to secrete antibodies, eradicate pathogens, activate macrophages, and kill infected cells, as well as for immunosuppression. The immunomodulatory role of vitamin D on the adaptive arm of immune system depends on its direct effect on cell proliferation, differentiation, as well as apoptosis of T and B lymphocytes.

5.1 B Lymphocytes

Former studies show that resting B lymphocytes express VDR at low levels; however, the expression can be upregulated during activation. Moreover, Chen et al. (2007) have shown that both 1,25(OH)2D3 and activating signals regulate VDR expression in B cells. Vitamin D may exert opposite effects on activated and resting B cells. Recent studies have reported that B cells also express proteins involved in vitamin D modification into its metabolites, including 1-α-hydroxylase and 24-hydroxylase, which indicates that B cells are capable of autocrine/intracrine responses to 1,25(OH)2D3. Further, 1,25(OH)2D3 inhibits proliferation and differentiation of plasma cells and post-switch memory B cells. However, data concerning 1,25(OH)2D3 action in B cells are contentious. On the one hand, 1,25(OH)2D3 may indirectly inhibit function of B cell as a result of alterations in CD4 T-cell responses or suppression of cytokine secretion by monocytes/macrophages (Muller et al. 1991). On the other hand, there are reports that 1,25(OH)2D3 directly affects human B cells inhibiting IgE production (Heine et al. 2002).

Further, expression of 24-hydroxylase in B cells is upregulated by 25(OH)2D3, but it is unaffected by activation of these cells. In contrast, 1-α-hydroxylase, which is also expressed in B cells, can be further induced by activating signals, but not by 1,25(OH)2D3 (Chen et al. 2007). Thus, these results indicate that B cells hydroxylate 25(OH)D3 by themselves into 1,25(OH)2D3 and become a reservoir for the extra renal 1,25(OH)2D3, similarly to macrophages and dendritic cells (Overbergh et al. 2000; Hewison et al. 2003). Interestingly, it has been demonstrated that 1,25(OH)2D3 inhibits the generation of plasma cells and post-switch memory B cells, without much affecting B-cell differentiation Thus, 1,25 (OH)2D3 therapy may be of potential benefit in maintaining B-cell homeostasis in autoimmune diseases, such as SLE, where the activated B cells proliferate.

5.2 T Lymphocytes

T lymphocytes can be divided into two groups. The first one consists of cytotoxic T (Tc) cells expressing CD8 antigen, and the second is composed of T helper (Th) cells characterized by the presence of CD4 antigen. Among Th cells, currently, we can distinguish five subpopulations: Th1, Th2, Th9, Th17, and Th22 (Boyse and Cantor 1977). Th1 cells secrete interleukin (IL)-2 and interferon (IFN)-γ and participate in the cell-type response directed against intracellular pathogens, while Th2 cells secrete IL-4, IL-5, IL-10, and IL-13 and are responsible for supporting the humoral response, associated with the synthesis of immunoglobulins and with the accumulation of eosinophils (Romagnani 2000). Therefore, adequate immune response is dependent on the balance between Th1 and Th2 cells (Berger 2000). Importantly, distorted balance may get Th1-dominated responses involved with the pathogenesis of organ-specific autoimmune disorders or Th2-switched responses involved with the initiation of the allergic cascade (Romagnani 1992). Studies suggesting the influence of vitamin D on T cells show that these cells express VDR and 1-α-hydroxylase at a low level, but the expression may be enhanced upon activation (Provvedini et al. 1983). Baeke et al. (2010a) have confirmed that T cells are a direct target for 1,25(OH)2D3. Further, expression of VDR and 1-α-hydroxylase increases in T cells subjected to the stimulators anti-CD3/anti-CD28, lectin, phytohemagglutinin, or ionomycin. Incubation of T cells with 1,25(OH)2D3 effectively enhances VDR signaling, which is confirmed by the induction of 24-hydroxylase, along with suppression of cytokine release. These observations are in line with other studies showing that 1,25(OH)2D3 leads to the inhibition of Th1 cell proliferation and differentiation and modulates their cytokine production by reducing the expression of pro-inflammatory IL-2, interferon-γ, and tumor necrosis factor-α (Lemire et al. 1995). On the other side, 1,25(OH)2D3 stimulates the formation of Th2 cells with upregulation of a specific transcription factor and production of anti-inflammatory Th2 cytokines. Recent studies show that *in vitro* treatment with 1,25(OH)2D3 suppresses the development of Th17 effector cells and inhibits the production of IL-17 (Chang et al. 2010). The 1,25(OH)2D3 exerts effects not only on effector T cells but also induces proliferation of regulatory T cells, known to inhibit pro-inflammatory responses of other immune cells, and this may be beneficial in autoimmune disease (Kalicki et al. 2017).

6 Conclusion

Increasing evidence indicates that vitamin D3, except for participation in calcium and bone metabolism, is an important regulatory factor for immunity. Current literature, based on numerous studies done on animal and human models, as well as genetic and epidemiological data, emphasizes the substantial role of vitamin D in maintaining immunological homeostasis. In particular, 1,25(OH)2D3, an active metabolite of vitamin D, exerts a multidirectional effect on both innate and adaptive arms of the immune system. This hormone is responsible for increasing the phagocytic and microbicidal activity of macrophages, for inhibiting the differentiation

and maturation of dendritic cells and B lymphocytes, and for suppressing Th1 and Th17 proliferation and their cytokine production. Current recommendations regarding vitamin D3 supplementation are based on bone-related studies and do not concern requirements for the properly functioning immune system. As the majority of immune cells express the VDR and vitamin D-activating enzymes, they are not only targets for 1,25(OH)2D3 but also are able to activate this vitamin locally. In addition, the issue is repeatedly brought up that an inadequate vitamin D level is related to immune abnormalities. Therefore, due to the immunomodulatory properties of vitamin D, there is growing attention to introduce this hormone or its analogs into the clinical setting for the prevention or treatment of autoimmune disorders and infections.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Cell Activation and Cytokine Release Ex Vivo: Estimation of Reproducibility of the Whole-Blood Assay with Fresh Human Blood

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Abstract

The whole-blood assay (WBA) with human fresh blood may provide insight into the features of an individual's innate immunity. To assess this, ex vivo cytokine release is measured after stimulation of whole blood with various stimuli, for instance, endotoxin in vitro. The aim of the present study was to evaluate WBA reproducibility with fresh blood using different calculation models. The blood was collected from 16 healthy volunteers on 6 different days. Ex vivo stimulation was performed in each individual's blood sample for 22 h, using different endotoxin concentrations. Interleukin (IL)-1β and IL-8 release were quantified using specific immunoassays in the cell-free supernatant. We found that a dose-response relationship between endotoxin and cytokine concentration could be verified for all blood donors in all tests. The median coefficient of variation of the repeated tests was 29% for IL-1β and 52% for IL-8. Upon stimulation with 40 pg/mL endotoxin, a confidence interval of 60–140% was calculated for IL-1β and 70–271% for IL-8 regarding test reproducibility. Furthermore, the classification into high or low responder was reproducible. We conclude that repeated blood collection offers an opportunity to evaluate the variability of WBA. Considering a high intragroup variability, an individual range assessment has been suggested to evaluate exposure effects.

Keywords

Cytokine release · Endotoxin · Pyrogenic effects · Test reproducibility · Whole-blood assay

1 Introduction

Human monocytes release cytokines, for instance, interleukin (IL)-1β and IL-8, in response to pathogen-associated molecular patterns, such as endotoxins. This feature is a basis of the whole-blood assay (WBA), which can be performed with fresh or cryopreserved human blood

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(Schindler et al. 2004, 2006; Kindinger et al. 2005; Wouters et al. 2002), to measure the pyrogenic activity of a sample, caused by an endotoxin or other pyrogenic contents, such as glucans or Gram-positive bacteria. Zucker et al. (2006) have established the WBA using fresh human blood as a replacement for the Limulus amebocyte lysate test for the endotoxin measurement. They have described the endotoxin activity of 17 bioaerosol samples from a duck-fattening unit using the test, as well as IL-1β release (with the WBA) into the whole blood of ten non-exposed volunteers. They have reported a significant correlation between the tests and suggested the use of WBA as a tool to characterize the inflammation-inducing potential of bioaerosols. However, performing the WBA using fresh human blood may be especially suitable to get information about a blood donor's individual immune reaction instead of information on the sample investigated (Liebers et al. 2009, 2012; Wouters et al. 2002).

Since cryopreserved blood is used as a standardized tool for WBA, the measured values should be the preferred choice to characterize the pyrogenic or pro-inflammatory activity of a dust sample (Punsmann et al. 2013; Liebers et al. 2012). Quantitative and qualitative measurements of the cytokine profile using standardized cryopreserved blood may be indicative of the immunogenic relevance of a dust sample and may therefore help to describe the environmental or occupational conditions under which it was collected. In contrast, the effect of different exposure environments on the individual may be monitored by measuring several effect parameters, such as symptoms, body temperature, blood inflammation markers, and lung function of the exposed subjects (Monsé et al. 2018). Concerning the innate immunity, individual's ex vivo cytokine release with fresh blood in response to endotoxin exposure may provide further information on exposure effects. However, there is so far little knowledge on the stability of cytokine release from a single individual, and if the reproducibility of this bioassay is not defined, evaluation of effects will be difficult. The variability of a bioassay has to be differentiated from definite changes, for instance, due to certain exposure settings.

Regarding cytokine release from monocytes of individuals, several factors have to be considered. These include features of the blood donor, for instance, environmental and genetic background (Singh and Schwartz 2005) that may contribute to variation of the results (Wouters et al. 2002). Effects can only be verified in comparison to a normal range. However, the "normal" range of ex vivo cytokine release can be defined from different points of view. Variability or rather reproducibility can be presented differently, for instance, as a percent of a given test or using the median, absolute or percent deviation, confidence intervals, and the slope of dose-response relationships.

The aim of the study was to evaluate the reproducibility of results obtained with the WBA if fresh human blood was collected from the same individual and stimulated repeatedly. Therefore, blood samples from 16 healthy volunteers were collected six times, stimulated with different concentrations of endotoxin, and the release of IL-1β and IL-8 measured.

2 Methods

2.1 Study Group

The study was approved by the Ethics Committee of the Ruhr-Universität Bochum (No. 4929–14) and was conducted in accordance with the Declaration of Helsinki. All study participants gave written informed consent to the study protocol. Heparinized blood was drawn from eight females and eight males, aged 19–42 (median 26 years). Participants were non-smokers and without any known health-related complaints. Median total IgE was 31 kU/L. Blood was drawn on 6 different days, with at least a 7-day interval between venipunctures, but not exceeding a maximum of 132 days (median 20 days). The WBA was initiated within 3 h after venipuncture which was performed between 8 and 10 a.m.

2.2 Human Whole-Blood Assay

The WBA is a two-step assay. Step 1: blood is incubated for 22 h with or without endotoxin. Step 2: cytokines in the supernatant are measured using a specific cytokine ELISA. Fresh whole blood was incubated with five different concentrations (1, 10, 40, 100, and 1000 pg/mL) of endotoxin (*E. coli*; Haemochrom Diagnostica, Essen, Germany, CSE *E. coli* O113:H10) as well as with the medium without endotoxin (RPMI 1640 supplemented with glutamin and Hepes (Gibco; Thermo Fisher Scientific, Darmstadt, Germany)). Incubation was performed in a total volume of 1 mL for 22 h at 37 °C (800 μL RPMI +100 μL endotoxin +100 μL blood). After centrifugation (2 min at 10,000 × *g*), supernatants of fresh blood were frozen at −70 °C until analysis. Total cell count and a differential cell profile were performed in EDTA blood according to a standard protocol.

2.3 Measurement of Cytokines

To quantify the released cytokines in the cell-free supernatant, IL-1β and IL-8 were measured using monoclonal "sandwich" enzyme immunoassay kits (IL-1β, DuoSet™; R&D Systems, Wiesbaden, Germany; IL-8, Becton Dickinson, Heidelberg, Germany) with a sensitivity range of 3.9–250 pg/mL for IL-1β and 3.15-200 pg/mL for IL-8, according to the manufacturers' recommendations. All samples were measured in two to three different dilutions, and results were accepted if the coefficient of variation (CV) was below 25%; otherwise the measurements were repeated.

2.4 Data Analysis

A total of 576 samples (6 blood samples from 16 subjects, stimulated with 6 different endotoxin concentrations) were analyzed with respect to IL-1β and IL-8. The median, CV, and other percentiles were used to describe the results. One-sided ANOVA and linear regression models were performed to test differences between cytokine releases after different endotoxin stimulations. Spearman's correlation coefficients (r) were calculated to predict the monotone association between parameters. The problem of multiple comparisons was tackled using the Bonferroni correction, by dividing the overall desired statistical significance level α by the number of hypotheses tested. Confidence intervals of repeated cytokine measurement were calculated, defining the first measurement as 100%. Rank-order tables were developed to give a further estimate of variability. The classification into high and low responders was based on the strength of cytokine release. For each WBA and each endotoxin concentration used for stimulation, a ranking position was identified for each subject. The sum of rankings for each blood donor defines the individual's classification. High and low responders were divided according to the median ranking position.

Descriptive analysis was performed for each variable stratified by the endotoxin. We proposed specific confidence levels for IL-1β and IL-8 with median ± double median absolute deviation (MAD) (Hampel 1974) for each individual subject and different endotoxin concentrations. Values above or below these intervals will be interpreted as probable changes of the immune reaction. Calculations were performed with a commercial statistical package of SAS v9.4 (SAS Institute Inc., Cary, NC) or GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA).

3 Results

3.1 Cellular Blood Composition and Correlations

Differential cell count was within the normal range for all subjects and at all times of blood drawing (leukocytes 2800–10,900 per μL blood). The median percentages of cells from the 16 subjects were as follows: 36.5% (range 24–46) lymphocytes, 8.5% (5.5–10) monocytes,

51.1% (40–68) neutrophils, 2.5% (1–8) eosinophils, and 1% (0–1) basophils. Without endotoxin stimulation, blood from all donors elicited IL-1β levels that were below the detection limit. After stimulation with endotoxin, a positive correlation was observed between IL-8 and IL-1β release: $r = 0.53$ for stimulation with 10 pg/mL endotoxin and $r = 0.40$ for 40 pg/mL endotoxin ($p < 0.0001$). The association between IL-8 and IL-1β release for 100 and 1000 pg/mL endotoxin was marginally positive ($r = 0.21$; $p < 0.037$). Furthermore, there was a clear positive correlation between the percentage of monocyte count and IL-1β release ($r = 0.66$, $p = 0.006$), but not IL-8 release, upon stimulation with 10 pg/mL endotoxin. No correlation between monocytes and cytokine release could be verified regarding the absolute cell count.

3.2 Dose-Response Relationship of Cytokine Release

IL-1β Release *E. coli* endotoxin induced IL-1β release from fresh blood of single donors in a dose-dependent manner as exemplified in Fig. 1a. Overall, median (n = 6 tests) IL-1β release ranged from the detection limit (<3.9 pg/mL) to 1,044 pg/mL in the 16 subjects. The maximum IL-1β release was measured after stimulation with either 100 pg/mL (n = 2) or 1,000 pg/mL endotoxin (n = 14).

The slope and range of IL-1β release were described for each endotoxin concentration, estimating a regression that included the 16 subjects and the 6 blood drawings (Table 1a). IL-1β release was significantly different in response to 1, 10, 40, 100, and 1,000 pg/mL endotoxin ($p < 0.0001$). In summary, the dose-response relationship reveals a 2.5–3-fold increase in IL-1β release in response to a fourfold (10–40 pg/mL) or 2.5-fold (40–100 pg/mL) raise in the stimulating endotoxin concentration.

IL-8 Release IL-8 release was measured in the same cell-free supernatant of fresh blood akin to IL-1β, and a dose-dependent reaction was found in response to *E. coli* endotoxin (Fig. 1b). In contrast to IL-1β, IL-8 was measured in a range of 45–525 pg/mL in the supernatant of non-stimulated blood. Overall, the median (n = 6 tests) IL-8 release ranged from 153 pg/mL without endotoxin stimulation to 6,340 pg/mL when stimulated with 1,000 pg/mL endotoxin. The maximum IL-8 release was reached after stimulation with 1,000 pg/mL endotoxin in eight subjects. However, it was found to be already at a maximum after stimulation with 100 and 40 pg/mL endotoxin in four subjects, respectively.

Estimating the regression factors, the slope and range of IL-8 release were described for each endotoxin concentration (Table 1b), and significant differences in release were measured in response to 1, 10, 40, 100, and 1,000 pg/mL endotoxin ($p < 0.0001$). In summary, the dose-response relationship revealed a 2.2-fold increase in IL-8 release in response to a fourfold raise (10–40 pg/mL) in endotoxin concentration. An increase in endotoxin concentration to 1000 pg/mL did not further increase the IL-8 release.

3.3 Intra- and Interindividual Variability of Whole-Blood Assay (WBA)

Variance of results was expressed using coefficient of variation (CV), percent (% of test 1), and ANOVA variance analysis. For IL-1β release, results of spontaneous cytokine release as well as after stimulation with 1 pg/mL endotoxin were not included in the analysis since the data were mostly at the detection limit (< 3.9 pg/mL).

IL-1β Release CV of the repeated tests for IL-1β release ranged from 14% to 54% (median 29%). If the reproducibility of IL-1β release is expressed as a percentage (test 1 = 100%), the five repetitions of test 1 were in the median range from 34% to 422% (median inter- and intraindividual 98%, which means a 2% deviation in comparison to test 1). Figure 2 shows the percentage of reproducibility with regard to

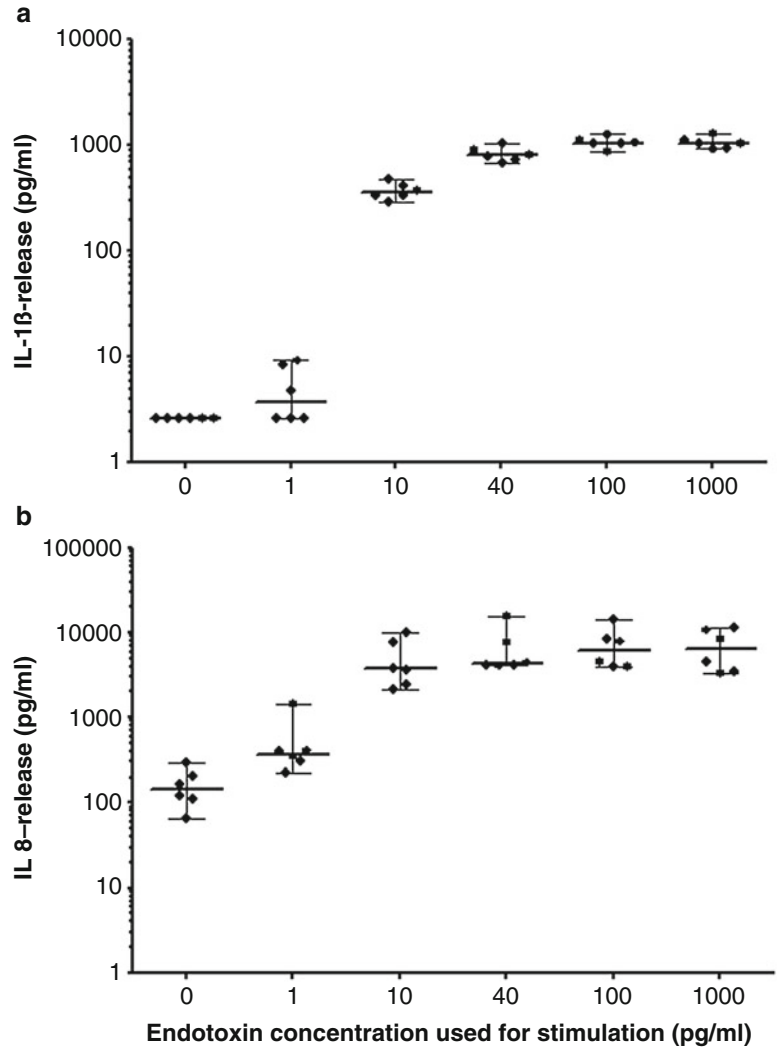

Fig. 1 Dose-response relationship between endotoxin stimulation and IL-1β release (**a**) or endotoxin stimulation and IL-8 release (**b**), as illustrated using data from subject No. 3. Median and range of whole-blood assay (WBA), where each dot represents one test result

stimulation with 10 and 40 pg/mL endotoxin. Slight differences in reproducibility were found depending on the concentration of the endotoxin stimulus, with medians between 84% and 116%. Estimating reproducibility of the test with ANOVA variance analysis resulted in a significant interindividual variance for all endotoxin concentrations, whereas intraindividual variance was not significant (Table 2).

IL-8 Release For IL-8, the median CV of the repeated tests was calculated as 54% (range 27–80%), including all endotoxin stimulations, as well as non-stimulated blood. If reproducibility of IL-8 release is expressed as a percentage (test 1 = 100%), the five repetitions of test 1 ranged from 40% to 604% (median intraindividual 132%; median interindividual 156%, indicating 32–56% deviation in comparison to test 1). The reproducibility for the different endotoxin concentrations was between 100% and 156%. No significant interindividual variance was found upon estimating reproducibility of the test

Table 1 Slope of dose-response relationship for IL-1β (**a**) and IL-8 (**b**)

Endotoxin concentration used for stimulation (pg/mL)	Factor Exp(β) (x-fold of blank)	95% CI of Exp(β)
Blank (0)	Defined as 1	
(**a**)	**IL-1β**	
1	1.30	1.15–1.47
10	47.72	42.08–54.12
40	155.93	137.49–176.85
100	240.03	211.63–272.23
1,000	281.96	248.61–319.79
(**b**)	**IL-8**	
1	1.81	1.52–2.16
10	18.07	15.20–21.48
40	39.79	33.47–47.31
100	43.08	36.23–51.23
1,000	43.44	36.53–51.65

Slopes of IL-1β release and IL-8 release described as x-fold of blank (= cytokine release without endotoxin stimulation). The blank concentration is defined as 1. A total of 6 whole-blood assays (WBA) from 16 subjects were included in the regression model. *CI* confidence interval

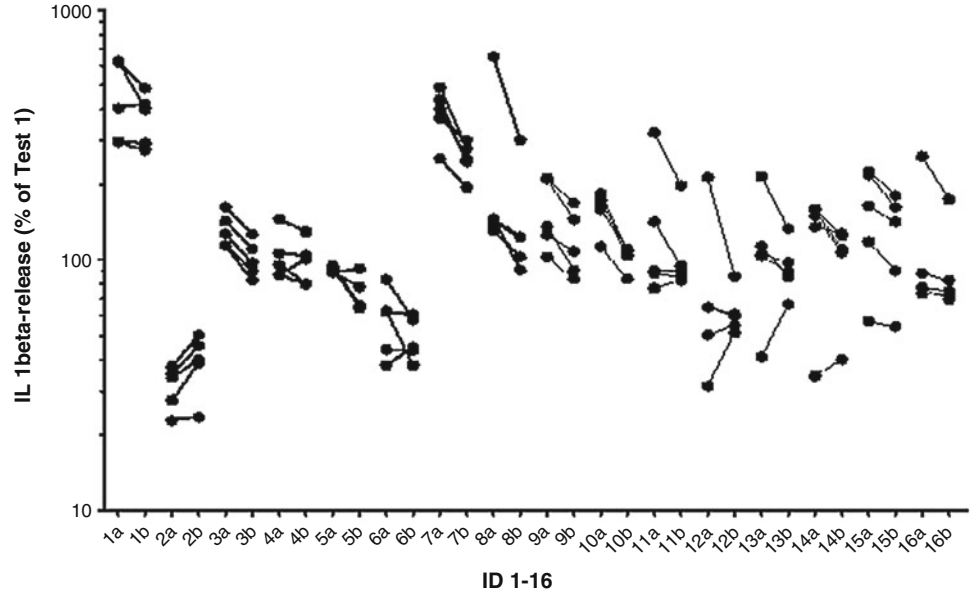

Fig. 2 Variation of IL-1β release from repeated whole-blood assays (WBA), calculated as a percentage of test 1. The median of five repetitions of test 1 ranged from 34% to 422%

with ANOVA variance analysis. In contrast to IL-1β, interindividual variance was lower for all endotoxin concentrations compared to the intraindividual variance (Table 2).

3.4 Confidence Intervals of IL-1β and IL-8 Release for Repeated WBA

Confidence intervals (CI) were calculated as a percentage (test 1 = 100%) and only after

Table 2 Intra- and interindividual variance of cytokine release

	IL-1β Individual variance		IL-8 Individual variance	
Stimulation with endotoxin (pg/mL)	Inter	Intra	Inter	Intra
0	–	–	35.9	44.1
1	33.8*	24.4	56.8	61.2
10	30.6*	19.1	21.1	37.3
40	13.7*	9.8	8.4	28.1
100	11.3*	7.2	4.8	19.1
1,000	9.6*	6.6	3.7	19.4

Estimating reproducibility of whole-blood assay (WBA) (6 tests of 16 subjects) with ANOVA analysis; *$p < 0.0005$ (F-test significant interperson variance)

stimulation with 10 and 40 pg/mL endotoxin since a distinct dose-response relationship was only detected in this range. The median IL-1β release ranged from 65% to 194% upon stimulation with 10 pg/mL endotoxin with 95% confidence interval. This range changed to 60%–140% when 40 pg/mL endotoxin was used. The median IL-8 -release ranged from 45% to 382% with 10 pg/mL endotoxin (probability of 95%) and from 70% to 271% with 40 pg/mL endotoxin.

3.5 Reproducibility of High and Low Responder Status

Subjects can be classified as high and low responders due to their cytokine release and the relative position within a group as measured with the WBA. In our study, high or low responder status was identical in 75% of the subjects with respect to IL-1β or IL-8 release. For instance, six of the eight individuals who were categorized as high responders, due to their IL-1β release, could also be categorized as high responders due to their IL-8 levels.

IL-1β Release For each whole-blood assay, subjects were ranked according to the amount of IL-1β release after stimulation with different endotoxin concentrations. Thus, a position between 1 and 16 was allocated for each condition. Accordingly, the sum of ranking position was between 4 (position 1 for all four concentrations of endotoxin) and 64 (position 16 for all four endotoxin concentrations) and was calculated for the 6 repetitions. High and low responders were divided according to the median ranking position (above or below 36). As shown in Figs. 3a and b, status of low/high responding was reproducible in 90% of the six repeated tests.

IL-8 Release Ranking of the WBA results was performed as done for IL-1β release. One difference was that results arising without endotoxin stimulation (spontaneous release) and after stimulation with 1 mg/mL endotoxin were also used for classification. Thus, the sum of ranking position was between 6 (position 1 for all six concentrations of endotoxin) and 96 (position 16 for all six endotoxin concentrations). Status of low/high responding was reproducible in 79% of the six repeated tests.

3.6 Range of Cytokine Release Using Double Median Absolute Deviation (MAD)

Individual range of cytokine release was calculated based on MAD. Plus/minus double MAD of the six repeated WBA was suggested as the normal range for each individual. Furthermore, an average normal range was calculated based on the MAD of the group.

IL-1β Double MAD of ex vivo IL-1β release for each subject representing the individual range of reactivity is shown in Fig. 4a and b. The average normal range for stimulation with 10 pg/mL endotoxin was 9–436 pg/mL IL-1β (5th and 95th percentile) or 50–304 pg/mL (25th and

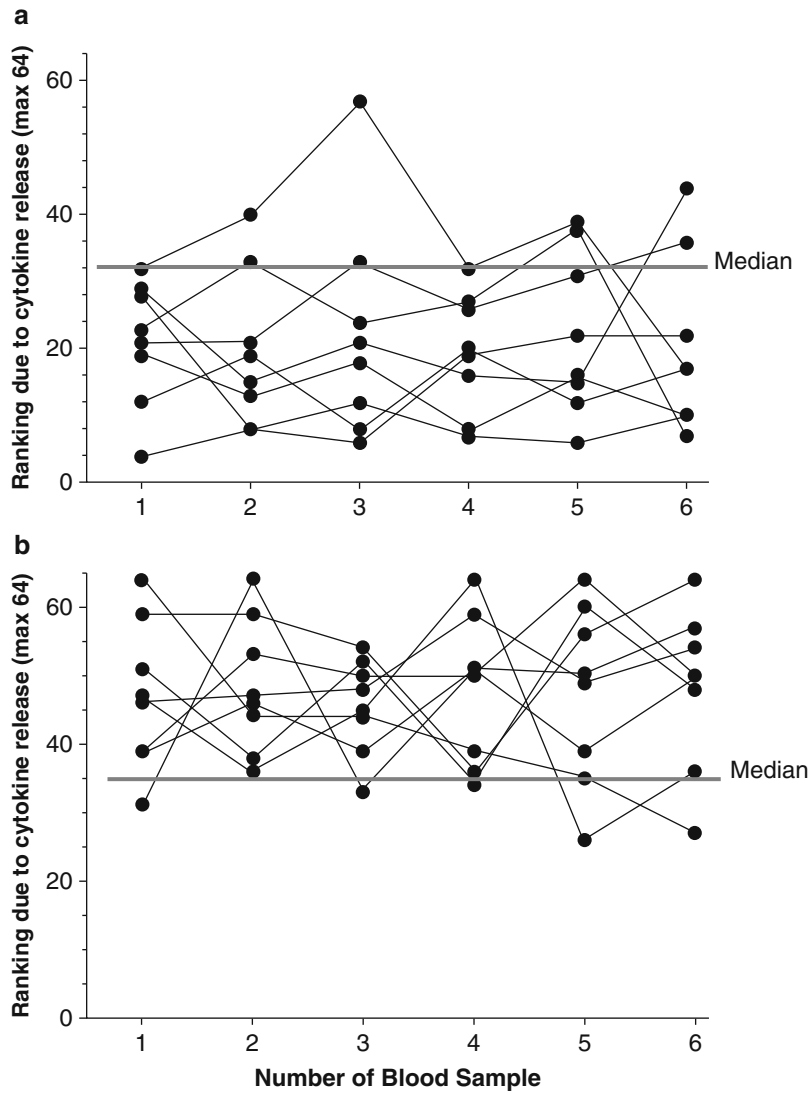

Fig. 3 A ranking position within the study group (n = 16) was allocated for each subject due to the strength of IL-1β release. Ranking was performed as a result of stimulation with 10, 40, 100, and 1,000 pg/mL endotoxin separately, and the sum of ranking was calculated. Low (**a**) and high (**3**b) responders were divided according to the median ranking position. The graph shows the respective ranking position in each of the six tests

75th percentile). The estimated average normal range for IL-1β release was 162–1,100 pg/mL (5th and 95th percentile) and 292–819 pg/mL (25th and 75th percentile) upon stimulation with 40 pg/mL endotoxin.

IL-8 Double MAD of ex vivo cytokine release was calculated for IL-8, considering each subject separately (Fig. 4c and d), as done for IL-1β. The average normal range upon stimulation with 10 pg/mL endotoxin was 0–9,630 pg/mL IL-8 (5th and 95th percentile) and 372–6,136 pg/mL (25th and 75th percentile). Upon stimulation with 40 pg/mL endotoxin, the estimated average normal range for IL-8 release was 0–24,681 pg/mL (5th and 95th percentile) and 1,291–10,861 pg/mL (25th and 75th percentile).

4 Discussion

Our data describe intra- and interindividual variability of the whole-blood assay using human fresh blood, with IL-1β and IL-8 release

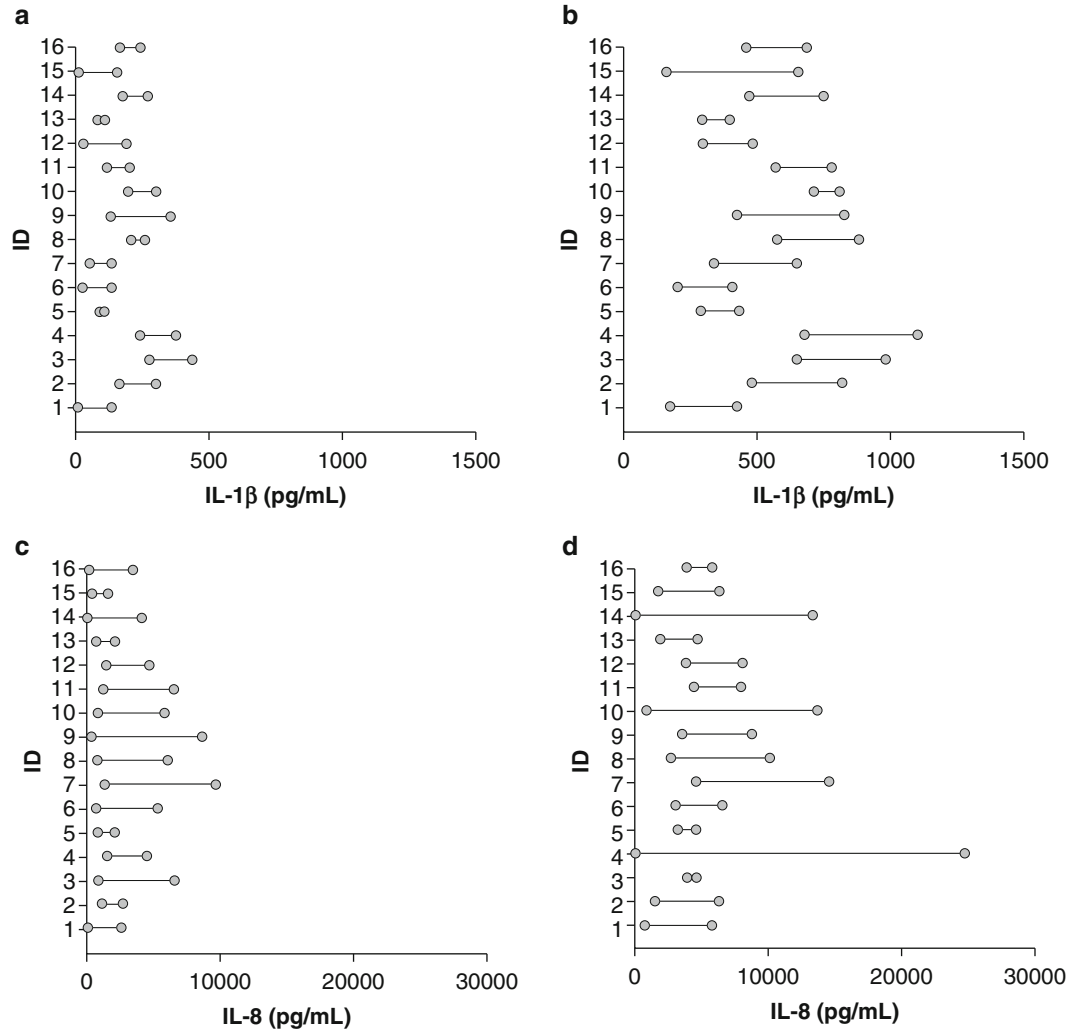

Fig. 4 Individual IL-1β (panels a and b) and IL-8 (panels c and d) release after stimulation with 10 pg/mL (panels a and c) or 40 pg/mL endotoxin (panels b and d). Double median absolute deviation (MAD) was used to calculate the individual range. The dots represent the calculated upper and lower limit of cytokine release for each individual. ID = subject number

as the measured outcome. Data are based on six repeated tests per individual in a group of 16 healthy blood volunteers. The dose-response relationship of cytokine release was a stable feature during all tests for all blood donors. IL-1β results were reproducible with a median deviation of 2%; the median coefficient of variation overall was 29%. However, even if the deviation of the median within the whole group was very low, it should be noted that in single tests, variations in cytokine release were up to fourfold. After stimulation with 40 pg/mL endotoxin, a confidence interval of 60–140% was calculated for the test repetition. Variance for IL-8 levels was markedly higher than for IL-1β, and a pronounced cytokine release was measurable even without endotoxin stimulation. Classification as low or high responder was a stable characteristic in 90% (IL-1β) or 79% (IL-8) of each donor with the six tests. Considering the differences among the

subjects regarding their reactivity to endotoxin using the whole-blood assay, individual ranges were suggested using the double median absolute deviation.

The measurement of IL-1β release was deemed more appropriate, since this pyrogenic cytokine has low background levels and its variability with the WBA is lower than that for IL-8. IL-1β measurement has already been favored in several whole-blood assay applications (Hasiwa et al. 2007; Liebers et al. 2007; Daneshian et al. 2006; Zucker et al. 2006). One reason is that no other cytokine induces fever in humans as potently as IL-1, especially IL-1β, and has therefore been the focus of immunologists for many years (Dinarello 2015). IL-1β is a powerful pro-inflammatory cytokine that is crucial for host-defense responses to infection and injury. It is produced and secreted by a variety of cell types, although in the present setting with heparinized blood, the vast majority of IL-1β will originate from monocytes (Lopez-Castejon and Brough 2011). IL-8 is also produced by monocytes and may also originate from endothelial or epithelial cells. Its main task is to regulate chemotaxis activity which helps recruit leukocytes, especially neutrophil granulocytes, to the "hot spot" of inflammation. If fresh blood is used, several variables of this biological test have to be taken into account. Composition of blood may be influenced by factors such as circadian rhythm, genetic background, and polymorphisms of molecular patterns, like TLR4 (Böttcher et al. 2004). Furthermore, age (Pinato et al. 2013), stress, sport (Clark and Mach 2016), or smoking (Mikuniya et al. 1999) may play a role. Consequently, variations in cytokine release have to be expected even if blood from the same donor is investigated.

In the present study, blood was consistently drawn between 8 and 10 a.m. in order to avoid the effect of circadian rhythm. Furthermore, only non-smokers were included, and the group was equally composed of men and women. No health complaints were reported by the blood donors, and cell counts were within normal range for each participant, thus excluding subjects with acute inflammation. However, state of stress and daily exercise, as well as diet, were not documented and may all strongly influence the gut microbiome and in turn transmitter release and systemic health (Clark and Mach 2016).

The reproducibility of WBA has been previously investigated for its use as a measure of individual susceptibility for bioaerosol-induced airway inflammation (Wouters et al. 2002). Those authors used the WBA twice to test the effect of endotoxin and curdlan as stimuli on the release of IL-6, IL-8, IL-1β, and TNF-α using blood from ten healthy volunteers. They described that WBA produced similar dose-response curves for both stimulatory agents when performed using blood from the same donor on two different occasions. Furthermore, marked differences could be verified between dose-response curves of different subjects with regard to IL-6 and IL-1β. Since interperson variance was mostly lower than interindividual variance for TNF-α and IL-8, Wouters et al. (2002) have suggested to focus on the measurements of IL-6 and IL-1β. In addition, they noted that blood should be used as soon as possible after venipuncture since they could show an increase in intraindividual variance over time. In the present study, blood was used in WBA within 3 h after venipuncture. A higher reproducibility also was observed for the measurement of IL-1β, whereas intraindividual variance exceeded interindividual variations for IL-8. In contrast to Wouters et al. (2002), we refer to a sixfold repetition of the test within a group of 16 subjects. Smit et al. (2009) have also found that intraindividual variation in IL-8 release exceeded the interindividual one and excluded this cytokine from their study. However, since IL-8 is an important module in inflammation, it may still serve as a meaningful marker once the high variability is acknowledged. For instance, Negherbon et al. (2017) have found a lower IL-8 content in asthmatic children compared to controls after stimulating whole blood with dust samples. Elisia et al. (2017) have used IL-8 ex vivo release from human blood as a powerful tool to describe the effects of age on chronic inflammation.

If the cytokine profile in fresh human blood is determined, it is recommended to consider that

subjects may be classified as high and low responders. This classification has been used in several studies (Smit et al. 2009; Brazova et al. 2005; Wurfel et al. 2005; Wouters et al. 2002). However, the definition of high and low responder is not fixed to absolute values but is defined due to the median and/or ranking within a group. In the present study, the definition of high and low responder was performed for each subject considering the different concentrations of endotoxin stimulation and the six repetitions of WBA. This strategy enables the recognition of precisely high (or low) responders. Regarding blood cell count, the percentage of monocytes in the blood of low responders was 8% vs. 11% for the high responders (median), indicating a potential relationship between the number of monocytes and cytokine release.

In accordance with Wouters et al. (2002), our data indicate that a feature of high or low responders is a stable characteristic for each individual within a period of up to 4 months. Reaction to exposure scenarios might be different in high and low responders. For instance, in a group of 412 agricultural workers, Smit et al. (2009) could show that subjects with above median responses to lipopolysaccharide in WBA had a higher prevalence of airway symptoms than low responders. Considering the high variability of this biological assay, evaluation of an average normal range of reactivity may under- or overestimate the effect of several exposure scenarios. Therefore, in the present study, we developed an individual assessment scheme by calculating the double median absolute deviation for each participant and cytokine (Fig. 4).

In conclusion, this study is to our knowledge the first to evaluate data from six repeated WBAs from one individual in a group of 16 healthy volunteers. We described variability of WBA with different calculation strategies, focusing either on the dose-response relationship, percentage of variance, low/high responder state, or median, including the double median absolute deviation. The range of cytokine release measured in this group of healthy volunteers, as well as for each individual, may serve as a basis to evaluate exposure effects in future studies.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Adverse Reactions in Antifolate-Treated Toxoplasmic Retinochoroiditis

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Abstract

This study seeks to define factors affecting the development of adverse reactions to intensive therapy of toxoplasmic retinochoroiditis with antifolate agents (pyrimethamine/sulfadoxine) and antibiotics followed by secondary antifolate prophylaxis. The study was of retrospective and observational nature. Medical files were reviewed of 551 patients suffering from ocular toxoplasmosis during 1994–2013. All patients were treated with the same protocol: 3-week intensive pyrimethamine/sulfadoxine plus antibiotic/steroid therapy. Three hundred and fourteen out of the 551 patients qualified for the subsequent 6-month long secondary antifolate prophylaxis. The type and occurrence rate of adverse reactions were taken into account. The probability of an adverse reaction during the intensive therapy phase was 33.4%. Hypertransaminasemia was the most common event observed in 24.6% of the patients, but it assumed a severe character in just 0.9%, with male gender and age over 25 years being the predisposing factors. Less common adverse effects included thrombocytopenia (8.3%), hypersensitivity skin reactions (3.0%), and abdominal pain (1.4%). The adverse effects of secondary antifolate prophylaxis, most commonly hypersensitivity skin reactions and hypertransaminasemia, followed by thrombocytopenia and abdominal pain, were observed in 4.9% of the patients. Ten of them (2.7%) had to discontinue the treatment while eight others continued with pyrimethamine alone without further adverse effects, which suggests that discontinuation of the sulfonamide decreased the propensity for adverse reactions. The treatment strategy in these patients

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differed from previous reports in that it used lower doses of pyrimethamine/sulfonamide, with no folinic acid supplementation. Nonetheless, the rate and severity of adverse events were no greater than those noticed with traditional regimens, with higher antifolate doses and folinic acid supplementation. We conclude that the dose and drug-mitigated treatment strategy we employed deserves consideration as a promising alternative to traditional treatments for ocular toxoplasmosis.

Keywords
Adverse reactions · Antifolates · Ocular toxoplasmosis · Retinochoroiditis · Risk factors · Secondary prophylaxis · Treatment

1 Introduction

Ocular toxoplasmosis is the most common infectious disease affecting the posterior eye segment. It is a relapsing-remitting disease and each inflammatory attack results in permanent retinal lesions which may impair vision. The negative effects of repeated inflammatory attacks may accumulate leading to irreversible damage of the macula area of the retina and blindness. A curative treatment for ocular toxoplasmosis has not yet been established. At present, therapy aims to minimize the effects of ongoing inflammation and to decrease the frequency of recurrences. The most effective treatment is difficult to design as there are no double-blind placebo-controlled clinical studies on large patient groups (Harrell and Carvounis 2014; Gilbert et al. 2002). Due to a potential risk of blindness, such studies cannot be conducted due to ethical issues. There are a number of available antiparasitic agents that are prescribed by clinicians who manage ocular toxoplasmosis. The 1991 survey conducted among physicians, the members of the American Uveitis Society, has shown that nine drugs or combinations thereof are used (Holland and Lewis 2002; Cantaluppi et al. 1984). This prescribing practice has not been much changed since then. The most commonly used antiparasitic agent is pyrimethamine, a folic acid antagonist and a dihydrofolate reductase inhibitor. The drug is given together with sulfonamides such as sulfadiazine, sulfadoxine, or trimethroprim/sulfamethoxazole combination, which also inhibit the sequential steps in the synthesis of tetrahydrofolic acid, with macrolide antibiotics such as spiramycin or azithromycin, and with clindamycin and atovaquone.

Prophylaxis with folinic acid during pyrimethamine therapy is prescribed to prevent myelosuppression and corticosteroids are used to reduce inflammation (Butler et al. 2013; Holland 2004; Derouin et al. 1992; Araujo et al. 1991). The arguments against a routine use of antibiotics for acute and chronic ocular toxoplasmosis include their questionable effectiveness and increased risk of adverse effects (Wakefield et al. 2011; Gilbert et al. 2002). The most frequently reported adverse effects consist of myelosuppression with the resulting thrombocytopenia and leukopenia, hypersensitivity skin reactions, including Stevens-Johnson syndrome in isolated cases, hepatotoxicity with elevated alanine aminotransferase (ALT), high serum creatinine, nausea, and abdominal pain (Balaskas et al. 2012; Wakefield et al. 2011; Bosch-Driessen et al. 2002).

This study is complementary to an earlier evaluation of short-term, intensive antifolate treatment of toxoplasmic retinochoroiditis with pyrimethamine, sulfadoxine, and antibiotics, followed by the long-term, secondary antifolate prophylaxis whose effectiveness was assessed from the rate of recurrences (Borkowski et al. 2016). The present ramification of the study consisted of describing the adverse effects and frequency of retinochoroiditis recurrence during such treatment regimen, administered without routine supplementation with folinic acid.

2 Methods

Patients described in the study were treated at the Department of Zoonoses and Tropical Diseases of Warsaw Medical University in Warsaw, Poland, during 1994–2014.

2.1 Study Design

A cohort of patients investigated in this study consisted of 551 subjects, mean age of 34 ± 15 (SD) and range of 14–83 years. There were 362 females (66%), mean age 36 ± 16 years, and 189 males, mean age 32 ± 14 years. The 551 patients received intensive 21-day treatment, which was the first phase of the study. The adverse effects that occurred during this treatment and the following 3-day interval (Days 1–24), before the commencement of secondary antifolate prophylaxis, were included in the evaluation. Three hundred and fourteen out of the 551 patients were enrolled into the secondary antifolate prophylaxis, which constituted the second phase of the study. In this latter group, adverse effects noticed from Day 25 till the end of prophylaxis were included in the evaluation. Not all the patients who received the first intensive treatment qualified for the secondary antifolate prophylaxis. Those who experienced persisting adverse reactions of skin hypersensitivity, abdominal pain, or increases in aminotransferase enzymes could not go on with the secondary antifolate prophylaxis. Patients who failed to fully comply with the medication regimen due to dementia or otherwise also were excluded. Thrombocytopenia, on the other side, usually reverted by omission of a few antifolate doses, was not considered as a reason for exclusion. However, adverse effects of long-lasting prophylaxis (see below) were assessed in all the patients who started it, regardless of whether the patient managed to complete a full course of it or not. If the patient experienced a late recurrence of retinochoroiditis during the course of prophylaxis, only the first treatment phase and the time till the recurrence were included in the evaluation. The cohort of patients above outlined did not include those who discontinued treatment for a reason other than adverse effects, immunocompromised patients, and those with autoimmune disorders, thrombocytopenia, increased aminotransferase levels, or decreased platelet (PLT) count prior to treatment.

During the treatment phase, patients were hospitalized on Days 0–3 and Days 19–21, with one follow-up outpatient visit scheduled about Day 10. Subsequently, patients visited the hospital outpatient clinic at 3 and 6 months, ending the secondary prophylaxis time. In addition, patients were seen by local ophthalmologists at least once a month for the first 6 months after treatment or as required.

2.2 Treatment

The basic 21-day treatment regimen consisted of pyrimethamine 25 mg/sulfadoxine 500 mg twice daily for the first two days, followed by once daily on Days 3–21. Spiramycin was started on Day 2 and was given at a dose of 3 m IU three times a day for 10 days, followed by azithromycin 500 mg once daily for another 6 days. Additionally, oral prednisone was started on Day 2 at varied doses that depended on the severity of exudation and the location of an inflammatory focus. Usually, the initial dose of prednisone was 40 mg taken in the morning and it was gradually tapered off over 4–6 weeks depending on the resolution of inflammation and the associated exudate. Concomitantly, ranitidine or omeprazole was started to prevent the possible gastrointestinal adverse effects of prednisone.

For the secondary antifolate prophylaxis, a pill consisting of a combination of pyrimethamine 50 mg/sulfadoxine 1000 mg was given twice a week for the following 6 months.

2.3 Laboratory Investigations

The diagnosis of toxoplasmosis was set on the basis of immunoserology tests. At the study onset, an immunofluorescence assay for IgG and an immunosorbent agglutination assay for IgM were used, which was substituted with an enzyme-linked fluorescent assay (VIDAS immunoanalyzer; bioMerieux, Marcy l'Etoile, France) as of 1999 (see details in Borkowski et al. 2016). Before starting antimicrobial therapy, the following other blood and biochemical tests were performed: complete blood cell count, serum levels of aminotransferase enzymes (AST and ALT), urea, creatinine, sodium, and potassium. Blood cell counts and serum biochemical indices were reinvestigated in the second and third weeks

of treatment. During the 6-month long secondary antifolate prophylaxis, platelet and leucocyte counts and aminotransferase tests were scheduled at monthly intervals. Blood test results were interpreted using the reference range provided by the hospital laboratory that performed the test. Thrombocytopenia was defined as a platelet count below 120,000 per μL. The aminotransferase ALT activity was measured using a kinetic method with nicotinamide adenine dinucleotide reduced (NADH) and phosphate buffer (DGKC) until August 2004, with the upper cut-off limit of 40 units/L for both genders. Thereafter, the ALT measurement was performed using a Vitros analyzer (Orto Clinical Diagnostics; Raritan, NJ), with the nom of up to 52 units/L for females and 70 units/L for males.

2.4 Statistical Evaluation

Continuous variables were presented as means ±SD and categorical variables as frequency and percentages. Comparisons of differences in frequency of adverse effects between genders and age groups were done using a Chi-square or Fisher exact test. All statistical computations were done using R 3.3.1 software.

3 Results

3.1 Adverse Effects of Intensive 21-Day Long Treatment

Overall, 637 recurrent episodes of toxoplasmic retinochoroiditis were recorded (418 in females and 219 in males), ranging from one to four *per* patient (Table 1). Adverse reactions occurred in 213 out of the 637 courses of treatment, i.e., 33.4% (95% CI 28.9–36.0). The occurrence of adverse reactions by gender and age is presented in Table 2. Adverse reactions were significantly less frequent in the youngest age group with no difference between genders. The most frequent adverse reactions consisted of the elevated ALT, decreased PLT count, hypersensitivity skin reactions, and abdominal pain (Table 3). Elevated ALT levels (Table 4) were found in 24.6% of all treated patients, but they were strongly elevated over 200 units/L in just 0.9% of patients, with the highest ALT recorded at 460 units/L. The ALT elevation of up to 100 units/L was found in 19.3% of patients. Thrombocytopenia of various intensities was seen, overall, in less than 10% of patients with toxoplasmic retinochoroiditis during the intensive 21-day long treatment phase (Table 5). Platelet counts indicative of thrombocytopenia (PLT+), ranged from 100,000 to 120,000 per μL in 3.3% of patients, 50,000–100,000 per μL in 3.3%, 25,000–50,000 per μL in 0.6%, and were below 25,000 per μL in 0.8% of patients. The lowest platelet count recorded was 12,000 per μL. Hypersensitivity skin reactions (HSR+) were observed in 19 (3%) patients (Table 6). In most cases it consisted of

Table 1 Number of toxoplasmic retinochoroiditis episodes in the study cohort

Recurrence (n)	Patients affected n (%)
1	483 (87.7)
2	52 (9.4)
3	14 (2.5)
4	2 (0.4)

Table 2 Absence (AR–) and presence (AR+) of adverse reactions during intensive treatment by gender and age

	AR (−) n (%)	AR (+) n (%)	p
Gender			
Male	143 (65.3%)	76 (34.7%)	0.688 (Chi^2)
Female	281 (67.2%)	137 (32.8%)	
Age (year)			
<25	179 (75.8%)	57 (24.2%)	0.001 (Chi^2)
25–50	179 (61.1%)	114 (38.9%)	
>50	66 (61.1%)	42 (38.9%)	

Table 3 All adverse reactions (AR) during the course of 21-day long treatment of toxoplasmic retinochoroiditis[a]

	n (%)[a]
All AR	213 (33.4%)
Elevated ALT	157 (24.6%)
Decreased PLT	53 (8.3%)
Hypersensitivity reaction	19 (3.0%)
Abdominal pain	9 (1.4%)

[a]Percentages do not sum up to a total of 32.3%, since more than one adverse reaction could occur in one patient. *ALT* alanine aminotransferase, *PLT* platelet count

Table 4 Patients with elevated aminotransferase (ALT) enzyme by gender and age during the course of 21-day long treatment of toxoplasmic retinochoroiditis

	ALT (−) n (%)	ALT (+) n (%)	p
Gender			
Males	152 (69.4)	67 (30.6)	0.015 (Chi^2)
Female	328 (78.5)	90 (21.5)	
Age (year)			
<25	201 (85.2)	35 (14.8)	0.001 (Chi^2)
25–50	204 (69.6)	89 (30.4)	
>50	75 (69.4)	33 (30.6)	

Table 5 Adverse reaction consisting of low platelet count (PLT+) by gender and age during the course of 21-day long treatment of toxoplasmic retinochoroiditis

	PLT (−) n (%)	PLT (+) n (%)	p
Gender			
Male	205 (93.6)	14 (6.4)	0.261 (Chi^2)
Female	379 (90.7)	39 (9.3)	
Age (year)			
<25	217 (91.9)	19 (8.1)	0.667 (Chi^2)
25–50	266 (90.8)	27 (9.2)	
>50	101 (93.5)	7 (6.5)	

PLT– without decrease in platelet count, *PLT*+ with decrease in platelet count

Table 6 Hypersensitivity skin reaction (HSR+) by gender and age during the course of 21-day long treatment of toxoplasmic retinochoroiditis

	HSR (−) n (%)	HSR (+) n (%)	p
Gender			
Male	218 (99.5)	1 (0.5)	0.014 (Chi^2)
Female	400 (95.7)	18 (4.3)	
Age (year)			
<25	229 (97.0)	7 (3.0)	1.000 (F)
25–50	284 (96.9)	9 (3.1)	
>50	105 (97.2)	3 (2.8)	

HSR– without skin hypersensitivity reaction, *HSR*+ with skin hypersensitivity reaction

Table 7 Abdominal pain (AP) by gender and age during the course of 21-day long treatment of toxoplasmic retinochoroiditis

	AP (−) n (%)	AP (+) n (%)	p
Gender			
Male	219 (100)	0 (0.0)	0.030 (F)
Females	409 (97.8)	9 (2.2)	
Age (year)			
<25	234 (99.2)	2 (0.8)	0.320 (F)
25–50	289 (98.6)	4 (1.4)	
>50	105 (97.2)	3 (2.8)	

mild, morbilliform eruptions. In some cases, however, these reactions assumed a more severe form of the Stevens-Johnson syndrome (Figs. 1 and 2).

There were significant gender differences in the occurrence of adverse reactions. The elevated ALT occurred more frequently in men ($p = 0.015$), while abdominal pain and hypersensitivity skin reactions were more common in women ($p = 0.031$ and $p = 0.014$, respectively); none of the male patients experienced abdominal pain (Table 7). Also, elevated ALT was less frequent in patients aged 25 and below ($p = 0.001$).

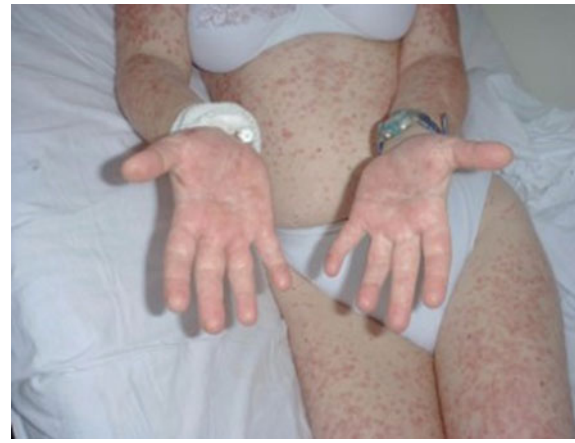

Fig. 1 Stevens-Johnson syndrome – severe skin hypersensitivity reaction

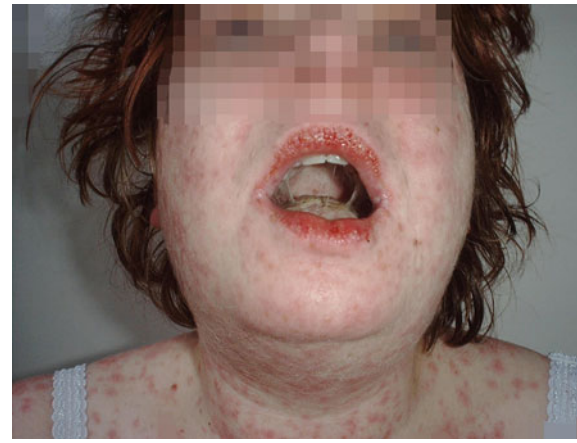

Fig. 2 Stevens-Johnson syndrome with the involvement of mucous membranes – same patient as in Fig. 1

The 21-day long treatment was discontinued due to adverse reactions in 20 cases (3.1% of the study population; 19 women and 1 man). The most common cause of discontinuation was a hypersensitivity reaction, which occurred in 16 women and 1 man. Two patients developed a serious hypersensitivity Stevens-Johnson syndrome. The skin-related adverse events concerned 0.3% of all patients treated for 21 days before 2014 when the study was completed.

Less common causes of treatment discontinuation were elevated ALT (2 women), leukopenia (2 women), abdominal pain (2 women), and thrombocytopenia (1 woman). One female patient experienced three adverse reactions, i.e., abdominal pain, leukopenia, and thrombocytopenia. In one thrombocytopenic patient with the lowest platelet count recorded in the study population, the drug dose was reduced by half. The patient managed to continue with her treatment and was not considered a non-completer. Of the 20 patients with mild adverse reactions, as many as 5 completed this stage of treatment without further complications, taking pyrimethamine pill alone. However, in severe cases of adverse reactions, treatment was discontinued.

3.2 Adverse Effects of 6-Month Long Secondary Antifolate Prophylaxis

Three hundred and fourteen patients were included into the safety evaluation of 6-month long secondary antifolate prophylaxis. The number of records in this dataset amounted to 366, with each record *per* recurrence of an adverse reaction. The adverse reactions were noticed in 18 (4.9%) patients and treatment was discontinued in 10 (2.7%) patients, due to elevated ALT and hypersensitivity skin reactions in 4 (1.1%) patients each, and abdominal pain and thrombocytopenia in 1 patient each. In one half of the patients with mild hypersensitivity skin reactions (4 persons) and slight hypertransaminasemia (another 4 persons), sulfadoxine was discontinued, and these patients completed their treatment without further adverse reactions on pyrimethamine alone. One patient experienced a recurrence of retinochoroiditis during the secondary prophylaxis and was then prescribed another course of intensive treatment. In this case, the second episode of intensive treatment was not subjected to statistical evaluation. Nor was the role of gender and age evaluated in the occurrence of adverse reactions above outlined due to the paucity of patients.

4 Discussion

4.1 Adverse Effects of 21-Day Intensive Treatment

4.1.1 Thrombocytopenia and Myelosuppression: Relationship to Folinic Acid Supplementation

Most authors describing antiparasitic treatment for ocular toxoplasmosis with pyrimethamine and sulfonamides are acutely aware of the risk of folic acid deficiency which may cause myelosuppression, manifest at first as thrombocytopenia and of the way to counteracting it with folinic acid supplementation. However, a combination of pyrimethamine with sulfonamide was routinely used without folinic acid supplementation for the treatment and prevention of malaria at our department in the 1990s and thrombocytopenia was very rarely observed. Based on that experience, we decided not to include folinic acid in the treatment regimen used in ocular toxoplasmosis.

The patients described in the present study were treated with pyrimethamine and sulfadoxine. This synergistic combination inhibits the synthesis of folic acid in the same way as does pyrimethamine and sulfadiazine, but the half-life of the two sulfonamides differs considerably, with about 7 days for sulfadoxine and 10 h for sulfadiazine. The half-lives of pyrimethamine and trimethoprim/sulfamethoxazole are 4 days, and 11 and 10 h, respectively. We herein compare the proportion, type, and severity of adverse reactions with the studies where a combination of pyrimethamine and sulfadiazine was used as well as with fewer studies employing the trimethoprim/sulfamethoxazole treatment (Weidekamm et al. 1982; Thiermann et al. 1978).

In folic acid deficiency, low platelet counts are observed first because the time required for platelets to achieve maturity is shortest. A decrease in white blood cell counts described by Rothova et al. (1993) occurs later with more severe or longer lasting myelosuppression, while iatrogenic anemia is not usually observed. In the present study, to monitor patients for thrombocytopenia and leukopenia, full blood cell counts were performed three times in the course of intensive treatment and six times at monthly intervals during secondary prophylaxis, with a particular focus on platelet counts.

Thrombocytopenia below 120,000 per μL was seen in 8.6% of patients, and this proportion was similar to the 9% reported by Bosch-Driesen et al. (2002). Those authors, however, described the platelet counts of 136,000 and 181,000 per μL as thrombocytopenia, while we defined thrombocytopenia as a platelet count below 120,000 per μL, so that the actual rate of thrombocytopenia we noticed might have been relatively lower. Rothova et al. (1993) have reported 1 out of 35 patients (2%) with a platelet count below

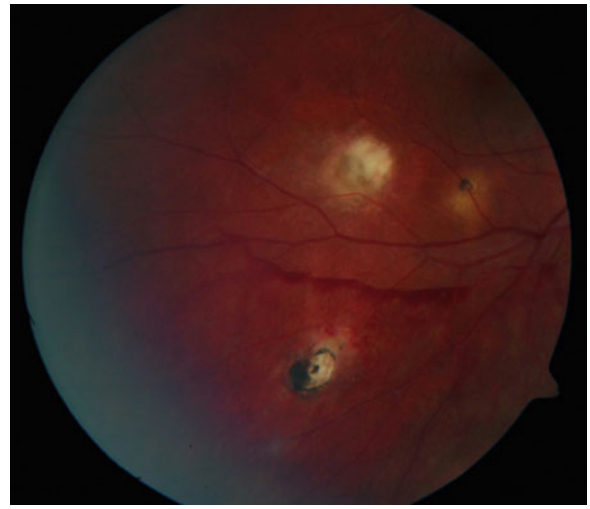

Fig. 3 Retinal hemorrhage in patient with severe folate deficiency; platelet count (PLT) – 1000

9,000 per μL. The overall thrombocytopenia rate in the present study was lower than that, as the lowest platelet count we noticed was 13,000 per μL and the counts below 25,000 were seen in 0.8% of patients. The platelet counts usually decreased to 50,000–100,000 per μL rather fast, over 3–6 days. With weekly blood studies, low platelet counts were promptly detected and specific treatment was initiated. The pyrimethamine 25 mg/sulfadoxine 500 mg combination was withdrawn for 3–5 days, folinic acid was administered with oral etamsylate, and a prednisone dose was increased. With such management, the platelet counts returned to normal within 3–7 days and the patients could continue the previous treatment. Thrombocytopenia as a solitary adverse effect was never the cause of permanent treatment discontinuation. In one patient with thrombocytopenia of 110,000 per μL and moderate leukopenia of 3700 per μL, and with abdominal pain, treatment was discontinued due mainly to the latter complaint.

The most severe thrombocytopenia was observed in an 18-year-old woman initially treated with pyrimethamine alone at a dose of 50 mg/day who first presented for an evaluation after 36 days of unsupervised treatment. This episode was not included in the present statistical analysis. The patient had thrombocytopenia of 1000 per μL with mild leukopenia with the leukocyte count of 3000 per μL. Additionally, the patient developed symptoms, as yet unreported in the literature, related to impaired regeneration of mucosal epithelium such as inflammation of the oral mucosa manifested as severe burning and odynophagia due to esophagitis, and dysuria. She developed a subretinal hematoma associated with a focus of resolved inflammation, which was also related to thrombocytopenia (Fig. 3). In addition to the standard management described above, she received platelet transfusions. She suffered a recurrence of toxoplasmic retinochoroiditis 2 years later. There was a drop in the platelet count to 12,000 per μL in the fourth week of routine treatment, i.e., in the course of secondary prophylaxis, which was the lowest platelet count recorded in the study population. Thrombocytopenia was not accompanied by any previous symptoms of folic acid deficiency. The patient successfully completed the secondary prophylaxis taking one half of the usual pyrimethamine 25 mg/sulfadoxine 500 mg dose. This recurrent episode was included in the present statistical analysis. This and other cases of thrombocytopenia, hypertransaminasemia, and abdominal pain recurring during toxoplasmosis treatment suggest the presence of individual susceptibility to adverse drug reactions. Such patients require special care and monitoring during subsequent

treatments. The mechanism of this susceptibility is unclear, but it may be due to altered drug metabolism or pharmacokinetics in some individuals.

Steroids counteract thrombocytopenia secondary to administration of folic acid antagonists, reduce inflammation, and have other beneficial effects as they increase the number of immature neutrophils in peripheral blood. We observed two cases of leukopenia in the present study, usually in the course of treatment with steroids, white blood cell counts increased to more than 10,000 *per* μL. There is a study that questions the use of steroids in ocular toxoplasmosis (Bosch-Driessen and Rothova 1998). In that study, pyrimethamine was given at a dose two times higher and sulfonamide at a dose eight times higher than those used in the present study, which failed to appreciably shorten the time to inflammation resolution. Nonetheless, it is hard to establish the exact time point of a complete resolution of inflammation as it is a dynamic process spreading over weeks. Generally, in the present population of patients, retinochoroiditis tended to resolve after 3–4 weeks of treatment. *Toxoplasma gondii* can synthesize folic acid and possibly could salvage folate from the host. In malaria caused by infection with *Plasmodium* protozoa, biologically akin to *Toxoplasma gondii*, folinic acid supplements have been documented to decrease the antiparasitic effect of pyrimethamine/sulfonamide. It may be assumed that in the study of Bosch-Driessen and Rothova (1998), where folinic acid was used concomitantly with pyrimethamine and sulfonamides, higher doses of antifolates had to be used to achieve the required therapeutic effect, which is liable to facilitate the appearance of adverse effects. For instance, sulfonamide hypersensitivity is related to the cumulative dose administered. It also has been suggested that excessive amounts of folic acid may blunt natural killer cell cytotoxicity and may accelerate the evolution of drug resistance in microorganisms (Meadows et al. 2015; Nzila et al. 2014; Smith et al. 2008; Carter et al. 2005; Garcia-Doval et al. 2000; Van der Ven et al. 1996). Considering all of the above, we find routine supplementation with folinic acid during antifolate treatment unnecessary, providing that there is a close monitoring of the platelet count, with appropriate management when required.

4.1.2 Hypertransaminasemia

Hypertransaminasemia was the most common adverse effect observed in 24% of patients treated for 21 days. It was more frequent in male patients, which might possibly be related to a higher alcohol consumption in men. In 19.3% of the patients with hypertransaminasemia, ALT levels were not higher than 100 units/L and the patients continued their treatment. They were advised to follow a special diet recommended in liver disease and take hepatoprotective agents, such as herbal preparations containing milk thistle (*Silybum marianum*) extract as well as L-ornithine-L-aspartate and choline. In most cases, ALT returned to normal when antibiotic treatment was completed and prednisone dose was reduced. Clinically significant damage (ALT > 200 units/L) occurred in six (0.9%) patients, whereas toxic liver damage was diagnosed in one woman.

In the study of Bosch-Driessen and Rothova (1998), transaminasemia of various severity has been observed in 9% of patients, ALT, elevated over 30% of the upper limit was found in 5% of patients. In the present study, there were 14.4% of patients with this level of ALT elevation; a greater percentage most probably resulted from a larger number of drugs used in combination. In all cases, weekly monitoring allowed for detection of the problem in its early stage and either changing or temporary or permanent discontinuation of the treatment.

4.1.3 Hypersensitivity Skin Reactions

Hypersensitivity skin reactions were the third most common adverse effect that occurred in 3% of the study population. For comparison, Bosch-Driessen and Rothova (1998) have reported such adverse effects in 4.5% of patients or 9% if itching and exanthema are included. In a study of Balaskas et al. (2012), hypersensitivity skin reactions occurred in 11% (1 out of 9 patients) and in that of Aleixo et al. (2016) in 3% of patients. In both studies, treatment with pyrimethamine 50 mg/day and sulfadiazine

3–4 g/day for 45 days was used. In a study of Soheilian et al. (2005), hypersensitivity reactions have occurred in about 2.9% of patients treated as above outlined or with sulfamethoxazole/trimethoprim. A less frequent occurrence of hypersensitivity skin reactions in the present study population might be accounted for a lower dose of pyrimethamine/sulfonamide we prescribed (over twofold lower for pyrimethamine and eightfold lower for sulfonamide) compared, for instance, to a study of Bosch-Driessen and Rothova (1998). It has been postulated that the adverse effects of sulfonamides are related to a concentration-dependent mechanism stemming from a cumulative dose (Garcia-Doval et al. 2000; Van der Ven et al. 1996). Overall, in the present study, the rate of hypersensitivity skin reactions was similar to or lower than those reported by other authors (Roujeau et al. 1995).

We are unable to explain why hypersensitivity skin reactions were more frequent in women, but an association with the use of cosmetics and skin care products cannot be excluded. Initially, with mild hypersensitivity reactions, the routine treatment regimen was not discontinued as we expected that after completion of the intensive treatment program, the reaction would resolve. Occasionally, we blamed allergens in food and cosmetics the patients used. However, the approach was changed when in two female patients in 2001, mild allergic reaction developed in the third week of treatment, evolved into Stevens-Johnson syndrome within 3–5 days (Figs. 1 and 2). From then on, in each case of any hypersensitivity skin reaction, irrespective of its severity, a combination of pyrimethamine 25 mg/sulfadoxine 500 mg was invariably replaced by pyrimethamine alone. The hypersensitivity reaction then subsided within a few days and no other case of Stevens-Johnson syndrome was ever observed, which confirms the appropriateness of the approach.

We consider drug hypersensitivity, quite often underestimated in the literature, as a potentially serious adverse effect. Yet these reactions are relatively predictable with the reported treatment regimen, they usually occur around the 3rd week of treatment and are triggered by sulfonamides. When a sulfonamide is discontinued once the first signs of skin hypersensitivity appear, the reaction no longer poses any serious problem, which confirms earlier observations that early withdrawal of causative drugs decreases the risk of the development of Stevens-Johnson syndrome (Garcia-Doval et al. 2000).

4.1.4 Abdominal Pain

Abdominal pain was the fourth most common adverse effect, which affected female patients only. In most cases, abdominal pain was reported soon after the beginning of treatment, and in some patients, it subsided when prednisone dose was reduced. Increased doses of the histamine H2-receptor antagonist ranitidine or its replacement with the proton pump inhibitor omeprazole were prescribed, which in some cases relieved pain. In two (0.5%) patients, abdominal pain was severe enough to necessitate the discontinuation of treatment. We linked abdominal pain to the use of steroids and antibiotics, but we fail to explain why it was reported by women only, although different symptom sensitivity might possibly be at play. Both antibiotics and antifolate agents are likely responsible for both elevated ALT and abdominal pain.

Overall, the intensive treatment phase was discontinued in 3.1% of the study patients versus 14% reported in a study of Bosch-Driessen and Rothova (1998) or 26% in other studies (Bosch-Driessen et al. 2002; Rothova et al. 1993). The difference may be explicable by our decision to continue the treatment in cases of thrombocytopenia and in some cases of hypertransaminasemia and abdominal pain. We observed that all patients who had any of the adverse reactions above outlined during the intensive treatment phase invariably experienced the same during treatment for recurrences.

5 Adverse Effects of Secondary Antifolate Prophylaxis

A 6-month long administration of low doses of antifolate drugs should be considered as secondary prophylaxis. Adverse effects of prolonged therapy were serious in one case only, when a female patient

developed toxic hepatitis after 3 months of treatment, with ALT of 1000 units/L. However, soon after discontinuation of treatment, the ALT level normalized. During the following recurrence of ocular toxoplasmosis in this patient, liver damage developed in the initial phase of treatment, with ALT of 460 units/L, yet the symptoms subsided after replacing the pyrimethamine 25 mg/ sulfadoxine 500 mg with pyrimethamine alone. The corollary is that the sulfonamide may have caused the liver damage in this particular case. It should be noted that as even low doses of antifolate agents were hepatotoxic in 0.8% of the study population, the mechanism of liver damage does not seem dose-dependent. Overall, adverse effects of the 6-month long treatment with pyrimethamine/ sulfadoxine were similar to those observed during the intensive treatment phase, but they were less frequent and severe hypersensitivity reactions did not occur.

A limitation of the present study is that we compared our treatment strategy to other treatment regimens that used similar but not same sulfonamides, which entails the possible differences in elimination half-lives, dose, and duration of administration.

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Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the Ethics Committee of the Warsaw Medical University.

Statement on the welfare of animals: This article does not contain any studies with animals performed by any of the authors.

Informed consent: Due to the retrospective nature of the work, the consent of patients is not required, the anonymity of all patients data was maintained. Additional informed consent was obtained from all individual participants for whom identifying information is included in this article.

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Antibody Response to Trivalent Influenza Vaccine in the Northern and the Southern Hemisphere in Elite Athletes

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Abstract

Being frequent travelers, the elite athletes are advised to undergo an influenza vaccination. The aim of the study was to describe the antibody response to repeated trivalent, inactivated, split influenza vaccine, of different antigenic content, recommended for the Northern and the Southern Hemisphere, administered to sportsmen before the Olympic Games in Brasil in 2016. Fourteen athletes were included in the study. For both A/California/7/209/pdm09 A/H1N1/ antigen and A/Switzerland/971593/2013/A/H3N2/ antigen, higher seroconversion rates were obtained after the first than the second vaccination (10.2 vs. 1.5 and 10.6 vs. 3.0, respectively; $p < 0.05$ both). Conversion rates for B/Phuket/3073/2013, B/Brisbane/60/2008, and A/HongKong/4801/2014/A/H3N2/ antigens were lower. Nonetheless, the protection rate was greater than 70% for all antigens contained in both vaccines. The proportion of individuals demonstrating a high level of both protection rate and response rate was greater after the first than the second vaccination. We conclude that the immunological response after influenza vaccination is good in elite athletes and remains so after a second influenza vaccination required due to a different vaccine composition recommended for different hemispheres.

Keywords

Antibody response · Antigen · Athletes · Influenza · Travelers · Trivalent vaccine · Vaccination

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1 Introduction

According to WHO estimations, every season 290,000–650,000 deaths occur globally due to influenza complications (WHO 2017). Influenza vaccination is a safe and effective tool to prevent the disease and its complications. Although influenza vaccination is widely recommended, the vaccine coverage rate remains at a low unsatisfactory level in many countries and risk groups. The elite athletes are advised to undergo a routine influenza vaccination every season (Nitsch-Osuch 2017). Professional sportsmen often spend time training in large groups, take part in competitions in different climate and geographical zones, and travel frequently, which is why they are prone to influenza virus infection (Gartner and Meyer 2014). Influenza outbreaks during sport competitions have already been described, including the Winter Olympic Games in Calgary in 2002, when influenza infection was confirmed among both sportspeople and technical staff (Gundlapalli et al. 2006). Even a mild or moderate influenza course may cause the absence in training and competition for several weeks, which may lead to a loss of a sport season. Medical risk-benefit balance related to a disease is different in athletes than that in the general population as the athletes' performance and sport career explicitly depends on the persistent continuation and attending the training sessions as opposed to the lay people who can afford, to an extent, work absenteeism. Also, risks related to potential side effects of vaccination are aggravated in athletes as local reactions like swelling or pain may influence the training or competition schedules (Gartner and Meyer 2014). In the main, immunogenicity of vaccines in elite athletes is similar to that observed in the general population. However, intensive training and severe physical effort may exert an immunosuppressive effect (Hackney 2013).

The reports describing the immunological efficacy of influenza vaccination in athletes are scarce. Therefore, the present study seeks to define the antibody response after influenza vaccination in Polish athletes preparing for the Rio de Janeiro Summer Olympic Games in 2016. The Games created a unique opportunity to test and compare the effects of two influenza vaccines of different compositions, administered in same athlete, recommended for the Northern and the Southern Hemisphere.

2 Methods

The study protocol was approved by the Ethics Committee of Warsaw Medical University in Warsaw, Poland (permit no. KB/16/2016). Fourteen track-and-field athletes were included in the study (seven males and seven females; mean age 27 ± 4, range 22–35 years). The athletes were vaccinated twice at a 6-month interval; the first vaccine had the content recommended for the Northern Hemisphere and the second for the Southern Hemisphere. The compositions of the vaccine were different in the 2015/2016 season (Table 1).

The first vaccination was carried out from 7th to 23rd October 2015 with a vaccine available in Poland, containing 15 μg hemagglutinin A/California/7/2009 (A/H1N1/pdm09), 15 μg hemagglutinin A/Switzerland/971593/2013 (A/H3N2/), and 15 μg hemagglutinin B/Phuket/3073/2013. The second vaccination was performed from 8th to 27th June 2016 with a vaccine obtained from abroad with the consent of the Polish Ministry of Health. The vaccine contained 15 μg hemagglutinin A/California/7/2009 (A/H1N1/pdm09), 15 μg hemagglutinin A/HongKong/4801/2014 (A/H3N2/), and 15 μg hemagglutinin B/Brisbane/60/2008/.

In both cases, a trivalent, inactivated, split influenza vaccine from the same manufacturer (Sanofi Pasteur; Swiftwater, PA) was administered. Vaccines were given in a volume of 0.5 ml intramuscularly in the deltoid muscle of the nondominant limb. Prior to both vaccinations and 4–6 weeks after the first and second

Table 1 WHO recommended composition for influenza trivalent vaccine for 2015/2016 season (WHO 2016)

	Northern Hemisphere	Southern Hemisphere
Influenza A virus	A/California/7/2009 (H1N1)pdm09-like virus	A/California/7/2009 (H1N1)pdm09-like virus
	A/Switzerland/9715293/2013 (H3N2)-like virus	A/Hong Kong/4801/2014 (H3N2)-like virus
Influenza B virus	B/Phuket/3073/2013-like virus	B/Brisbane/60/2008-like virus

vaccination, a sample of 5 ml of blood was taken from the athletes. The blood was centrifuged and the serum stored at −20 °C until the conductance of a hemagglutination inhibition test (HI), which was done at the Influenza Virus Research Center at the National Institute of Public Health – National Institute of Hygiene in Warsaw, Poland. All antigens for HI test were grown in 11-day-old embryonated fowl eggs and prepared in accordance with the WHO recommendations (WHO 2011). The HI test was performed with the use of 8 hemagglutination units of the virus. Immediately before being used in the test, the serum was inactivated with the *Vibrio cholerae* enzyme.

The pre- and postvaccination anti-hemagglutinin antibody titers were determined for each athlete. Geometric mean antibody titers (GMT) were calculated to demonstrate the following vaccine efficacy indicators:

- Conversion rate (mean increase in the level of antibodies)
- Protection rate (proportion of individuals with antibody titer $\geq$1:40)
- Response rate (proportion of individuals with at least a fourfold rise in the concentration of antibodies after vaccination)

The results were interpreted using the criteria of the Committee for Proprietary Medicinal Products (CPMP) presented in Table 2.

The GMT after vaccination was compared with respective pre-vaccination values using the Wilcoxon signed rank test. The seroconversion rates in two groups were compared with a test of proportions and McNemar's test. A p-value of less than 0.05 defined statistically significant differences. Statistical analysis was performed with a Dell Statistica v13 package (http://www.statsoft.com/Products/STATISTICA-Features).

3 Results

3.1 Geometric Mean Titer (GMT)

The antibody response to vaccine antigens is presented in Table 3. The GMT for A/California/7/209/pdm09/A/H1N1/was lower before the first (Northern Hemisphere vaccine content) than the second vaccination (Southern hemisphere vaccine content), but the difference was not statistically significant (5.8 vs. 25.2; $p > 0.05$). A low GMT for A/California/7/209/pdm09/A/H1N1/ antigen before the second vaccination may indicate a fast waning immunity after the first vaccination. Generally, GMT for influenza A/H1N1/ and influenza A/H3N2/ antigens were lower than for influenza B antigens, both before and after vaccination.

3.2 Conversion Rates

The conversion rate for A/California/7/209/pdm09/A/H1N1/was significantly higher after the first compared to the second vaccination (10.2 vs. 1.5; $p < 0.05$); it was too low after the second vaccination to fulfil the requirements of CPMP. The conversion rate for A/Switzerland/971593/2013/A/H3N2/, a component present only in the Northern Hemisphere vaccine, was high enough (10.6), while that for B/Phuket/3073/2013 and B/Brisbane/60/2008 antigens were rather low and grossly akin to each other (2.2 vs. 2.6; $p > 0.05$).

3.3 Protection Rates

The required protective level ($\geq$70% of individuals studied) was obtained for all antigens contained in both vaccines. The protection rate

Table 2 Criteria for assessing the immunological efficacy of influenza vaccinations according to the Committee for Proprietary Medicinal Products (CPMP 1997)

Criterion	Persons aged 18–60	Persons >60 years
Conversion rate	≥ 2.5	≥ 2.0
Protection rate	$\geq 70\%$	$\geq 60\%$
Response rate	$\geq 40\%$	$\geq 30\%$

Table 3 Antibody responses to trivalent, inactivated, influenza vaccines for the Northern and the Southern Hemisphere in elite athletes in the 2015/2016 season

	Geometric mean titer (GMT)		Conversion rate	Protection rate (%)		Response rate (%)
Antigen/vaccine	Before vaccination	1 month after vaccination	1 month after vaccination	Before vaccination	1 month after vaccination	1 month after vaccination
Northern Hemisphere vaccine – first vaccination						
A/California/7/209/pdm09A/H1N1/	5.8	59.4	10.2	3/14 (21.4%)	13/14 (92.9%)	8/14 (57.0%)
A/Switzerland/971593/2013A/H3N2/	8.8	92.8	10.6	5/14 (35.7%)	12/14 (85.7%)	9/14 (64.0%)
B/Phuket/3073/2013 HB	69.0	152.3	2.2	14/14 (100%)	14/14 (100%)	5/14 (36.0%)
Sothern Hemisphere vaccine – second vaccination given after a 6-month interval						
A/California/7/209/pdm09/A/H1N1/	25.2	38.1	1.5	7/14 (50.0%)	17/14 (71.4%)	2/14 (14.0%)
A/HongKong/4801/2014/A/H3N2/	31.8	93.9	3.0	10/14 (71.4%)	12/14 (85.7%)	6/14 (43.0%)
B/Brisbane/60/2008 HB	62.5	160.0	2.6	13/14 (92.9%)	14/14 (100%)	7/14 (50.0%)

for A/California/7/209/pdm09 A/H1N1/was higher after the first compared to the second vaccination, encompassing 92.9% vs. 71.4% of the athletes, respectively, $p > 0.05$. A similar percentage of those studied (85.7%) got a protective level of antibodies for A/Switzerland/971593/2013 A/H3N2/ and A/HongKong/4801/2014/A/H3N2/antigens. The protection rate for influenza B antigens after both vaccinations was 100%.

3.4 Response Rates

The response rate for California/7/209/pdm09 A/H1N1/was higher after the first compared to the second vaccination (57% vs. 14% of individuals studied; $p < 0.05$). The rate for A/Switzerland/971593/2013A/H3N2/antigen was higher compared to that for A/HongKong/4801/2014/A/H3N2 (64.0% vs. 43.0%; $p > 0.05$). A reverse phenomenon was observed for influenza B virus antigens, where the response rate was higher after the second compared to the first vaccination (50.0% vs. 36.0% of individuals studied; $p > 0.05$).

4 Discussion

The aim of this study was to evaluate the antibody response, i.e., the immunological efficacy, of the influenza vaccine in a group of professional athletes. A selection of this study group seemed interesting due to the possible immunosuppressive effect of increased physical effort, quite typical for elite athletes (Gleeson and Williams 2013). The findings demonstrate that the influenza vaccination was effective and ensured a postvaccination protective antibody levels in 71–100% individuals, depending on the type of antigen

contained in the vaccine. Thus, the immunological efficacy of the trivalent, inactivated, split influenza vaccine was similar in professional athletes to that observed in the general population (ACIP 2017; Osterholm et al. 2012).

The advantage of this study was a unique situation when the individuals received two influenza vaccines with a different antigen composition at an interval of 6 months. That was incidentally possible due to the Olympic Games in 2016 taking place in Brasil during a typical peak influenza season (June–July) for the Southern Hemisphere. The administration of two influenza vaccines with a different antigen composition is recommended for travelers to the countries in the other hemisphere (Marti et al. 2008). However, this recommendation is very rarely followed due to a limited access to the vaccine recommended for the other hemisphere (Goeijenbier et al. 2017).

We noticed in the present study that the conversion rates for A/H1N1/ and A/H3N2/antigens were higher after the first than the second vaccination. A similar seroconversion pattern among health-care personnel vaccinated with the trivalent influenza vaccine has been reported in other studies as subjects with vaccination repeated each season have a higher pre-vaccination and lower postvaccination anti-hemagglutinin titer than those with a single vaccination (Huang et al. 2017).

In case of influenza B virus antigens, we found that the anti-hemagglutinin antibody titer was similar before the first and the second vaccination. The titer was considered protective after either vaccination, which may indicate a cross protection and may result from a previous infection or from a previous influenza vaccination. High pre-vaccination seroprotection levels for influenza B strains were also reported by Ross et al. (2014). Prior influenza vaccinations have been associated with lower dynamics of antibody growth after the following vaccination in the studies by Nuñez et al. (2017) and Ramsay et al. (2017). The exact cause of this phenomenon is unknown, although it is supposed that the antibody response to the current vaccine strains may be only one measure of vaccine-induced protection. Studies published in the 1970s and 1980s also signaled a concern that repeated influenza vaccinations could affect a vaccine protection (Powers and Belshe 1994). Currently, the antigenic distance hypothesis provides a theoretical framework for the explanation of the variability in repeated vaccination based on the antigenic similarity between successive vaccine components and the epidemic strain (Belongia et al. 2017). There also is a theory that persons with broader antibodies repertoires, having the antibody response against a greater number of historical influenza strains, may produce enhanced immune responses to vaccination, compared to persons with narrow repertoires (Andrews et al. 2015). Ramsay et al. (2017), in a systematic review and meta-analysis, have concluded that from the patient's perspective, vaccination in the current season is generally the best option, regardless of the prior season vaccination, while from the policy perspective, there is no evidence that repeated vaccination has a negative impact on vaccine effectiveness in the current season.

In the present study, differences were observed in immunogenicity dependent on the antigen composition of a vaccine. We also noticed a substantially diminished antibody response for the A/H1N1/ antigen after the second vaccination. Despite differences in the seroconversion rate, with lower values after the second vaccination, the protection rate for either dose of vaccine was high (>70%), meeting the criteria set for the immunological efficacy of a vaccine (CPMP 1997). In other studies, repeated vaccination blunts the hemagglutinin antibody response, for instance, found for the A/H3N2/antigen in the 2014/2015 season (Belongia et al. 2017).

A limitation in this study is a relatively small number of participants. Another potential limitation is the use of a trivalent vaccine, while currently the quadrivalent influenza vaccine is preferred, having a wider protection against influenza (Tisa et al. 2016). However, only a trivalent vaccine was available in Poland in the 2015/2016 season, and, for the sake of comparison, a matching vaccine was taken for the Southern Hemisphere. Yet another limitation may be the

lack of data on influenza vaccines used in previous seasons in the context of the influence of a prior vaccination on the level of anti-hemagglutinin antibodies produced after the following vaccination discussed above.

In conclusion, the present study shows that the immunological response after influenza vaccination is good in elite athletes and remains so when a second influenza vaccination is required due to a different vaccine composition recommended for different hemispheres.

Conflicts of Interest The authors declare no conflict of interests in relation to this article.

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Knowledge Regarding Influenza and Influenza Vaccination in General Population: Results of a National Survey in Poland

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Abstract

For the past 10 years, influenza vaccination coverage rate in Poland remains at a low 3% threshold. This low rate may be related to the unsatisfactory knowledge of vaccination, influenza, and misperception of health risks in the general population. To examine these issues, we used an online questionnaire consisting of 12 closed questions. The basic knowledge on influenza and vaccination was examined. The questionnaire was completed by 1669 persons, mostly young women. Generally, 73% of respondents passed the threshold of 70% correct answers, but important gaps in their knowledge were identified concerning the persons at risk of developing the infection (7.9% of correct answers) and the timing of vaccination (8.4% of correct answers). Although most respondents did identify the etiologic agent correctly (91.1% knew influenza is caused by a virus), only 12.3% knew that the vaccines registered in Poland contain fragments of viruses or its antigens, while 63.1% thought the vaccines contain live bacteria. In conclusion, the knowledge on influenza vaccination is deficient in the general population. Education on immunization should be prioritized to increase vaccination coverage rate in Poland.

Keywords

Anti-vaccination movement · Decision-making · General population · Influenza · Public health · Recommendations · Vaccination

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1 Introduction

Every year, approximately 5–20% of the general population (about 10% of adults and 20–30% children) acquires influenza (WHO 2015). The burden of morbidity and mortality makes influenza one of the largest public health concerns. In the USA (population of approx. 300 million), an average annual influenza-related deaths have been estimated as 23,607. In the EU, the annual number of influenza-infected individuals of all ages has been estimated at 25–100 million with approximately 38,500 deaths occurring each year (CDC 2016). In Poland (population of approx. 38 million), there were about 3.8 million cases of influenza and influenza-like illness (ILI) reported in the most recent 2016/2017 season, along with 13,000 hospitalizations and 24 deaths being attributed to influenza (National Influenza Center 2017).

In 1947, the WHO established the Global Influenza Program. It has been 70 years of international effort, aimed to limit the world influenza burden. Reaching a vaccine coverage rate threshold of 75% in all risk groups, especially among elderly and healthcare workers, is one of the priorities appointed by the WHO and the Council of the EU (WHO 2017). According to these aims, Polish and other national guidelines are updated regularly, and, in recent years, vaccine indications have been broadened to include such risk groups as pregnant women and children under 5 years of age (Grohskopf et al. 2016). In 2013, the National Program for Influenza Prevention was established in Poland. Over the past 4 years, independent medical experts affiliated with this organization made an effort to raise the knowledge of influenza and influenza vaccination in the general population and in healthcare workers. Annual educational campaigns in Poland included mostly posters, leaflets, and educational materials available on the Internet and in mainstream media (OPZG 2017). Despite these efforts, the vaccination coverage rate remains dismally low in Poland: approx. 2.2–3.4% in the general population, 9.0% in healthcare workers, 0.5–1% in children aged 6 months to 4 years, and 7.0–13.2% in the elderly aged over 65.

Vaccine hesitancy is a raising global problem that negatively influences the epidemiological situation of many infectious diseases, especially influenza in Poland. The insufficient knowledge regarding vaccine-preventable diseases and vaccinations has been pointed among the reasons of vaccine hesitancy in society (WHO 2014). To address this issue in the Polish society, we examined the basic knowledge of influenza and influenza vaccination in general population.

2 Methods

This survey-type study was approved by the Ethics Committee of Warsaw Medical University in Poland, and it was conducted according to the principles of the Declaration of Helsinki for Human Research of the World Medical Association. The population sample surveyed consisted of 1669 randomly gathered adult volunteers from Poland, mostly women (71.5%). Table 1 summarizes the basic demographic information and characteristics of the participants. An online questionnaire consisting of 12 closed questions was designed for the purpose of this study by the

Table 1 Sociodemographic characteristics of the study population

	n (%)	Total
Gender		1669
Female	1193 (71.5)	
Male	476 (28.5)	
Age group (yr)		1564*
18–25	693 (44.3)	
26–35	527 (33.8)	
36–45	223 (14.3)	
45–55	73 (4.7)	
>56	48 (2.9)	
Size of agglomeration (number of citizens)		1669
<10,000	444 (26.2)	
10,000–50,000	319 (19.1)	
50,000–100,0000	189 (11.3)	
100,000–500,000	307 (18.4)	
>500,000	410 (24.6)	

*some respondents have not disclosed their age

members of the Polish Expert Committee of the National Program for Influenza Prevention. The questionnaire was hosted at the website www.opzg.pl, was available online from August to September 2015, and was distributed to a varied group of respondents via social media and emails. The questionnaire questions, along with the responses provided by the interviewees, are presented in Table 2. The questionnaire was anonymous and voluntary, and the participants were informed about the study aims. Answers were scored as correct based on published literature and current recommendations of the Advisory Committee on Immunization Practices (ACIP) of the Centers for Disease Control and Prevention (CDC 2016).

3 Results

Questionnaire results are displayed in Table 3. The vast majority of respondents (73.0%) passed the threshold of 70.0% correct answers. Survey results were highly satisfying in questions related to the etiology of influenza (90.0% of correct answers) and modes and routes of transmission

Table 2 Survey results (single choice questionnaire)

Items (correct answers are underlined)	Correct answers n (%)	Incorrect answers n (%)
1. What is the etiological agent of influenza?	1520 (91.1)	149 (8.2)
Bacteria		
Virus		
Fungus		
Parasite		
2. What are the most typical symptoms of influenza?	1088 (65.2)	518 (34.8)
High fever		
Sudden beginning		
Muscle and joint aches and headache		
All of the above		
3. What are possible routes of influenza transmission?	1515 (90.8)	154 (9.2)
Airborne – via droplets		
Blood transmission		
Only person-to-person close contact		
Sexual transmission		
4. Why is influenza considered as dangerous disease?	1560 (93.5)	109 (6.5)
It spreads easily in population and causes serious complications		
Person with influenza feels unwell		
It is a seasonal disease		
People at any age can be infected		
5. For whom influenza is especially dangerous?	132 (7.9)	1537 (92.1)
People with chronic diseases		
Small children and the elderly		
People with impaired immunity		
All of the above		
6. Why vaccination against influenza should be administered every year?	1514 (90.7)	155 (9.3)
Such is the act of the minister of health		
Infection can be caused by different influenza viruses, so every season WHO verifies the vaccine composition		
Vaccine's effect is short lived		
Vaccines are little effective		

(continued)

Table 2 (continued)

Items (correct answers are underlined)	Correct answers n (%)	Incorrect answers n (%)
7. When can a person become vaccinated against influenza?	140 (8.4)	1529 (91.6)
Throughout the infectious season from September to March since the appearance of vaccines in pharmacies		
Only in autumn		
Only in winter		
In summer		
8. What is the minimal age of influenza vaccination in children?	945 (56.6)	724 (43.4)
6 years of age		
Since birth		
6 months of age		
Only adults can get vaccinated		
9. What is the content of influenza vaccine?	205 (12.3)	1464 (87.7)
Live viruses		
Live bacteria		
Only antigens of the influenza virus or viral fragments[a]		
Killed bacteria		
10. Why, despite vaccination against influenza, one can get common cold?	1281 (76.8)	388 (23.3)
Vaccine contains influenza viruses from another country		
Vaccine protects against influenza, not against any respiratory tract infection		
Vaccine is ineffective		
All of the above are wrong		
11. Choose the correct statement out of the following:	1435 (86.0)	234 (14.1)
Influenza vaccine also protects against so-called intestinal flu		
Vaccine can cause influenza		
Vaccine protects against influenza and its serious complications		
Persons who are healthy and have good immunity should not get vaccinated		
12. Choose incorrect statement out of the following:	1038 (62.2)	631 (37.8)
Influenza vaccine protects against common cold		
One of the symptoms after vaccination may be pain and swelling at injection site		
After vaccination, flu-like symptoms of mild intensity may occur		
Vaccine cannot cause influenza		

[a]No live vaccine is registered in Poland

(90.1% of correct answers). The results were less satisfying regarding symptoms of the disease (65.2% of correct answers). As far as immunization is concerned, respondents knew well the reasons for the need of annual vaccination (90.1% of correct answers) and the aim of vaccination (86.0% of correct answers), but they were not familiar with other issues. Only 7.9% of respondents were able to correctly identify all the risk groups. Most of the respondents (72.2%) singled out chronic diseases alone as a risk factor, but did not recognize the extremes of age as a risk factor. They did not know that small children, elderly people, and immunocompromised people were at risk. Proper time for influenza vaccine administration was another difficult question in the examined group. Only 8.4% of respondents answered this question correctly. The most surprising finding was that even though most of the respondents did identify the influenza causative agent correctly (91.1% knew it was a virus), only 12.3% of them knew that the vaccines

Table 3 Distribution of survey answers by specific items asked (single choice questionnaire)

Items (correct answers are underlined)	Answer (a) n (%)	Answer (b) n (%)	Answer (c) n (%)	Answer (d) n (%)
Influenza is especially dangerous for:	1205 (72.2)	101 (6.1)	231 (13.8)	132 (7.9)
(a) People with chronic diseases				
(b) Small children and the elderly				
(c) People with impaired immunity				
(d) All of the above				
When can a person be vaccinated against influenza: (a) Throughout the infectious season from September to march since the appearance of vaccines in pharmacies	140 (8.4)	29 (1.7)	246 (14.7)	1254 (75.1)
(b) Only in autumn				
(c) Only in winter				
(d) In summer				
What is the content of influenza vaccine:	149 (8.9)	1053 (63.1)	205 (12.3)	262 (15.7)
(a) Live viruses				
(b) Live bacteria				
(c) Only antigens of the influenza virus or viral fragments [a]				
(d) Killed bacteria				

*No live vaccine is registered in Poland.

registered in Poland contain fragments of the viruses or its antigens, while 63.1% answered that vaccines contained live bacteria.

4 Discussion

Influenza is one of the most concerning global problems in public health. Its annual burden in Poland is expressed not only in millions of infected people, several thousands of hospitalized patients, and dozens of deaths but also in high economic costs due to absenteeism at work, treatment costs, and lost years of life (National Influenza Center 2017). The basic annual cost of influenza for Polish budget has been estimated at about €10 m to €170 m depending on the epidemic season. Another category of loss related with influenza is harder to estimate as it results of disease-connected suspension of social relations and free time activities. Despite those threats and vaccine-preventable nature of influenza, the vaccine coverage rate is unacceptably low in Poland. One of the important reasons is that annual influenza vaccination is not refunded. Studies show that it is an important factor responsible for low vaccination coverage rate (Blank et al. 2009; Endrich et al. 2009). The other factor may be poor, inconvenient accessibility. A research conducted in Israel shows that providing vaccination at a workplace influences positively the vaccine uptake (Shahrabani and Benzion 2010). Immunization is defined by Polish law as a doctor's procedure and associated with time-consuming formal requirements. Immunization are generally delivered by primary care physicians, who are busy with other tasks, but several healthcare companies offering vaccination services for their customers operate on the Polish market, e.g., Luxmed, Medicover, Scanmed, or Falck, and together cover about 2 million people of the Polish population. Over the last few years, a growing number of private companies and corporations in Poland, understanding the need for influenza vaccination, have provided their workers with vaccines. Additionally, there are also some local initiatives of free vaccination for seniors. Regardless, the general vaccination coverage rate in Poland remains far below expectations. It amounted to approx. 2.5% of the general population during 2016/2017 influenza season, and it has never exceeded 10.0% of the population. Nonetheless, coverage is significantly better in selected risk groups, e.g.,

chronically ill patients. The highest (58.0%) rate of vaccination has been demonstrated in patients with pulmonary chronic diseases (Nitsch–Osuch et al. 2017).

A systematic review of influenza vaccine hesitancy has not indicated the lack of general access to influenza vaccines as a significant barrier to vaccination (Schmid et al. 2017). That means that patient's knowledge and attitude are likely pivotal factors. The patient knowledge also is important in the timing of vaccination; some patients miss the opportunity for immunization because they seek medical attention too early or too late during the influenza season. The knowledge of proper timing of influenza vaccination has not been examined. A lack of the ability to indicate appropriate time for influenza vaccination, revealed in our study, may be another practical barrier for immunization in Poland. The results of our survey reveal that although the knowledge of influenza infection was satisfying, the inability of recognition of risk groups was highly alerting. The knowledge of recommendations for vaccination is associated with a higher vaccine uptake (Lu et al. 2017; Tsai et al. 2014), and vice versa a lack of this knowledge is identified as a barrier for immunization (Schmid et al. 2017). Most of the studies have shown that people aged over 65 and patients with chronic diseases who have a higher vaccination rate also have a better knowledge of influenza and influenza vaccination. Nonetheless, even high-risk individuals do not consider themselves as susceptible to influenza infection, and they fear possible adverse events following immunization (Adadan Güvenç et al. 2017; Guthrie et al. 2017; Kuchar et al. 2017; Santos et al. 2017). A lack of knowledge and false assumption that healthy people do not need immunizations are important reasons that healthy adults fail to receive vaccinations (Johnson et al. 2017). Raising the awareness of the risk groups for influenza in society should be prioritized. Considering how numerous conditions predispose to influenza complications, almost everyone has someone belonging to that group in close contacts, e.g., housemates or co-workers. The awareness that every infected person is a possible source of infection for those most vulnerable could positively influence vaccination coverage in society. It has been shown that social pressure positively influences the decision to get vaccinated (Schmid et al. 2017).

Fear of side effects, concerns about influenza vaccine effectiveness, and belief that vaccine spurs the infection are another most often indicated reasons for refusing vaccination, revealed in the present and other previous studies (Adadan Güvenç et al. 2017). The majority of articles reviewed by Schmid et al. (2017) report that the myth that the vaccine can cause influenza constitutes a significant barrier for vaccination. Although most of the respondents in the present survey did know that it is a false statement, it might subconsciously influence their personal perception of risk and consequently bear on the decision not to vaccinate. We identified another worrisome phenomenon that may indicate a complete lack of understanding of the idea of vaccination. While the most of the respondents knew that influenza is caused by the virus, they believed the vaccine contains live bacteria. That false opinion may be associated with a popular myth that "you can become ill due to influenza vaccination." All the authorities agree that vaccines have become the victims of their own success. With a reduction of vaccine-preventable infectious diseases in developed countries, the sense of vaccination need has fainted in society (Ołpiński 2012). We assume that in the era of rising vaccine hesitancy, the basic information on the vaccine and the mechanisms of the immunity gained after vaccination should be taught at schools. The results of our survey could have been worse if it were not for educational campaigns often organized on the issue. In 2013, the National Program for Influenza Prevention was established in Poland. It is the largest association of Polish medical experts on influenza, whose aim is to promulgate the knowledge of influenza and influenza vaccination in healthcare workers and in society. Every year, scientific events are organized, and current information for patients is prepared and shared (OPZG 2017). In 2015, the year when the present survey was conducted, 943 printed materials and articles

branded by the program were published, and 1862 mass-media materials about influenza prevention were broadcast. It was estimated that the information reached 39 million receivers, which means that on average, every Polish citizen must have heard it at least once (OPZG 2015).

5 Conclusions

This study identified the lack of knowledge about influenza and influenza immunization in society as one of the most important reasons for a low influenza vaccine coverage in Poland. We determined the most important gaps in the public knowledge as the lack of awareness about the risk groups and the misunderstanding of the vaccine composition and of proper timing for influenza immunization. The knowledge of influenza outweighed that of vaccination per se. To increase the vaccination coverage rate, educational efforts focused on the rationality and benefits of immunization should be prioritized.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Virological Situation in Poland in the 2016/2017 Epidemic Season Based on Sentinel Data

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Abstract

The influenza Sentinel surveillance system is a source of valuable data about the dynamics of epidemic seasons in Poland. During the epidemic season 2016/2017, more than 1,000 samples were examined, of which 48% were positive for influenza diagnosis. The predominance of influenza A/H3N2/ was confirmed. Influenza B viruses were noted in only 1% of the samples tested. After the analysis in age groups, the highest number of positive samples was observed in the group of 26–44 years. Infections caused by influenza-like viruses were confirmed only in 3% of cases. The Sentinel surveillance system makes it possible to evaluate the spread of the influenza virus in each epidemic season.

Keywords

Epidemic season · Infection · Influenza · Surveillance · Virology

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1 Introduction

The virological and epidemiological surveillance system Sentinel was introduced in Poland in 2004. The system is coordinated by the Department of Influenza Research, National Influenza Center of the National Institute of Public Health – National Institute of Hygiene in Warsaw, Poland (Wozniak–Kosek and Brydak 2013). The system is based on the cooperation of family physicians, 16 Voivodship Sanitary Epidemiological Stations (VSES), and the National Influenza Center. Family physicians collect nasal and throat swabs and send the specimens to the VSES for the molecular diagnostic workout. The samples are reanalyzed in the virological laboratory of the Department of Influenza Virus Research of the National Influenza Center in Warsaw to confirm the presence of the genetic material of the influenza and influenza-like viruses. The results are reported to the Sentinel system on a weekly basis and are posted on the platform of the European Surveillance System (TESSy) (Brydak 2008). Data from all European countries are the basis for weekly reports assessing the current situation of influenza spread in Europe (Cieślak et al. 2017).

Samples are also subtyped and then sent to the WHO Collaborating Center in London for sequencing of the viral genome using genetic tests, which is the basis of subsequent phylogenetic analyzes (Bednarska et al. 2015).

The influenza virus is a virus that very easily modifies. Consequently, it is necessary to continually monitor the emerging strains in the world. There are currently 143 National Influenza Centers worldwide (WHO 2017a). Each of them collects data about the virological and epidemiological situation in a country.

2 Methods

The study was approved by an institutional ethics committee, and it was conducted in accordance with the Declaration of Helsinki for Human Research. In the 2016/2017 epidemic season, 1283 nasal and throat swabs were collected within the framework of the Sentinel system. The samples were analyzed in 16 Voivodship Sanitary Epidemiological Stations across Poland. Based on the innovations introduced in the influenza surveillance, samples were analyzed in seven age groups: 0–4, 5–9, 10–14, 15–25, 26–44, 45–64, and 65+ years of age (Bednarska et al. 2016a).

Viral RNA isolation was carried out using the Maxwell 16 Viral Total Nucleic Acid Purification Kit (Promega Corporation, Madison, WI) according to the manufacturer's instructions. The material was suspended in saline. The final volume of the eluted sample was 50 μl.

Real-time reverse-transcription polymerase chain reaction (qRT-PCR) was performed to define the presence of influenza virus in the swabbed samples, using a Light Thermocycler 2.0 System (Roche Diagnostics, Rotkreuz, Switzerland) according to the manufacturer's instructions. Starters and probes were obtained from the International Reagent Resources (IRR), part of the Centers for Disease Prevention and Control in the USA. RNA isolated from the viruses that were selected for the 2016/2017 vaccine (A/California/7/2009(H1N1)pdm09, A/Hong Kong/4801/2014, and B/Brisbane/60/2008) were used as positive controls. Negative control was RNase-free water.

3 Results

Positive samples for influenza infection were found in 616 (48%) out of 1283 tested in the epidemic season 2016/2017. The largest group of patients who acquired were those aged 26–44 (30%), and the smallest were in the age group 10–14 (5%) (Fig. 1). There was a clear prevalence of influenza A virus, which accounted for 96% of all confirmed cases of influenza and influenza-like viruses. Only was the A/H3N2/ subtype of influenza found, confirmed in 26% of cases. Influenza B virus was confirmed in 1% of infections (Fig. 2).

Stratified by the age groups, the greatest number of infections caused by the influenza A subtype A/H3N2/ and unsubtyped virus was found in the age groups 26–44 and 45–64, amounting to 54 cases of influenza A/H3N2/ and 128 cases of unsubtyped influenza A and 35 cases of influenza A/H3N2/ and 125 unsubtyped influenza A virus in the respective age groups. A small number of confirmed cases in the three youngest age groups, i.e., children below 14 years of age, were reported (Fig. 3).

There were 16 cases of influenza-like illness found during in the season, which accounted for 3% of all confirmed cases. The greatest number of these infections was recorded in the group of 45–64 years (6 confirmations). The only group with no influenza-like illness was that of 5–9 years. The predominant influenza-like virus was respiratory syncytial virus (RSV) (6 confirmations) (Table 1).

4 Discussion

Thanks to the data collected in the Sentinel system, it is possible to evaluate the spread of the influenza virus in Poland in each epidemic season. These data are extremely important because they are necessary for the epidemiological surveillance by the international organizations, such as the European Center for Disease Prevention and Control (ECDC) and the World Health Organization (WHO), and are later used later to choose the virus strains for the vaccine of the next epidemic season.

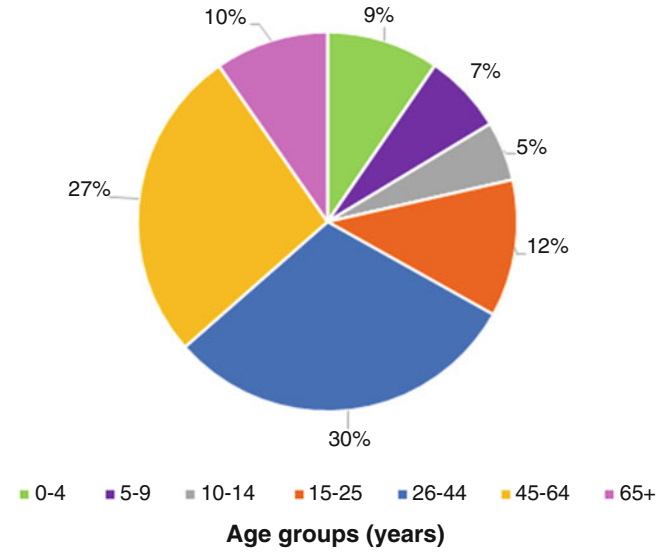

Fig. 1 Percentage of samples tested for influenza infection in different age groups in the 2016/2017 epidemic season

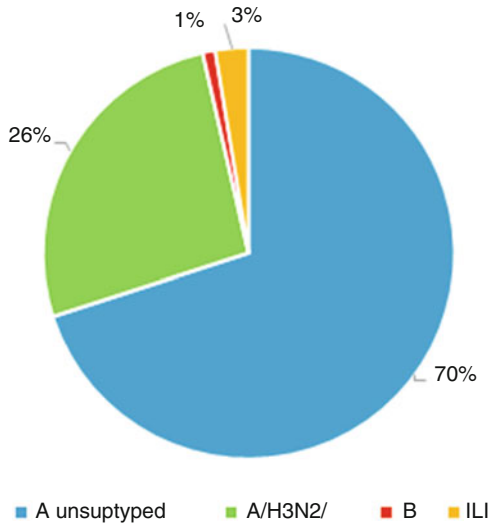

Fig. 2 Percentage of influenza and influenza-like viruses in the 2016/2017 epidemic season

In the 2016/2017 epidemic season in Poland, there was a clear prevalence of influenza A virus in all studied age groups. Similar data were obtained in other European countries, for instance, Germany, Denmark, and Italy (WHO 2017b). This trend is different from the preceding season where the A and B virus co-predomination was recorded. The 2015/2016 predominant subtype had been A/H1N1/pdm09, whereas in the current epidemic season, we observed a clear predominance of A/H3N2/ (Cieślak et al. 2017). The A/H3N2/ subtype predominated 2 years back in the 2014/2015 season as well (Bednarska et al. 2016b).

In the 2016/2017 epidemic season, positive samples accounted for 48% of all samples tested. That is an increase of the number of confirmed influenza infections by 10% points compared to the preceding season. The increase may reflect a better organized sample collection and diagnostic procedures, a greater number of samples, and a more dynamic morbidity course in the preceding season. However, the greatest number of influenza A infections recorded in the age group of 26–44 in the 2016/2017 season is in line with the epidemiologic picture recorded in the preceding season (Cieślak et al. 2017).

There were only six cases of influenza B reported in Poland in 2016/2017, accounting for 1% of all positive samples. These data distinguish the season from the preceding season, when the number of confirmed cases of influenza B was comparable to that of influenza A (Cieślak et al. 2017). A small number of influenza B infections in 2016/2017 distinguished Poland internationally, since an increase in the incidence of

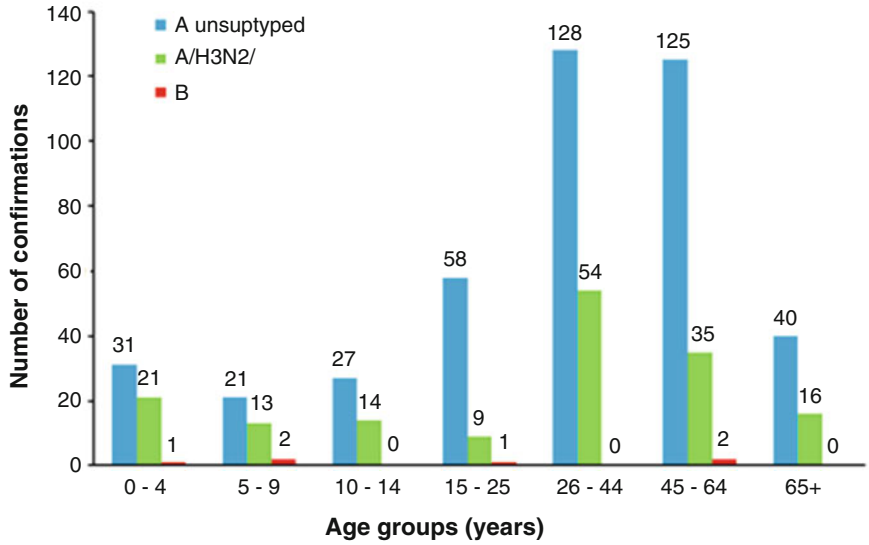

Fig. 3 Confirmed influenza virus infections in the 2016/2017 epidemic season

Table 1 Confirmations of influenza-like viruses in different age groups in the 2016/2017 epidemic season

Age group (year)	ADV	RSV	PIV1	PIV2	PIV3	CoV	RV	hMPV	Total
0–4	0	3	0	0	0	0	0	0	3
5–9	0	0	0	0	0	0	0	0	0
10–14	0	1	0	0	0	0	0	0	1
15–25	1	0	1	0	0	0	0	0	2
26–44	0	0	2	0	1	0	0	0	3
45–64	0	1	1	1	2	0	1	0	6
65+	0	1	0	0	0	0	0	0	1
Total	1	6	4	1	3	0	1	0	16

influenza B cases was observed worldwide as of Week 5 of the season (WHO 2017a). The Sentinel system for influenza surveillance enables to gather high-quality epidemiological data, which apparently has transformed the way researchers monitor the infection and which is essential for setting the antigen composition for each next seasonal vaccine.

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Conflicts of Interest The authors declare no conflict of interests in relation to this article.

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Antibodies to Influenza Virus Hemagglutinin in the 2016/2017 Epidemic Season in Poland

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Abstract

The aim of the study was to determine the level of antibodies against hemagglutinin of influenza viruses in the sera of 1,050 patients stratified into 7 different age groups during the 2016/2017 epidemic season in Poland. The method consisted of using the hemagglutination inhibition test (HAI). The findings confirmed the presence of anti-hemagglutinin antibodies against the following influenza virus antigens: A/California/7/2009 (H1N1) pdm09, A/Hong Kong/4801/2014 (H3N2), and B/Brisbane/60/2008. The level of anti-hemagglutinin antibodies differed across the age groups investigated, with the highest values in patients aged 10–14 years. We also found that the protection factor was highest for B/Brisbane/60/2008 antigen. It amounted to 72.7% in 10–14-year olds and to 69.3% in both 15–25 and 26–44-year olds. Taking into account a dismally low percentage of vaccinated population in the 2016/2017 epidemic season in Poland, amounting to barely 3.33%, the findings should be interpreted as representing the patients' immune system response to influenza virus infection.

Keywords

Antibodies · Epidemics · Hemagglutinin · Influenza · Protection factor · Vaccine · Virus

1 Introduction

The purpose of seasonal vaccinations against influenza is to produce a protective concentration of antibodies. According to the recommendations of the Advisory Committee on Immunization Practices (ACIP 2016), there are no deadlines for influenza vaccination, but it is recommended that people at risk be vaccinated before onset of influenza activity in the population. Not only the WHO but more than 15 International Scientific Societies appeal to increase vaccination coverage rate.

Antibodies found in unvaccinated individuals are indicative of past infection (Brydak 2008). The peak of influenza and influenza-like illnesses in the epidemic season 2016/2017 occurred in the fourth week of January 2017. There were 363,583 cases of influenza and influenza-like illnesses recorded during the week, and the average daily incidence was 105.0 *per* 100,000 population (NIPH-NIH Report 2017).

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Overall, in the 2016/2017 epidemic season, there were 4,373,588 cases of influenza and influenza-like illnesses recorded, and the incidence was 1,507.0 *per* day. The predominant strain of influenza virus during the season, both in Poland and Europe, was A/H3N2/ (ECDC 2017). Respiratory syncytial virus (RSV) predominated among the influenza-like viruses. The only effective form of protection against influenza infection is vaccination. Unfortunately, the percentage of vaccinated population gets lower from season to season. In the 2016/2017 epidemic season, only 3.33% of the population (NIPH-NIH Report 2017) was vaccinated in Poland.

The aim of this study was to determine the level of specific antibodies against influenza virus hemagglutinin in the serum samples of infected patients during the 2016/2017 influenza season in Poland.

2 Methods

This study was approved by the Institutional Review Board for Human Research, and it was conducted in accordance with the principles for human research set by the Declaration of Helsinki. Serum samples of individuals in seven age groups (0–4 years, 5–9 years, 10–14 years, 15–25 years, 26–44 years, 45–64 years, and $\geq$ 65 years) were collected by the employees of the Voivodship Sanitary Epidemiological Station the Department of Influenza Research, National Influenza Center in the National Institute of Public Health – National Institute of Hygiene. In total, 1,050 randomly selected serum samples, i.e., 150 samples in each age group, were tested. Samples were stored at −80 °C until testing.

The level of antibodies against the following specific antigens, listed in Table 1, was assessed. The method consisted of a hemagglutination inhibition test (HAI) conducted with 8 hemagglutination units according to the WHO's recommendations (WHO 2011). The antigens were prepared in-house and covered those set for the 2016/2017 epidemic season (WHO 2016). Prior to the analysis, the serum was inactivated using the *Vibrio cholerae* enzyme and was serially diluted from 1:10 to 1:1,280 according to the method of Brydak et al. (2003).

3 Results and Discussion

The geometric mean titers (GMT) of anti-hemagglutinin antibodies in the serum taken from patients were calculated for different age groups during the epidemic season 2016/2017 in Poland. The results are shown in Fig. 1. The level antibodies against hemagglutinin H1 was highest in the group 5–9 years: GMT amounting to 143.4. Lower antibody levels were detected, in descending order, in the groups 0–4 (GMT-53.8), 10–14 (GMT-63.1), 45–64 (GMT-48.6), 15–25 (GMT-35.0), and $\geq$ 65 (GMT-39.2) years. The lowest level of GMT was recorded in the group 26–44 (GMT-25.2) years of age.

For hemagglutinin H3, GMT was highest in the group 10–14 years, amounting to 70.5. A lower level of hemagglutinin H3 was obtained in the groups 0–4 (GMT-55.8), 5–9 (GMT-62.9), and $\geq$ 65 (GMT-55.5) years. In the groups 15–25 and 45–64 years, GMT was 44.7 and 35.2, respectively. The lowest GMT value for hemagglutinin H3 was found in the patients of 26–44 years of age (GMT-23.7).

For type B hemagglutinin, the observed level of antibodies were similar in the age groups: 0–4 (GMT-33.5), 5–9 (GMT-32.7), 45–64 (GMT-35.2), and $\geq$ 65 (GMT-30.1) years. Higher values were obtained in the patients 10–14 (GMT-51.6) and 26–44 (GMT-42.3) years of age, while the highest GMT value was present in those aged 15–25 (GMT-71.7).

The proportion of people with the titers of antibodies against immunodominant head domain of influenza virus hemagglutinin of at least 1:40 present after vaccination or prior infection is referred to as a protective factor (Brydak et al. 2003). It should be noted that this proportion, to be optimally protective, should reach >60% in people over 60 years of age and > 70% in those aged 18–60 (Brydak 2008). In the epidemiological season 2016/2017, the highest levels of

Table 1 Strains of influenza viruses in the 2016/2017 epidemic season

A/H1N1/	A/H3N2/	B
A/California/7/2009-(H1N1)pdm09-like virus	A/Hong Kong/4801/2014-(H3N2)-like virus	B/Brisbane/60/2008-like virus-Victoria lineage

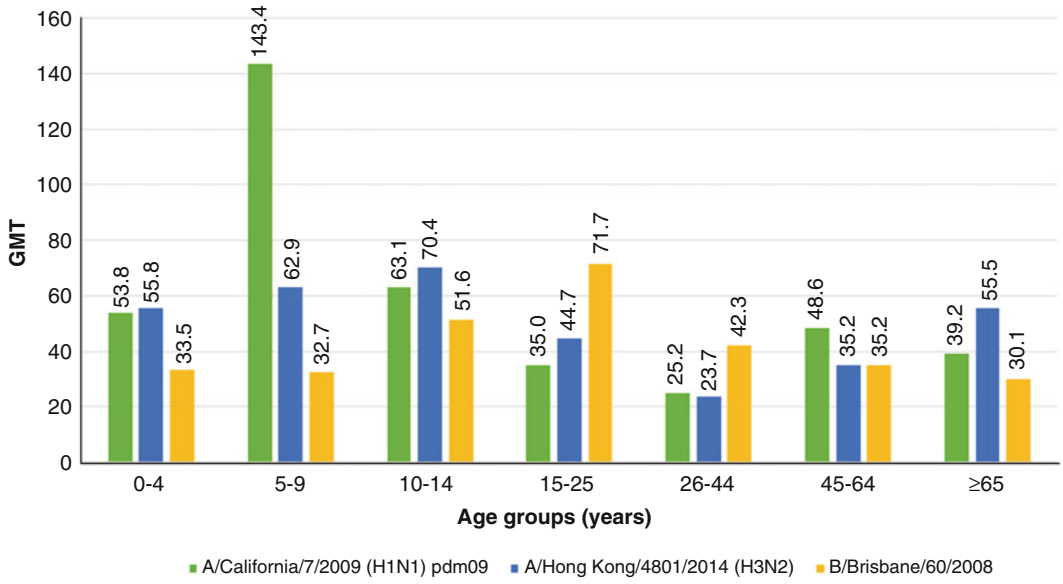

Fig. 1 Geometric mean titers (GMT) of anti-hemagglutinin antibodies in serum of people in different age groups in the epidemic season 2016/2017 in Poland

protection were recorded for type B hemagglutinin of the strain B/Brisbane/60/2008 in the age groups: 10–14 (72.7%), 15–25 (69.3%), and 26–44 (69.3%) years. In other age groups, clearly lower levels of protective factor were recorded: 0–4 (35.3%), 5–9 (20.7%), 45–64 (46.7%), and ≥ 65 years (30.7%) (Fig. 2).

For hemagglutinin H1 of the strain A/California/7/2009 (H1N1) pdm09, the highest level of the protective factor was recorded in the group 10–14 (63.3%) years and significantly lower in the 45–64 years of age (40.7%) ($p < 0.05$). The remaining age groups showed a comparable percentage of the protective factor in a range of 20–30%, 0–4 (20.0%), 5–9 (25.3%), 15–25 (28.7%), 26–44 (21.3%), and ≥ 65 years (29.3%), all significantly lower than that of the 45–64 age group.

For hemagglutinin H3 of the strain A/Hong Kong/4801/2014 (H3N2), the highest level of the protective factor was recorded in the group 10–14 (60.0%) years, followed by 56.0% in 15–25 years of age. In the other age groups, the value of the protective factor was significantly lower: 0–4 (22.0%), 5–9 (14.0%), 26–44 (14.7%), 45–64 (33.3%), and ≥ 65 years (26.7%).

Of the three analyzed hemagglutinins considered together, the highest percentage of the protective factor was present in the group 10–14 years, H1–63.3%, H3–60.0%, and B–72.7%, while the lowest was in the group ≥65 years: H1–29.3%, H3–26.7%, and B–30.7%.

Each year there were modifications in the antigenic composition of the tertiary influenza vaccine; in the epidemic season 2016/2017, compared to the preceding season, the change consisted of substituting A/Hong Kong/4801/2014 (H3N2) for A/Switzerland/9715293/2013 (H3N2) and B/Brisbane/60/2008 for B/Phuket/3073/2013 (Table 2). The A/California/7/2009 (H1N1) pdm09 antigen remained unchanged (WHO 2015).

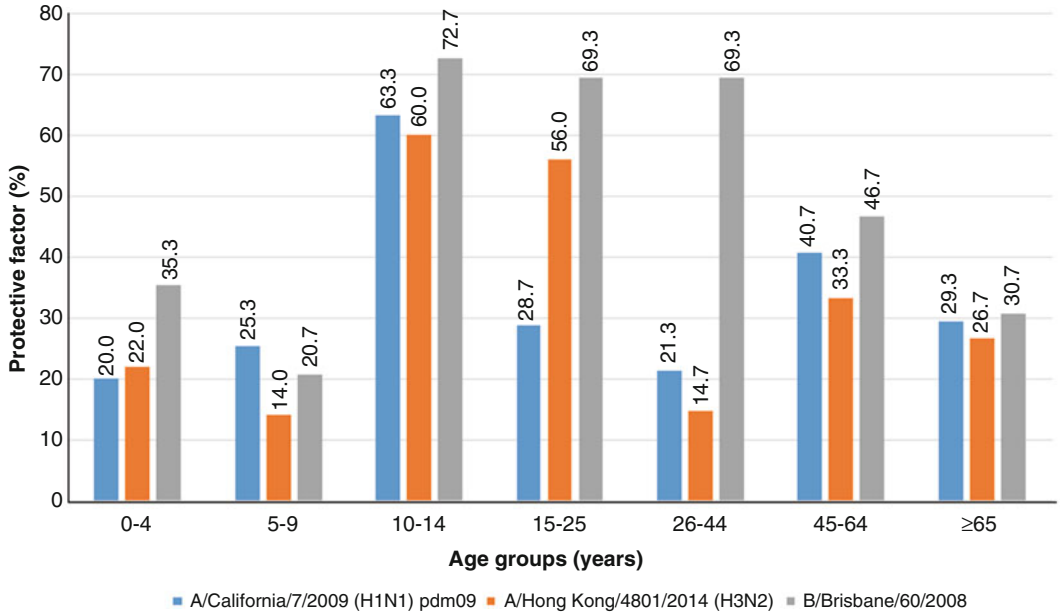

Fig. 2 The percentage of people with protective factor of anti-hemagglutinin antibodies in age groups in the epidemic season 2016/2017 in Poland

Table 2 Antigen composition of influenza vaccines in recent successive epidemic seasons

Epidemic season 2014/2015	A/California/7/2009 (H1N1)pdm09
	A/Texas/50/2012 (H3N2)
	B/Massachusetts/2/2012
Epidemic season 2015/2016	A/California/7/2009 (H1N1)pdm09
	A/Switzerland/9715293/2013(H3N2)
	B/Phuket/3073/2013
Epidemic season 2016/2017	A/California/7/2009 (H1N1)pdm09
	A/Hong Kong/4801/2014 (H3N2)
	B/Brisbane/60/2008

There also were seasonal differences in the levels of specific antibodies titers and, consequently in protective factors, in the age groups investigated. Considering the value of the B-type hemagglutinin protective factor, it reached its maximum and minimum in the children groups. In the currently analyzed 2016/2017 season, its lowest value of 20.7% was in the group of 5–9 years and the highest of 72.7% in 10–14 years of age. In the preceding 2015/2016 season, conversely, protective factor was lowest (8.7%) in the group 0–4 years, highest (61.3%) in 5–9 years, and intermediate (42.7%) in 10–14 years of age. In the past 2014/2015 season, protective factor was lowest (22.7%) in the group 10–14 years of age. On the other hand, protective factor was highest (76.7%) in the group ≥65 years of age in the 2014/2015 season (Kowalczyk et al. 2017), whereas it dropped to 30.7% in the current 2016/2017 season. For comparison, vaccination rate in this age group amounted to 7.65% in 2014; it fell to 6.97% in 2015 and down to 6.87% in 2016 (NIPH-NIH Report 2017).

For subtype A/California/7/2009 (H1N1) pdm09, protective factor was highest (49.3%) in the group 15–25 years and lowest (10%) in ≥65 years of age in the 2014/2015 season. In

the following 2015/2016 season, protective factor was highest (46.0%) in the group 5–9 years and again lowest (12.7%) in ≥65 years of age. In the currently analyzed 2016/2017 season, protective factor was highest (63.3%) in the group 10–14 years and lowest (20%) in 0–4 years of age.

For subtype A/H3N2/, protective factor was highest (31.3%) in the group 5–9 years and lowest (6.7%) in 40–64 years of age in the 2014/2015 season. In the following 2015/2016 season, protective factor again was highest (52.7%) in the group 5–9 years and lowest (12.7%) in the ≥65 years of age. In the currently analyzed 2016/2017 season, protective factor was highest (60.0%) in the group 10–14 years and lowest (14.0%) in 5–9 years and (14.7%) in 26–44 years of age.

In synopsis, protective factors against both subtypes of the influenza A antigen were several-fold higher in the age groups 10–14, 45–64, and ≥ 65 years in the 2016/2017 epidemic season compared to the 2014/2015 season. Comparing the 2016/2017 season to the 2015/2016 season, protective factors also were higher in all age groups except 5–9 years, albeit the increase in protection was here rather meager. In the group 5–9 years of age, protective factor was reduced in all tested influenza virus antigens. Protection against B-type hemagglutinin, generally, in the percentage terms was higher compared to that against A-type virus, also tended to be enhanced in the consecutive season, albeit to a much less extent (Table 3). A low level of protection against influenza antigen seems to reflect, in all likelihood, a dismally low vaccination coverage rate which, for instance, in the children aged 5–14 hovered around just 1% and in seniors aged ≥65 around 7% yearly in 2014–2015 (NIPH-NIH Report 2017).

4 Conclusions

Serological screening of serum collected from people of different ages during the 2016/2017 influenza epidemic season confirmed the circulation of the following three antigenic strains of the virus: subtypes A, A/California/7/2009 (H1N1) pdm09 and A/Hong Kong/4801/2014 (H3N2), and type B, B/Brisbane/60/2008. A calculated protective factor against the viral strains above outlined, having to do with previous infection or vaccination, appeared higher in the 2016/2017 epidemic season compared with the two past seasons. Protection was also greater against type B than type A influenza virus. Nonetheless, inadequate protection reflects a persistently low level of influenza vaccination coverage rate, fluctuating around 3% in the general population, with 1% in children and 7% in persons aged over 65, on the yearly basis.

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Table 3 Comparative display of the protective factor values in recent successive epidemic seasons

Antigens	Age groups (years)							Epidemic seasons
	0–4	5–9	10–14	15–25	26–44	45–64	≥65	
A/H1	30.7	30.7	16.7	49.3	25.3	16.0	10.0	2014/2015
	16.7	46.0	45.3	28.0	13.3	20.0	12.7	2015/2016
	20.0	25.3	63.3	28.7	21.3	40.7	29.3	2016/2017
A/H3	20.7	31.3	8.7	24.7	20.7	6.7	14.7	2014/2015
	18.0	52.7	34.0	20.0	13.3	19.3	12.7	2015/2016
	22.0	14.0	60.0	56.0	14.7	33.3	26.7	2016/2017
HB	62.0	40.0	22.7	72.7	43.3	39.3	76.7	2014/2015
	8.7	61.3	42.7	44.7	41.3	56.7	27.3	2015/2016
	35.0	20.7	72.7	69.3	69.3	46.7	30.7	2016/2017

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Respiratory Infections with Particular Emphasis on Influenza Virus Activity in Persons Over 14 Years of Age in the Epidemic Season 2016/2017 in Poland

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Abstract

Influenza viruses cause respiratory infections every epidemic season regardless of the patient's age. The aim of this study was to determine the activity of respiratory viruses in the epidemic season 2016/2017 in Poland, with particular emphasis on influenza viruses among people aged over 14. There were 2982 clinical samples taken from patients from four age groups: 15–25, 26–44, 45–64, and ≥ 65 years tested under the Sentinel and non-Sentinel surveillance programs. The presence of influenza viruses was confirmed in more than 40% of cases, the predominant type was influenza A virus unsubtyped, followed by subtype A/H3N2/. The results for the four age groups indicate that the highest number of confirmed respiratory viruses was recorded in individuals ≥65 years of age, slightly less, in the decreasing order, in the age groups 45–64 years, 15–25 years, and 26–44 years. Influenza type B infections were observed only in sporadic cases. Given the epidemiological data, epidemic season 2016/2017 was characterized by similar dynamics compared to the previous season in Poland and also in most of the European countries. More than 4 million cases and suspected cases of influenza and influenza-like viruses have been reported in Poland, more than 16,000 hospitalizations, and 25 deaths, of which 20 cases occurred among people ≥65 years of age in the epidemic season 2016/2017.

Keywords

Epidemic season · Infectious diseases · Influenza · Influenza virus activity · Molecular diagnostics

1 Introduction

Infections caused by influenza A and B viruses are observed each season, and the course of illness may differ from one another (Brydak 2008). In each case, however, quick confirmation of the presence of the influenza virus is important since inappropriately treated infections can lead to sometimes deadly complications, especially among people from

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the risk groups (Fiore et al. 2011). Generally, the diagnostic process is carried out using molecular biology methods (Bednarska et al. 2016b), which allows the obtaining of results promptly. The use of antiviral treatment with neuraminidase inhibitors reduces the risk of complications, also often emanating from the respiratory tract (Bednarska et al. 2015; Brydak 2015).

The epidemic season 2016/2017 in Poland was characterized by a high incidence of influenza and influenza-like illness (ILI) (4,919,110) and of the number of hospitalizations (16,890). The number of deaths due to complications amounted to 25 cases in persons over 14 years of age, out of which 20 cases were reported in persons older than 65 years (NIPH-NIH 2017). The influenza activity was rather moderate with the peak incidence of ILI at week 4 of 2017, whereas the highest number of confirmed influenza viruses was observed at week 3 of 2017. Not without significance remains the fact that the proportion of population in Poland vaccinated against influenza is declining in Poland season by season. In 2016, only 2.48% people aged over 14 and 6.87% people aged over 65 were vaccinated, the proportions comparable to those in the preceding year (EPIMELD 2016). The dismal influenza vaccination coverage rate is particularly relevant in the face of a clearly increasing incidence of respiratory infections in adult persons in recent years. The aim of the present study was to evaluate the activity of influenza and influenza viruses in individuals in the age bracket of 14–65 years in the epidemic season 2016/2017 in Poland.

2 Methods

The study was approved by an institutional ethics committee, and it was conducted in accordance with the Declaration of Helsinki for Human Research. The study material consisted of 2982 clinical specimens taken from patients from four age groups (15–25, 26–44, 45–64, and $\geq$ 65 years) within the Sentinel and non-Sentinel surveillance programs. The specimens were nasal and throat swabs, and bronchial alveolar lavage fluid (BALF), collected during the epidemic season 2016/2017. All laboratory investigations were performed in the Department of Influenza Research, National Influenza Centre in the National Institute of Public Health-National Institute of Hygiene (NIC NIPH-NIH) in Warsaw and in 16 Voivodeship Sanitary Epidemiological Stations (VSES) in Poland. The tests investigated in VSES were reinvestigated in the reference laboratory in the (NIC NIPH-NIH).

Viral RNA was isolated using the Maxwell 16 Viral Total Nucleic Acid Purification Kit (Promega Corp; Madison, WI) according to the manufacturer's instructions. The isolates were eluted in 50 μL of RNase-free water. To determine the presence of influenza viruses, real-time RT-PCR (qRT-PCR) was performed in capillary tubes using a Roche LightCycler 2.0 System (Roche Diagnostics; Rotkreuz, Switzerland) and SuperScript ® III/Platinum ® Taq Mix (Invitrogen by Life Technologies-Thermo Fisher Scientific; Carlsbad, CA). Primers and probes for the analysis were obtained through the International Reagent Resources (IRR), part of the Centers for Disease Prevention and Control in the USA. Positive controls consisted of the RNA isolated from the reference viruses A/California/7/2009 (H1N1) pdm09, A/Hong Kong/4801/2014 (H3N2), and B/Brisbane / 60/2008 as recommended by the WHO for the epidemic season 2016/2017. The RNase-free water was used as a negative control.

A conventional multiplex RT-PCR was performed to confirm the presence of influenza-like viruses, using a RV15 OneStep ACE Detection Kit (Seegene; Seoul, South Korea). Specimens were tested for the following respiratory viruses: influenza A and B; adenoviruses (ADV); respiratory syncytial viruses (RSV) A and B; human metapneumovirus (HMPV); human coronavirus (HcoV); human parainfluenza viruses 1, 2, 3, and 4; human bocavirus (HboV); and enteroviruses.

3 Results

In total 2882 clinical specimens were tested in people aged over 14. The presence of influenza viruses and influenza-like infections (ILI) was

confirmed in 46.5% of cases. The percentages of respiratory viruses stratified by age groups and virus types are shown in Figs. 1 and 2, respectively. The highest percentage of infections was noticed in the age group ≥65 years (49.4%), slightly less in 45–64 years of age (44.9%), while in the 15–25 and 26–44 years of age, the percentage was at a comparable level of about

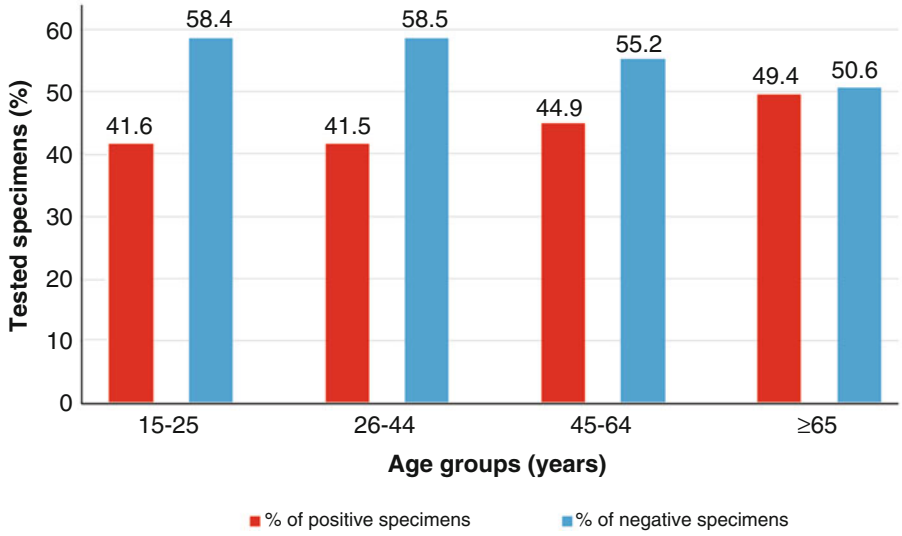

Fig. 1 Percentage of confirmed cases of influenza and influenza-like viruses in people over the 14 years of age in the epidemic season 2016/2017 in Poland

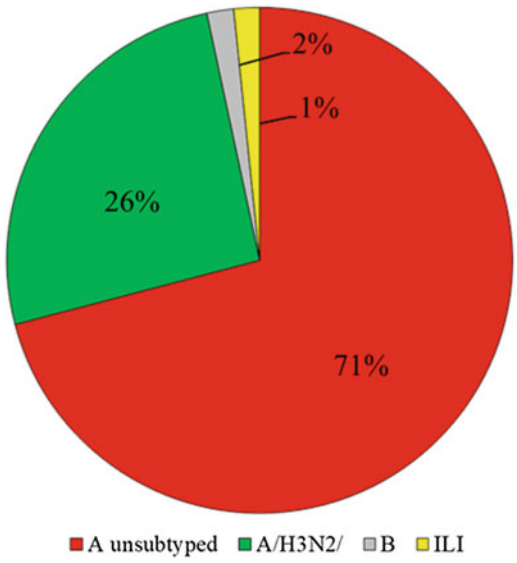

Fig. 2 Percentage of influenza and influenza-like virus infections in persons over 14 years of age in Poland in the epidemic season 2016/2017

41.5%. Taking all age groups combined, 71% of confirmed infections were caused by influenza A unsubtyped, 26% by A/H3N2/ subtype, and 2% by influenza B virus. Other respiratory viruses were confirmed in 1% of cases (Fig. 2).

In the season 2016/2017, infections caused by influenza A virus remained prevalent (Fig. 3), with the highest number of confirmed cases noted in the age group ≥65 (n = 356). In age groups 45–64 and 26–44 years, infections caused by influenza A unsubtyped amounted to 279 and 230 cases, respectively. The lowest number of confirmed influenza A unsubtyped cases was observed in the age group 15–25 years (n = 86). The presence of influenza virus subtype A/H3N2/ was observed across all age ranges. The highest number of confirmed cases of A/H3N2/ subtype was observed in the age groups 26–44 and 45–64 years, 105 and 104, respectively, slightly lower in the age group ≥65 years (n = 91), and the lowest in 15–25-year-old subjects (n = 45). There were single cases of influenza B virus, but

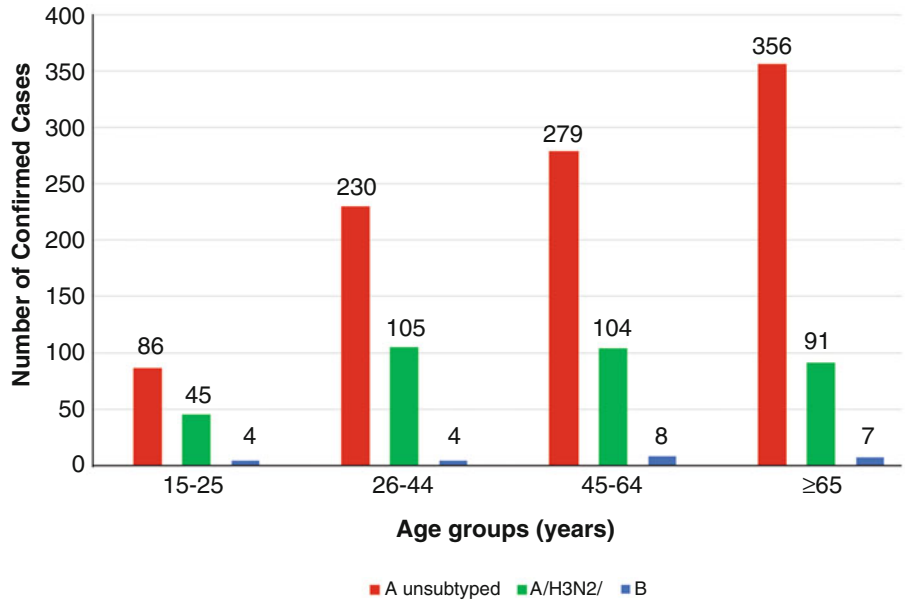

Fig. 3 The number of confirmed cases of influenza virus infection in people over 14 years of age in Poland in the epidemic season 2016/2017 in age groups

no A/H1N1/pdm09 (Fig. 3). During the epidemic season 2016/2017, there were only 22 cases of ILI caused by other influenza respiratory viruses reported in people aged over 14, with the predominance of RSV (n = 11).

4 Discussion

Comparing epidemiological data, the epidemic season 2016/2017 was characterized by similar intensity to the preceding 2015/2016 season. The number of cases and suspected cases of influenza and influenza-like viruses and the number of hospitalizations were akin to each other in both seasons, exceeding 4 million cases (NIPH-NIH 2017). However, there was almost sixfold fewer deaths due to postinfection complications in the season 2016/2017 (Table 1).

During the epidemic season 2016/2017, the number of specimens analyzed to confirm influenza infections was lower than that in the previous seasons. However, the percentage of confirmations among people aged over 14 was higher (45.0%) compared with the seasons 2015/2016 (40.2%) and 2014/2015 (21.2%) (Kowalczyk et al. 2017a; Bednarska et al. 2016b). Akin to the season 2014/2015, the influenza A unsubtyped (71%) virus prevailed in 2016/2017, followed by the A/H3N2/ subtype (26%) (Hallmann-Szelińska et al. 2016). While in the season 2015/2016 influenza A/H1N1/pdm09 was the prevailing subtype, there were no confirmed cases of this subtype among people aged over 14 years in the season 2016/2017 (Kowalczyk et al. 2017a).

Concerning the age-dependent differences in the type of influenza virus causing infections (Fig. 1), the highest number of confirmations in 2016/2017 was observed in persons over 65 years of age (49.4%), which was different from 2015/2016 when the highest number was in those in the age brackets of 45–64 and 26–44 (Kowalczyk et al. 2017a). The results are consistent with the data from other EU countries concerning influenza virus infections; the prevalence of A/H3N2/ subtype was noted in the season2016/2017, especially in persons aged over 65, who were most vulnerable to infection, according to the ECDC risk assessment (Flu News Europe 2017; ECDC 2016). An additional factor that differentiates the epidemic seasons 2014/2015 and 2015/2016 from

Table 1 Epidemiological indicators for influenza and influenza-like virus infections in the 2015/2016 and 2016/2017 epidemic seasons in Poland (according to NIPH-NIH)

Epidemic season	ILI cases (n)	Morbidity (n)	Hospitalizations (n)	Deaths (n)	Specimens (n)
2015/2016	4,107,077	10,685.2	15,969	140	8542
2016/2017	4,811,501	12,519.2	16,602	25	4078

NIPH-NIH National Influenza Center of the National Institute of Public Health-National Institute of Hygiene in Warsaw, Poland

2016/2017 is a pronounced decrease in the number of influenza B virus infections in the latter season. In 2014/2015, the presence of influenza B virus was reported in 34.1% of cases (Bednarska et al. 2016b). In 2015/2016, B virus was the dominant type in the 15–25 and 26–44 age groups; its presence was confirmed in 201 cases in the latter group. In contrast, in the season 2016/2017 there were only single confirmed cases of influenza B virus (Fig. 3).

Comparing confirmed ILI infections among individuals aged over 14 in the epidemic seasons 2015/2016 and 2016/2017, the number of confirmations was at a comparable level ($n = 16$ and $n = 22$, respectively). In both seasons, also in 2014/2015, RSV infections were predominant (Kowalczyk et al. 2017b; Bednarska et al. 2016a).

Given the increase in the number of infections among the elderly over 65 years of age in the analyzed season 2016/2017, a low percentage of people vaccinated against influenza in Poland appears to be an important factor. Although in many voivodships vaccination for this age group is paid by the marshal's offices, 6.87% of them have been vaccinated (NIC 2017). There also was an alarming increase in the number of deaths due to complications among the elderly, 20 out of the 25 cases in 2016/2017, compared with the 56 out of the 140 influenza-related deaths in the preceding season. This dramatic increase in the proportion of influenza-related deaths in the elderly should signal an urgent need for intensified attempts to increase the vaccination coverage in Poland. According to the recommendations issued by WHO, 15 academic medical societies, and 143 national influenza centers, vaccination is the only and most effective method of preventing influenza complications and deaths (NIC 2017; Grohskopf et al. 2016; Brydak et al. 2012; Brydak 2008).

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Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Influenza: Underestimated in Children Below 2 Years of Age

A. Wrotek, M. Czajkowska, E. Zawłocka, and T. Jackowska

Abstract

Children under 2 years of age may receive antiviral therapy when influenza is suspected. Signs of influenza are frequently unclear and testing is indicated. The aim of the study was to assess the usefulness of clinical signs and the rapid influenza diagnostic test (RIDT) in diagnosing influenza and in choosing the appropriate treatment. In the 2015–2016 influenza season, 89 children under 2 years of age (56.7% of 157 children diagnosed with influenza) were hospitalized. There were 74 RIDT and 70 reverse transcription polymerase chain reactions (RT-PCR) performed for the purpose of diagnosis, either test per child. Eighty-three percent of children (74/89) presented with fever, 55.1% (49/89) with cough, and 39.3% (35/89) with both cough and fever. The RIDT was positive in 31.1% (23/74) of cases. The highest percentage of positive RIDT was within the first 24 h of disease, decreasing dramatically thereafter (70% vs. 13–17%, respectively). The RIDT shortened the time to diagnosis by 43.8 h/patient (an average €149 gain in treatment costs). The mean delay for RT-PCR-based diagnosis was 33.5 h/patient (an average €114 loss in treatment costs). We conclude that clinical signs have a low diagnostic sensitivity in children under 2 years of age. Likewise, RIDT is of low sensitivity, being diagnostically useful only in the first 24 h. The PCR is recommended for the diagnosis, but that requires a constant access to the method.

Keywords

Children · Diagnostics · Costs · Infants · Influenza · Rapid diagnostic test · Reverse transcription polymerase chain reaction · Sensitivity

1 Introduction

Influenza is a serious global health concern and especially younger children are at a higher risk of complications. There are various prevention strategies and treatment approaches, but the majority of them is consistent in identifying children under 2 years of age as a high-risk group as stated by the Committee on Infectious Diseases of the American Academy of Pediatrics (AAP 2017). According to the Centers for Disease Control and Prevention (CDC 2018), antiviral treatment should be implemented, inter alia, in all children younger than 2 years with a suspicion of influenza. Polish recommendations also identify

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children under 2 years of age as a high-risk group, in which antiviral treatment should be administered as early as possible (Jackowska 2016). It is crucial for the effectiveness of the treatment that it is implemented immediately – optimally, within the first 48 h of disease onset. A major problem is a proper diagnosis and the time limit when it should be made. From the epidemiological standpoint, "an influenza-like illness" (ILI) is defined as fever of at least 37.8 °C and sore throat or cough. Influenza, on the other hand, is defined as a disease with acute onset, high fever, cough, headache, and myalgia. Referring to these symptoms and definitions, the diagnosis may be problematic, especially in younger children who cannot verbally communicate symptoms. Thus, more sophisticated diagnostic tools are required, among which viral culture, real-time reverse transcription polymerase chain reaction (qRT-PCR) test, and antigen detection such as a rapid influenza diagnostic test (RIDT) are of increasing research and clinical interests. These methods may be performed on samples obtained from nasopharyngeal or nasal swabbing, aspirate, or lavage. A drawback of viral cultures is that it takes time to get a result, usually from 3 to 10 days. Therefore, cultures are mainly used for the epidemiological purpose or as a verification of other methods. The qRT-PCR has high sensitivity and specificity (of approx. 86–100% and 99%, respectively) (Frisbie et al. 2004) but requires more sophisticated and pricey equipment, and qualified personnel, which makes it of limited availability. In contradistinction, RIDT is inexpensive, easy to perform, and not requiring specialized equipment or highly trained personnel and gives a quick result in up to 15 min. On the downside, RIDT has suboptimal sensitivity, which is related to a higher probability of false-negative results. Sensitivity of RIDT in young children is another issue. The available studies show sensitivity of 47–70% (Eggers et al. 2015), but there are also studies that suggest that it may be as low as 23% (Koul et al. 2015). On the other hand, specificity of RIDT is satisfying, reaching 98–100% (Avril et al. 2016).

The present study seeks to assess the frequency of signs and symptoms of influenza, deemed typical, and the treatment time and costs gained/lost using the RIDT vs. RT-PCR diagnostics.

2 Methods

The study was approved by the Ethics Committee of the Medical Center for Postgraduate Education in Warsaw, Poland. The study has a retrospective character. In the 2015/2016 influenza season, 163 children at the Bielanski Hospital in Warsaw were diagnosed with influenza, including 157 hospitalizations. Out of them, 89 children (56.7%; 51 male and 38 female children) were younger than 2 years of age and were included in this study. The mean age of children was 7.4 ± 6.4(SD) months, and their number by the age groups was as follows: 9 neonates (0–1 months; 4 male and 5 female), 22 children aged 1–2 months (15 male and 7 female), 17 children aged 3–6 months (13 male and 4 female), 16 children aged 7–11 months (7 male and 9 female), and 25 children aged 12–23 months (12 male and 13 female).

When influenza was suspected, RIDT, PCR, or both were used for diagnosis. Altogether, 74 RIDT and 70 PCR were performed. In 55 patients both methods were used. In general, the PCR was performed only in case of a negative RIDT result, but in six patients, the PCR was performed after obtaining a positive RIDT result. Seventy-two patients were diagnosed with influenza type A and ten patients with influenza type B, and there were seven coinfections of type A and B. The children's parents/guardians were asked about the typical influenza signs and symptoms, including fever, cough, coryza, difficulties in breathing, apnea, seizures, headache, myalgia, chest pain, malaise, and altered mental status or anxiety.

Complications occurred in 52 patients. In most cases complications consisted of lower respiratory diseases, such as pneumonia (29 cases), bronchitis with obturation (25 cases), bronchitis without obturation (6 cases), and laryngitis (3 cases); otitis media also was a frequent complication (10 cases). One case of encephalitis was

noted as well. Some patients had several coexisting complications; each single complication was treated separately, but the patients were not further analyzed in terms of single/multiple complications.

In order to calculate the time gained or lost as a result of positive or negative RIDT test, certain assumptions need to be presented: a minimal time of 5 h or 24 h, including the time required for the transport of samples to the external laboratory cooperating with the hospital, needed to obtain the PCR result during working days (Monday to Friday). The maximum time to get the PCR results is 72 h for patients admitted to the hospital on Friday, as the first available date for the transport of samples performing PCR was Monday morning. Thus, in general, for patients who were admitted between Friday noon and Saturday, the mean time needed to obtain the PCR result was 72 h or 24 h for those admitted on Sunday. A gain in time was calculated for patients who had a true-positive result of RIDT, but when RIDT was false negative, the time needed to obtain the PCR result was treated as a loss of time. The exact admission time was omitted in the analysis, as it varied in each case, but the mean time should be around the time estimated above.

To calculate the costs generated with the use of RIDT, a simple assumption was made: the earlier the result, the faster the implementation of treatment, meaning a faster discharge and shorter hospitalization. For these calculations, we used the time gained or lost (as above outlined) and multiplied it by the officially published full medical cost of a patient-*per*-day hospitalization at the pediatric ward of the Bielanski Hospital in Warsaw, which amounted to €81.5. Since we analyzed the influence of a diagnostic method on treatment costs, false-negative RIDT results were also taken into calculations, as they may generate an additional and unnecessary cost. Both RIDT and PCR used showed positive or negative results separately for influenza type A and type B. The specific costs of tests were as follows: RIDT *ca* €9 and PCR *ca* €50.

Additionally, four theoretical diagnostic models were created, based on the study results: (1) RIDT in each child, followed by PCR in those who had a negative result (the percentage of true-positive RIDT results was extrapolated to the whole group of 89 children); (2) PCR in each child without a prior RIDT test; (3) use of PCR if it were available at the hospital's laboratory every day around the clock, with the provision, as in the first model above outlined that RIDT comes first, and when negative it would be followed by PCR; and (4) PCR in each child available every day around the clock, without a prior RIDT.

Data distribution was analyzed with the Shapiro-Wilk test, and means ±SD or medians with upper-lower centiles were given in case of normal or non-normal distribution, respectively. The independent-samples *t*-test or Mann-Whitney U test were used accordingly. A p-value of 0.05 defined the statistically differences. The evaluation was performed with a commercial statistical package Statistica v12 (Statsoft, Tulsa, OK).

3 Results

Seventy-four RIDT were performed and were positive in 25 cases, including 14 cases of influenza type A, 10 cases of influenza type B, and 1 case positive for both influenza type A and B. In six patients, initially diagnosed with influenza type A in two cases and type B in four cases, samples were further sent for a PCR diagnosis, which confirmed two cases of influenza type A infection, but among four patients diagnosed with type B, only one case was confirmed, and one turned out to be a mixed infection (type A and B), while the other two were diagnosed with the PCR as influenza type A (instead of type B). These last two cases were considered false-positive results in further analysis, although they confirmed the influenza infection as such. Finally, true-positive RIDT results were obtained only in 23/74 (31.1%) tests performed, corresponding to 25.8% of patients (23/89 children). There was a group of 15 patients in whom the RIDT was not performed and the diagnosis was based only upon the PCR.

The PCR was performed in 70 patients, including the 15 patients without the RIDT. The

diagnosis was based upon the PCR method in 66 patients (73.3%): 15 patients without a prior RIDT, 49 patients with a false-negative RIDT result, and 2 false-positive RIDT results. In one patient, the PCR helped verify the diagnosis as the RIDT was positive only for type B infection, while there turned out a mixed type A and B infection.

According to the age groups, not a single positive RIDT result was observed in neonates (0/5), 53% of positive results in children 1–2 months old (10/19), 14% in children aged 3–6 months (2/14), 36% in children aged 7–11 months (5/14), and 27% in children aged 12 months and more (6/22) (Table 1).

There was no difference between the group of patients who had the RIDT performed and those who had not in terms of age, duration of fever before hospital admission, the total feverish period, and length of hospitalization, nor the duration of signs and symptoms before hospital admission. However, the only difference observed was between the groups of patients with a true-positive RIDT result and a true-negative/false-positive RIDT result, and it was statistically significant for the duration of signs and symptoms before admission (median value 1 vs. 3 days, $p = 0.001$). This is supposedly related to a higher percentage of positive RIDT results in children who had been presenting with signs/symptoms for a shorter period of time (Table 2). For the sake of a practical purpose, the results are also shown as a growing number of patients and a growing duration of influenza signs/symptoms (Table 3). The highest percentage of positive RIDT results was observed in children who had been presenting with influenza signs/symptoms for up to 24 h (70%), and then the percentage was decreasing (from 41% in patients with symptoms lasting no more than 48 h to 31% in the whole group, including patients who had been sick for even more than 7 days). A great difference was observed between the first 24 h of disease onset and the rest of the group (70% vs.13–17%). Due to a low number of cases, the higher percentage (33%) of positive results in children who had been presenting symptoms for 121–144 h may be treated as a bias and omitted in further analysis.

The patients presented with the following signs and symptoms: the most frequent was fever in 74/89 (83.1%) children, followed by cough (49/89 children, 55.1%), coryza (46/89; 51.7%), difficulties in breathing (21/89; 23.6%), seizures (8/89; 9%), and apnea (3/89; 3.4%). Due likely to young age, no headache, myalgia, or chest pain were reported. The parents also reported malaise observed in 19 (21.3%) and altered mental state/anxiety in 14 (15.7%) cases. A typical clinical picture, i.e., fever and cough, was seen only in 35 patients (39.3%).

The performance of RIDT and PCR was a clinical practice, not belonging to theoretical models proposed above. The cost of 74 RIDTs and 70 PCRs in this study group was €4,153 with a mean of €46.7/patient. The mean hospitalization time was 9.2 days. This value was used as a reference in calculations presented below. The mean hospital treatment cost (excluding diagnostics) was €751.

In patients with a positive RIDT result, the mean time gained was 43.8 ± 21.3 h. The gain amounted to 36.4 ± 29.1 h in case of the shorter 5-h delay in obtaining the PCR result. The time gained thanks to the positive RIDT corresponded to the gain of €148.7 ± 72.2 thanks to the theoretically shorter hospitalization. In the 5-h delay model, the RIDT positive result gained €123.5 ± 99.5 (Table 4).

In patients with a negative RIDT result, when the PCR was needed to make a diagnosis, the mean time lost was 33.5 ± 20.5 h or 21.0 ± 27.2 h in the 24-h and 5-h delay models, respectively. The PCR use and the delay related to this corresponded to the loss of €113.8 ± 69.4 or €71.3 ± 92.3, respectively. The total costs of a PCR-related delay and a PCR-based diagnosis was €170.6 ± 68.4 or €128.1 ± 91.3, respectively (Table 4).

Had the RIDT been performed in each patient (not just in 74 out of the 89 patients, i.e., in 83%), then the time and money gained in the 24-h and 5-h delay models would have been 44.8 ± 23.0 h or 34.7 ± 32.2 h and €152.7 or €117.6, respectively. It needs to be underlined that there still is a low probability of a positive RIDT result (31% in the

Table 1 Rapid influenza diagnostic tests (RIDTs) performed in different age groups

Age group	No. of patients	No. of RIDT	No. of positive RIDT	% of positive RIDT
0–1 month	9	5	0	0
1–2 months	22	19	10	53
3–6 months	17	14	2	14
7–11 months	16	14	5	36
12–23 months	25	22	6	27

Table 2 Relation between the percentage of positive rapid influenza diagnostic tests (RIDT) and the length of influenza signs and symptoms

Duration of influenza signs/symptoms (hours)	No. of patients	No. of RIDT	No. of positive RIDT	% of positive RIDT
< 24	24	20	14	70
25 − 48	26	24	4	17
49–72	9	6	1	17
63–96	9	6	1	17
97–120	10	8	1	13
121–144	3	3	1	33
> 144	8	7	1	14

Table 3 Relation between the percentage of positive rapid influenza diagnostic tests (RIDTs) and the length of influenza signs and symptoms, shown as a growing number of patients and a growing duration of influenza signs/symptoms

Duration of influenza signs/symptoms (hours)	No. of patients	No. of RIDT	No. of positive RIDT	% of positive RIDT
Up to 24	24	20	14	70
Up to 48	50	44	18	41
Up to 72	59	50	19	38
Up to 96	68	56	20	36
Up to 120	78	64	21	33
Up to 144	81	67	22	33
Over 144	89	74	23	31

Table 4 Time and cost gained/lost in case of rapid influenza diagnostic test (RIDT)-based or polymerase chain reaction (PCR)-based diagnosis and in patients without RIDT

		PCR – 24-h delay	PCR – 5-h delay
Positive RIDT (n = 23)	Time gained (h)	43.8 ± 21.3	36.4 ± 29.1
	Money gained (€)	148.7 ± 72.2	123.5 ± 99.0
Negative RIDT, need for PCR (n = 51)	Time lost (h)	33.5 ± 20.5	21.0 ± 27.2
	Money lost (€)	113.8 ± 69.4	71.3 ± 92.3
	Total cost of PCR and its delay	170.6 ± 302.2	128.0 ± 99.0
RIDT in patients without RIDT* (n = 15)	Time to be gained (h)	44.8 ± 23.0	34.7 ± 32.2
	Money to be gained (€)	152.7	117.6

*Low probability of positive RIDT result (31% in the study group)

entire study group), so that the time and costs gained should be, roughly estimated, divided into three.

Theoretical models were created based on these categories. In the four theoretical models mentioned above, the estimated costs would present as follows:

First Model: RIDT in Each Child and PCR if RIDT Negative

The total cost of RIDTs in 89 children and PCRs in those with negative RIDT result (with the assumption of 31% of true-positive RIDT results, meaning that 61 patients would still require additional PCR testing) would be 16,980PLN/€3,842 (Table 5). The total mean time gained would be 43.8 h in the 24-h (1st model A) or 36.4 h in the 5-h PCR delay model (1st model B). This total time gained was used for calculating a reduction of hospital treatment costs. For the group of 28 patients with a theoretically positive RIDT, with the mean time of 1,226.4 h gained in the 24-h delay model or 1,019.2 h in the 5-h delay model, the fiscal gain would correspond to €4,162 or €3,459, respectively.

Second Model: Omission of RIDT, PCR in Each Child

The cost of 89 PCRs would be €4,430. The mean time lost was 33.5 h in the 24-h PCR delay model (2nd model A) or 21 h in the 5-h PCR delay model (2nd model B). This total time gained was used for calculating the increase of hospital treatment costs. For the whole group of 89 patients waiting for the PCR result, it would mean 2,981.5 h or 1,869 h (in the 24-h and 5-h delay models, respectively), which would correspond to hospital treatment costs being higher by €2,289 or €6,343. The lowest possible cost of diagnostics and treatment would be €712 *per* patient in the 1st model, assuming a shorter 5-h delay for the PCR result (Table 5). The cost in this case would be €63,376, which was used as a reference value for the next two models.

Third Model: RIDT in Each Child and then Rapid PCR in Those with a Negative Result

The percentage of true-positive RIDT results was extrapolated to the whole group of 89 children. The cost of RIDTs in all 89 children and 61 rapid PCRs would be €805 and 61x, where "x" stands for the unknown maximum tolerable price of a single rapid PCR testing, which is to be established (see Table 6). The total time gained, mean of 43.8 h in the 24-h PCR delay model (3rd model A) or 36.4 h in the 5-h PCR delay model (3rd model B), was used for calculating the reduction in the hospital treatment costs. The time would be gained in all patients, irrespectively, of the final diagnostic method, be it RIDT or PCR. The hospital treatment cost reduction would be €13,229 (model A) or €10,994 (model B).

Calculation of a Possibly Tolerable Rapid PCR Price (Called "x") The lowest total cost of the two models (1st and 2nd) was used as a reference value: €63,376. From this value the theoretical cost of €54,411 or €56,646 was subtracted, and then the result was divided by 61. The tolerable price range came out between €110 and €147.

Fourth Model: Rapid PCR in Each Child Without Prior Performance of RIDT

The cost of diagnostics in this model is unknown (89y, where "y" stands for the price of a single rapid PCR). When from the total cost of hospital treatment, shown in the 3rd model, the time and money gained were subtracted and the remaining part were divided by 89 rapid PCRs, the price range would emerge between €76 and €101 (Table 6).

3.1 Optimum Model

Based on the results presented above, the crucial aspects appear to be the patient's age and the time from onset of influenza symptoms to the performance of RIDT. Among the age groups, the null probability of a positive RIDT result was seen in neonates. The issue of perfect timing is also of a key importance; the shorter the disease duration, the higher the probability of a positive RIDT result. When the symptoms lasted for no longer than 24 h (in 24 patients), the probability of a

Table 5 Time and cost gained/lost in the 1st and 2nd diagnostic theoretical model

Theoretical model	No. of RIDT	Cost of RIDT (€)	No. of PCR	Cost of PCR (€)	Diagnostic cost (€)	Hospitalization cost (€)	Reduction/increase in hospital treatment costs (€)	Total cost (€)	Mean cost *per* patient (€)
1st A (24-h)	89	805	61	3,036	3,842	295,409	–4,162	66,514	747
1st B (5-h)	89	805	61	3,036	3,842	66,835	−3,459	63,376	712
2nd A (24-h)	0	0	89	4,430	4,430	66,835	10,118	76,953	865
2nd B (5-h)	0	0	89	4,430	4,430	66,835	6,343	7,3177	822

RIDT rapid influenza diagnostic test, *PCR* polymerase chain reaction. In each model, calculations were as follows: cost of RIDT = number of RIDTs multiplied by the cost of a single RIDT; cost of PCR = the cost of a single PCR multiplied by the number of PCRs needed to make a diagnosis; cost of hospitalization = number of patients (89) multiplied by 9.2 days, multiplied by the mean patient-*per*-day cost (€81.4); reduction of hospital treatment costs = 43.8 for 24-h or 36.4 for the 5-h delay model multiplied by the number of patients with positive RIDT (reduction of costs) multiplied by €3.4 (cost of patient-*per*-hour hospitalization). Reduction of costs was marked with the "–"symbol. In case of hospital treatment, the cost increase = number of patients with PCR, multiplied by the mean delay to obtain a diagnosis (33.5 h or 21.0 h in the 24-h and 5-h delay model, respectively) multiplied by €3.4 (cost of patient-*per*-hour hospitalization). The total cost was then calculated and divided by the number of patients to show the mean cost per patient

Table 6 Time and cost gained/lost in the 3rd and 4th diagnostic theoretical models

Theoretical model	No. of RIDT	Cost of RIDT (€)	No. of PCR	Cost of single rapid PCR (€)	Diagnostics cost (€)	Hospitalization cost (€)	Reduction in hospital treatment costs (€)	Total cost (€)	Mean cost *per* patient (€)
3rd A (24-h)	89	806	61	x	806 + 61x	66,835	−13,229	54,411 + 61x	x = 147
3rd B (5-h)	89	806	61	x	806 + 61x	66,835	−10,994	56,646 + 61x	x = 110
4th A (24-h)	0	0	89	y	89y	66,835	−13,229	54,411 + 89y	y = 101
4th B(5-h)	0	0	89	y	89y	66,835	−10,994	56,646 + 89y	y = 75.6

RIDT rapid influenza diagnostic test, *PCR* polymerase chain reaction. In each model, calculations were as follows: cost of RIDT = number of RIDTs multiplied by the cost of a single RIDT; cost of hospitalization = number of patients (89) multiplied by 9.22 days, multiplied by the mean patient-*per*-day cost (€81.4); reduction of hospital treatment costs = 43.8 for 24-h or 36.4 for 5-h delay model multiplied by the number of patients with positive RIDT (reduction of costs), multiplied by €3.4 (cost of patient-*per*-hour hospitalization). Reduction of costs was marked with the "–"symbol. The total cost after the reduction due to a faster diagnosis was calculated next and then divided by the required number of the rapid PCR tests to establish the PCR price

Table 7 Time and cost gained/lost in the optimum diagnostic model

Theoretical model	No. of RIDT	Cost of RIDT (€)	No. of PCR	Cost of PCR (€)	Diagnostic cost (€)	Hospitalization cost (€)	Reduction in hospital treatment costs (€)	Total cost (€)	Mean cost *per* patient (€)
Optimal A (24-h)	43	389	70	3,484	3,873	66,156	−2,824	64,010	719
Optimal B (5-h)	43	389	70	3.484	3,873	66,156	−2,347	64,488	725

RIDT rapid influenza diagnostic test, *PCR* polymerase chain reaction

positive RIDT was 70%; when they lasted for up to 48 h (in 50 patients), this probability declines to 41%. The optimum time cut-off mark seems 48 h, as it accounts for more than half of patients admitted to the hospital, and still the probability is higher than otherwise. The optimum diagnostic model would then only include patients with no more than 2-day-long disease duration, except of neonates. Hence, 50 out of the 89 patients would be eligible for RIDT. With the neonates excluded, the remaining 43 children would be eligible. In fact, 40 of them had the RIDT done, with 18 positive results (45%). Extrapolating these data, 43 RIDTs are performed and 19 patients are diagnosed. In the remaining 70 patients, the PCR would be the method of choice. The total cost of this diagnostic path would be €3,873, with an average of €43.5 *per* patient. The general cost of hospitalization would be €66,156, and after taking into account a reduced of hospital stay, the mean cost *per* patient would be in a range of €719–725 (Table 7).

4 Discussion

The American Academy of Pediatrics (AAP 2017) recommends that all children aged 6 months and older should be protected against influenza with vaccination. Moreover, all persons in the household who have contact with children younger than 5 years of age should also be vaccinated. Obviously, the preferred method of flu-fighting is vaccination, but when there is a suspicion of influenza, antiviral medications should be administered. The AAP recommendations for antiviral treatment include children hospitalized with a suspicion of influenza; children hospitalized for severe, progressive, or complicated influenza or influenza-related-disease; as well as patients from high-risk groups suspected of influenza, independently of the disease severity. Antiviral treatment should also be considered to any healthy child, especially when living at a household with other children under 6 months of age, i.e., being too small to be vaccinated. The need for prevention seems to be increasingly crucial as there are many problems with diagnosing influenza in children. Clinical signs and symptoms cannot be used in young children either to confirm or exclude influenza. The only sign that was present in a substantial percentage of children (83%) in the present study was fever, but fever as such is the most frequent sign in children hospitalized, so that it is a highly unspecific sign. Clinical findings are of little use in diagnostic utility concerning influenza (Call et al. 2005). Yet some studies suggest the opposite that experienced general practitioners may be able to correctly diagnose influenza on their judgment, from a constellation of typical symptoms, on par or even better than from the results of modern laboratory techniques (van Elden et al. 2001). The present study did not evaluate the sensitivity, specificity, and positive nor negative predictive value of the infection signs, but it shows that the signs or the lack of symptoms in children under the age of 2 years may be especially misleading. A strong emphasis should be put on keeping in mind influenza as one potential causative factor of fever in young children. That especially refers to children hospitalized.

The so-called suboptimal sensitivity of the RIDT seems to be even lower than expected in

children under 2 years of age. The percentage of true-positive results was very poor in the entire study group (31%), which is grossly in line with the surprisingly low 23% sensitivity shown in a study by Koul et al. (2015) and below the generally considered RIDT sensitivity. The most frequently mentioned factors that influence RIDT results are the age of patients, the time when RIDT was performed, and the virus type and viral load (Busson et al. 2014). Further, simple errors in obtaining specimens or test storage may cause false results. To avoid such problems, in the present study, only physicians trained in performing RIDT performed the patients' swabbing and collected samples that were stored in controlled laboratory conditions. The criteria in which patient the RIDT should be performed remain questionable, as they mainly rest on clinical decisions. The most important interfering factor was the time period between onset of infection signs and the performance of RIDT. The highest percentage of positive results (70%) noticed was too low to find it satisfying. This percentage is still considered suboptimal, and it was seen only in patients admitted within the first 24 h from onset of infection. Then, the percentage decreased dramatically. From the practical standpoint, patients are rarely referred to the hospital during the first day of influenza, which hampers the diagnostic investigation on influenza. A low number of patients with influenza type B and the lack of viral load measurements were limitations in the valuation of the present results.

Even after narrowing the eligible group of patients to those who had presented signs for no more than 48 h (neonates excluded), in our theoretical "optimal" model of diagnosis, the costs related to diagnostics and hospitalization are not much lower than those in the study group. A truly optimal diagnostic path would include PCR testing, available around the clock every day. New options are under development, and special attention is paid to expanding the availability and simplicity of molecular techniques, including multifactorial analyses of the most frequent respiratory tract pathogens (Pham et al. 2017; Malhotra et al. 2016). For the purpose of clinical practice, we presented the most tolerable price of a single PCR analysis. As of 2016/2017, the Bielanski Hospital in Warsaw, Poland, launched new diagnostic facilities with a 24 h a day availability of the rapid PCR (approx. 5 h from taking the sample to the final result) for children hospitalized, to diagnose the presence of influenza viruses and respiratory syncytial virus (RSV).

5 Conclusions

Clinical signs of influenza are often not present in children younger than 2 years of age, so there is a strong need for taking influenza into account as the possible etiological factor of an infection in the upper respiratory tract, particularly in hospitalized children. The rapid influenza diagnostic test shows low sensitivity in comparison to molecular biology techniques. Sensitivity of the test depends mostly on the time delay between onset of infection and the performance of test, reaching 70% only during the first 24 h. Neonates, in particular, are at higher risk of false-negative test results. Molecular diagnostic methods, seeking the determination of viral DNA, such as the polymerase chain reaction, are pricey and much time-consuming for the time being. The methodological advancements are underway to make these modern methods less expensive, rapid, and widely accessible.

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Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Infections with Influenza A/H3N2/ Subtype in Poland in the 2016/2017 Epidemic Season

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Abstract

The 2016/2017 epidemic season was characterized by a lower number of diagnostically tested samples of the nasal and throat swabs and bronchoalveolar lavage fluid, compared with the preceding season. The predominant influenza subtype found was A/H3N2/ which was notably diagnosed in patients over 25 years of age. This subtype was also often diagnosed in older people of 65+ years, which is in line with the risk assessment prepared by the European Center for Disease Prevention and Control (ECDC) at the beginning of the season. The A/H3N2/ subtype was most often diagnosed in the West Pomeranian and Warmian-Masurian Voivodeships. In this epidemic season, there were 11 coinfections of the A/H3N2/ subtype with other influenza and influenza-like viruses recorded in Poland. A different situation had occurred in the 2014/2015 season, when the subtype A/H3N2/ also was predominant, but the virus was most commonly diagnosed in children up to 14 years of age. In both seasons, the least confirmations were observed in patients between 15 and 24 years.

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Keywords

Epidemic season · Influenza · Influenza-like viruses · Respiratory infection · Throat swabs

1 Introduction

Influenza is a seasonal illness, but influenza viruses circulate in the environment at all times. Influenza is caused by influenza virus types A and B. These viruses are divided into subtypes according to their antigenic properties and surface hemagglutinin and neuraminidase antigens (Brydak 2012). In the Northern Hemisphere, the highest incidence of infection occurs in winter, and the subtypes causing the epidemic vary each season. In the 2013/2014 and 2014/2015 seasons, both A/H3N2/ and A/H1N1/pdm09 subtypes were observed in the population (Hallmann-Szelińska et al. 2016; Bednarska et al. 2015). The A/H3N2/ subtype was not recorded in the 2015/2016 season. The purpose of this study was to report which influenza virus exerted in controlling influence in the most recent 2016/2017 season in Poland, the epidemiological information essential for selecting the antigenic content of successive seasonal influenza vaccine by the World Health Organization agencies (WHO 2016).

2 Methods

2.1 Clinical Specimens

The study was approved by an institutional ethics committee, and it was conducted in accordance with the Declaration of Helsinki for Human Research. In the 2016/2017 epidemic season, over 3000 specimens consisting of the nasal and throat swabs and bronchoalveolar lavage fluid (BALF) were collected for testing. The collection was performed in 16 Voivodeship Sanitary Epidemiological Stations (VSES) across the country from week 40/2016 to week 39/2017 (October–September). Viral content of the samples was initially determined at VSES. The results were reported to the Department for Influenza Research in the National Influenza Center, National Institute of Public Health-National Institute of Hygiene in Warsaw, where they were reinvestigated by molecular methods for confirmation.

2.2 Extraction of Viral RNA

Viral RNA was isolated using a Maxwell 16 Viral Total Nucleic Acid Purification Kit (Promega Corporation, Madison, WI) from 200 μL of clinical samples in phosphate-buffered saline (PBS) in accordance with the manufacturer's instructions for low elution volume cartridges. The RNA was eluted with 50 μL of RNase-free water.

2.3 Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

The qRT-PCR was performed using a LightCycler 2.0 setup (Roche Diagnostics, Rotkreuz, Switzerland). The reaction mixture was in 20 μL capillaries, containing $MgSO_4$, BSA (bovine serum albumin), reaction buffer, RNA (RNase-free water), SuperScript® III/Platinum® Taq Mix purchased from the Invitrogen Life Technologies (Carlsbad, CA), and 0.5 μL probe and 0.5 μL (20 nM) primers. Probes and primers were obtained from the International Reagent Resources of the Centers of Disease Control (IRR-CDC), a US-based biological reagent depository. Five microliters of RNA was added to each prepared mixture. Negative control constituted RNA-free water, while positive control constituted RNA strains of the influenza vaccine viruses for the 2016/2017 season: A/H1N1/California/7/2009, A/H3N2/Hong Kong/4801/2014, and B/Brisbane/60/2008.

2.4 Conventional Multiplex RT–PCR

The use of RT-PCR enables the confirmation of the presence of respiratory viruses: influenza A and B viruses; RSV A and B (respiratory syncytial virus); hMPV (human metapneumovirus); AdV (Adenovirus); RV (rhinovirus); hCoV 229E/NL63 and OC43/HKU1 (human coronaviruses); PIV-1, PIV-2, PIV-3, and PIV-4 (human parainfluenza viruses); EV (enterovirus); and hBoV 1/2/3/4 (human bocaviruses). In this study an RV15 OneStep ACE Detection Kit (Seeplex, Seoul, South Korea) was used, according to the manufacturer's instructions. The resulting product was separated using a 2% agarose gel electrophoresis.

3 Results and Discussion

In the 2016/2017 epidemic season, 3977 samples were tested for the presence of respiratory viruses. A preliminary testing, performed by the country's Voivodeship Sanitary Epidemiological Stations, diagnosed influenza infection in 228 samples. These samples were reinvestigated at the National Influenza Center, National Institute of Public Health-National Institute of Hygiene in Warsaw, Poland, and the infection was confirmed in 205 specimens. Of the influenza viruses, unsubtyped type A accounted for 66.8% of all confirmed influenza infections. Subtype A/H3N2/ constituted 31.2% of all confirmations; type B amounted to 2.0% of confirmed infections (Fig. 1). In the 2016/2017 epidemic season, the

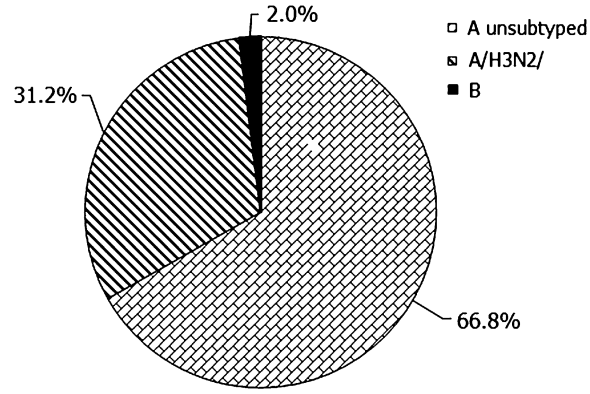

Fig. 1 Prevalence of individual types and subtypes of influenza viruses in the 2016/2017 season in Poland

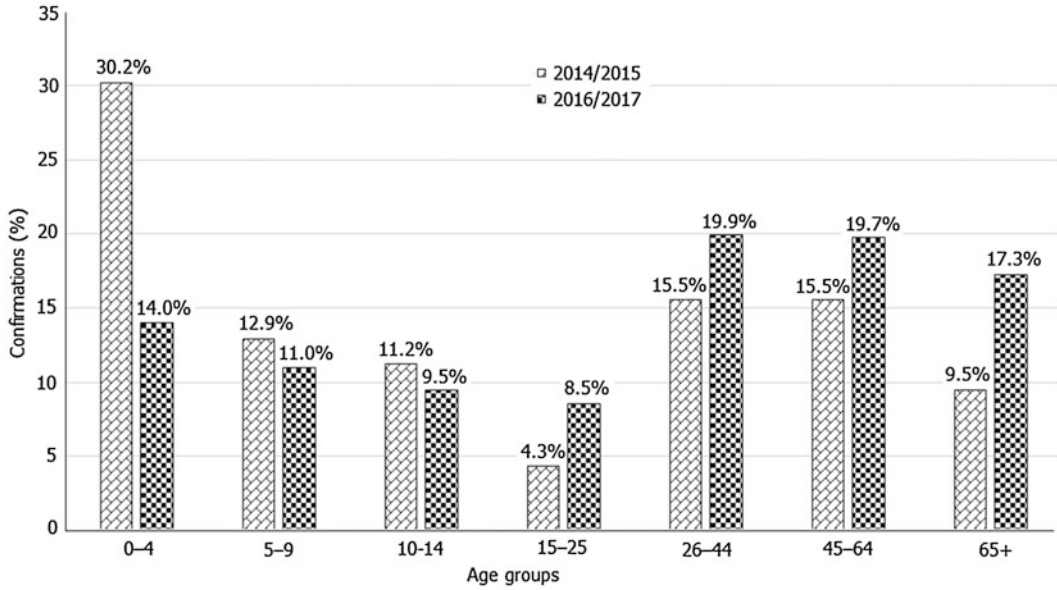

Fig. 2 Comparison of confirmed influenza infections with subtype A/H3N2/ in successive aged groups in the 2014/2015 and 2016/2017 epidemic seasons in Poland

A/H3N2/ subtype was most frequently diagnosed in people aged 26–44 years, accounting for 19.9% of all confirmed infections with this subtype, followed by 45–64 years, where it constituted 19.7% and 65+ years with 17.3%. In the children age groups of up to 14 years of age, the A/H3N2/ subtype accounted for about 10–14% of infections; the prevalence of infection was the lowest in adolescents. This pattern of infection distribution by the age groups was grossly similar to that in the 2014/2015 season, with two major exceptions. In that past season, prevalence of subtype A/H3N2/ was about twice greater for the age 0–4 and twice smaller for 65+ years compared with the 2016/2017 (Fig. 2).

An analysis of the prevalence of influenza subtype A/H3N2/ infections across Poland failed to show any relevant geographical pattern. The infection turned out most prevalent in the West Pomeranian, Mazovian, and Lower Silesian Voivodeships, distantly situated from one another. Likewise, there were no infection noticed in the diametrically distant Opolskie and Warmian-Masurian (Fig. 3). There also were nine

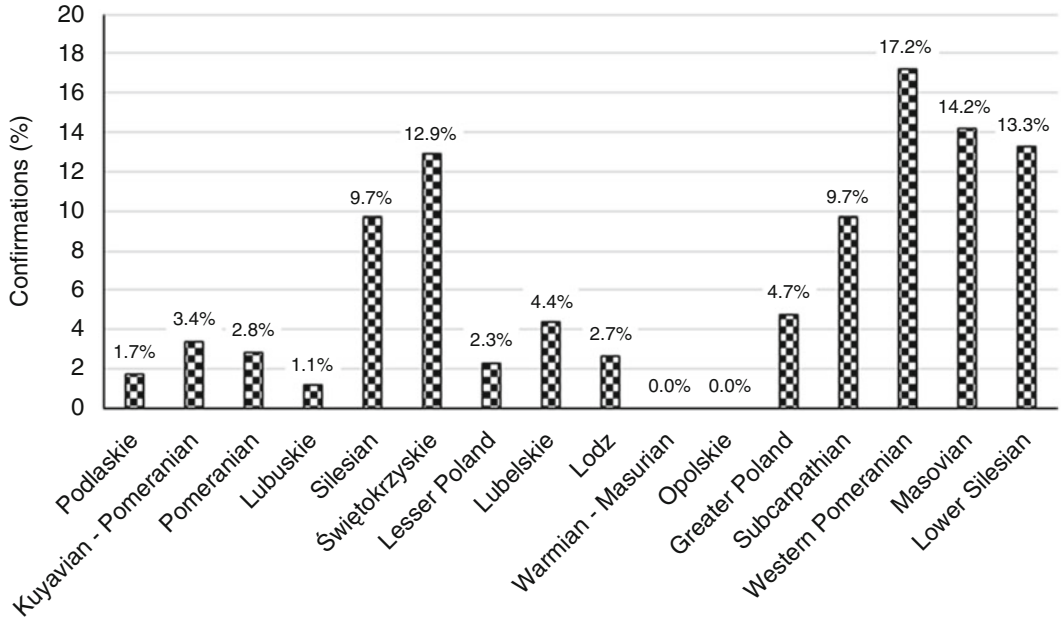

Fig. 3 Prevalence of the confirmed infections with influenza virus subtype A/H3N2/ in the Polish provinces in the 2016/2017 epidemic season

Table 1 Coinfections with influenza virus subtype A/H3N2/ in the 2016/2017 epidemic season in Poland

Viruses	Patient age (years)
A/H3N2/ + B	4
A/H3N2/ + B	8
A/H3N2/ + B	50
A/H3N2/ + B	56
A/H3N2/ + B	67
A/H3N2/ + B	70
A/H3N2/ + B	70
A/H3N2/ + B	70
A/H3N2/ + B	79
A/H3N2/ + RSV	4
A/H3N2/ + RSV	70

RSV respiratory syncytial virus

coinfections of A/H3N2/ subtype and influenza type B recorded, as well as two cases of influenza-like virus infections in the 2016/2017 season (Table 1).

The goal of this study was to describe seasonal variability in the influenza virus or its subtype circulating during recent epidemic seasons in Poland. We found that the A/H3N2/ subtype of influenza virus predominated in Poland during the 2016/2017 season. This finding contrasted with the preceding 2015/2016 season when, although somehow fewer samples were tested, the A/H1N1/pdm09 subtype predominated (Cieślak et al. 2017; Kowalczyk et al. 2017; Szymański et al. 2017a). However, the A/H3N2/ also was a leading subtype in the 2014/2015 season, although it circulated together with the A/H1N1/pdm09 subtype (Bednarska et al. 2016). Vice versa, in the still earlier 2013/2014 season, the A/H3N2/ circulated together with the then predominant A/H1N1/pdm09 (Bednarska et al. 2015). These findings suggest that the two influenza virus subtypes, A/H3N2/ and A/H1N1/pdm09, assume the leading epidemiological role alternately every other year.

The epidemiology of influenza infection shows a variable pattern in recent seasons. There were fewer confirmed cases of the A/H3N2 subtype in children up to the age 14 and more of these infections in the other age groups in the 2016/2017 season compared with the 2014/2015 season (Fig. 2). These results are in line with the

European Center for Disease Prevention and Control risk assessment published in January 2015, pointing to a more frequent diagnosis of infection caused by the A/H3N2/ subtype in children up to the age 4 years in the 2014/2015 season (ECDC 2015), with a high 75% confirmation rate of the presence of influenza and influenza-like virus genetic material (Hallmann-Szelińska et al. 2016). A reverse situation was observed in the 2016/2017 season, when the elderly were reported to be at the greatest risk for influenza virus infection in Poland (Fig. 2) (ECDC 2016).

In the 2016/2017 season in Poland, there were 11 coinfections of the A/H3N2/subtype with other influenza and influenza-like viruses, particularly found in the elderly and young children (Table 1). In contrast, in the preceding 2015/2017 season, the presence of the A/H3N2/subtype was not confirmed in any of the coinfections (Szymański et al. 2017a). For comparison, in the past 2014/2015 and 2013/2014 seasons, there were only two and four coinfections recorded with this subtype, respectively, in children up to 14 years (Hallmann-Szelińska et al. 2016; Bednarska et al. 2015).

The predominant circulation of the A/H3N2/ subtype in the 2016/2017 season has also been observed in the countries neighboring Poland as well as in other parts of the world (WHO 2017a). In Europe, two-thirds of the cases diagnosed as the A/H3N2/ infection were identified as belonging to subclass 3C.2a1 that is antigenically close to the 3C.2a, a subclass from which the vaccine was derived for the subsequent 2017/2018 epidemic season (Flu News Europe 2017, WHO 2017b). Data collected in this study point to a typical pattern of seasonal influenza epidemic, with the majority of infections in the age groups of 0–4, 5–9, and 65+ years, i.e., the most vulnerable age categories. The predominant influenza virus subtype seems irregularly variable in successive seasons. In 2013/2014 and 2014/2015, co-predomination of the A/H1N1/pdm09 and A/H3N2/ subtypes was observed, whereas A/H1N1/pdm09 predominated alone in 2015/2016 and A/H3N2/ in the 2016/2017 season. Nor could any regularity be substantiated in the geographical distribution of the most affected regions across the country in successive epidemic seasons (Szymański et al. 2017b).

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Conflicts of Interest The authors declare no conflicts of interests in relation to this article.

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Disorders of Humoral Immunity in Children with IgG Subclass Deficiency and Recurrent Respiratory Infections

Gerard Pasternak, Aleksandra Lewandowicz-Uszyńska, and Katarzyna Pentoś

Abstract

Respiratory tract infections in children are one of the most common causes for medical consultations. When the infections are of recurring nature, they are a major reason for the diagnostics for primary immunodeficiency that is in about 65% of cases underlain by disorders of humoral immunity. This study seeks to retrospectively evaluate the history of recurrent respiratory tract infections in children with humoral disorders and the associations among deficiencies in the immune system components. We evaluated 394 children aged 3 months to 18 years. We found 49.5% (195 cases) of children with IgG deficiencies, all of whom had normal IgE levels. There were 8.4% (33 cases) of IgA deficiency, 7.4% (29 cases) of IgM insufficiency, and 4.1% (16 cases) of CD19+ cells deficiency. The elevated level of CD19+ cells was found in 27.7% (109 out of the 394 children). Immunoglobulin deficiencies often coexisted with a deficiency in another immunoglobulin class above outlined. There was an interdependence between IgA abnormality and IgG, IgG3, and IgG4 abnormalities as well as between IgM abnormality and IgG and IgG1 abnormalities. We conclude that respiratory tract infections in children are often underlain by a convergence of IgG with both IgA and IgM abnormal states. The physiopathological meaning of this convergence for the infection course and resulting functional respiratory changes remains elusive.

Keywords

Children · Humoral disorders · Humoral immunity · IgG deficiency · Immune deficiency · Respiratory infections

1 Introduction

Immunodeficiency is a state of impaired immunity balance in the body, which usually predispose to a variety of infections. Congenital immunodeficiency is rather rare, but when untreated may have a severe course, sometimes leading to death. Diagnostic tests and treatment should be undertaken as soon as possible to avoid serious infections that could cause a permanent damage. Humoral immunity is associated with

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circulating antibodies, notably involving IgA, IgG, IgM, and IgE classes. The immune system in children develops relatively slowly, as it starts functioning in the mature way around the age of 12 years. Younger children are, in the main, vulnerable to develop a disease when in contact with infectious agents (Nicholson 2016). The problem is potentiated when there exists a background humoral immunodeficiency, giving rise recurrent, severe, and difficult-to-treat infections. Such disorders go beyond and over the usual 6–8 benign upper airway infections *per* year, the frequency assumed as a customary norm for healthy preschool children. Normally, with increasing age of a child, there is a decrease in the incidence of infections. However, in children with primary immunodeficiency, infections may occur dramatically, often one after another, making it hard to treat, and without regaining full health in-between. Immunodeficient children notably suffer from chronic sinusitis and bronchitis. Recurrent or chronic infections can inhibit the growth and development of a child.

Humoral immunity disorders constitute the most frequent group of primary immune deficiency accounting for about 65% of related diseases. The group encompasses isolated IgA deficiency, a common congenital immunodeficiency, variable immunodeficiency syndrome, X-linked agammaglobulinemia, IgG subclass deficiencies, specific antibody deficiency, hyper-IgM and IgE syndromes, and other rare occurrences such as insufficient IgM or IgE deficiency (Carroll and Isenman 2012). In pediatric practice, 50% of children consulted for frequent respiratory infections have a normally functioning immune system. Another 30% suffer from various allergies, 10% have anatomical defects or inborn errors of metabolism, and 10% of children have abnormalities in the immune system (Glocker et al. 2007).

Respiratory infections are one of the most common reasons for medical consultations. When recurring nature, such infections are a major reason for the diagnostics toward primary immunodeficiency (Raby et al. 1996). Serum immunoglobulins are routinely determined in clinical practice as they provide key information on the status of the humoral immune system. On the other hand, low levels of immunoglobulins may indicate the presence of some of the humoral immunity deficiencies. For the interpretation of laboratory data, it is important to determine population-based reference intervals of immunoglobulin levels. That is particularly important for the physician to distinguish between the healthy and diseased patient when significant differences are encountered due to age, gender, or other, for instance, environmental factors (Horn and Pesce 2003; NCCLS 2000; Sasse 1992). Nonetheless, studies focusing on the possible influence of these factors on serum immunoglobulin levels are limited, particularly in the children population. In adults, IgM level has been shown higher in women than men. The concentration of immunoglobulins in the serum increases with age (Ichihara et al. 2004; Giltay et al. 2000; Stoica et al. 1980; Maddison and Relmen 1976).

Studies devoted to the deficiency of individual immunoglobulins and of IgG subclasses in children are scarce. Therefore, in this study we set out to evaluate the history of recurrent respiratory infections underlain by deficiency of specific immunoglobulins and the possible mutual associations between deficient immunoglobulins in the pediatric population.

2 Methods

The study was approved by the Ethics Committee of the Medical University in Wroclaw, Poland, and it was conducted according to the ethical principles for medical research as set by the Declaration of Helsinki of the World Medical Association. This retrospective study consisted of the evaluation of clinical history of children suffering from recurrent respiratory tract infections (RTI), indices of humoral immunity, and the interdependence between the immune system components. The study group consisted of 394 patients, aged from 3 months to 18 years, including 152 (38%) females and 242 (62%) males, all of whom were hospitalized with suspicion of primary immune deficiency disorders as the underlying cause if RTI. The serum level of four major classes of

immunoglobulins, IgA, IgG, IgM, and IgE, was measured with an immunoturbidimetric analyzer (Architect c-System; Abbott Laboratories, Lake Bluff, IL). The level of IgG subclasses was measured with a nephelometric analyzer (BN ProSpec System; Siemens Healthcare GmbH, Erlangen, Germany). Both automatic analyzers use a set of reagents provided by the manufacturers. Further, interdependence between deficient immunoglobulins and the association of immunoglobulin deficiency with demographic factors, such as age and gender, also was assessed. Children with a history of allergy or elevated IgE immunoglobulin were excluded from the study.

Continuous data were reported as means ±SD and categorical data as percentages of patients with each immunoglobulin deficiency. Statistical analysis was based on the Kruskal–Wallis or Mann-Whitney U test, and on Chi-square test for the respective data types. A p-value <0.05 defined statistically significant differences. The analysis was carried out using a commercial statistical package of Statistica v10 (StatSoft, Tulsa, OK).

3 Results

Overall, there were 12.2% of children (48 out of the 394) with RTI who had a deficiency in total IgG (IgGt). There also were deficiencies of IgA – 8.4% (33 cases), IgM – 7.4% (29 cases), or CD19 + cells – 4.1% (16 cases), whose percentage was small, not exceeding 10% (Fig. 1). There were significant differences among the children counts regarding each immunological deficiency ($p < 0.05$; Chi^2). The level of the immunoglobulins investigated in the absolute terms, including IgG subclasses, is presented in Table 1.

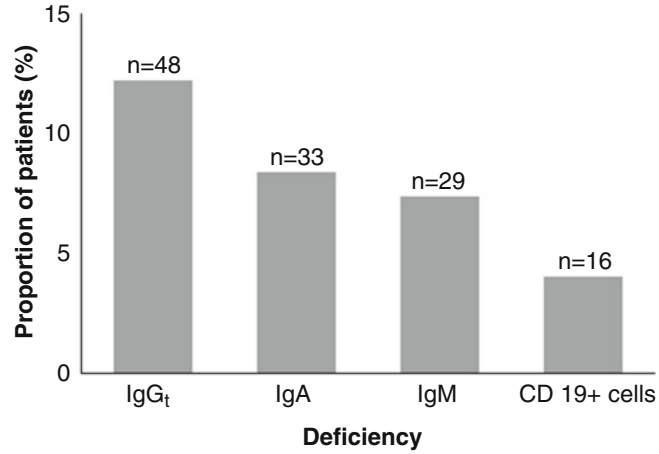

Fig. 1 Proportion of patients deficient in IgA, IgM, and CD19+ cells among the 394 children investigated

Table 1 Immunoglobulin levels (g/L) in children with respiratory tract infections

Immunoglobulin	Deficient	n	Non-deficient	n
IgA	0.06 ± 0.01	33	0.76 ± 0.46*	361
IgM	0.31 ± 0.06	29	0.81 ± 0.29*	365
IgGt	3.90 ± 0.87	48	7.30 ± 2.26*	346
IgG1	3.10 ± 0.95	108	5.51 ± 1.63*	286
IgG2	0.48 ± 0.38	24	1.46 ± 0.76*	370
IgG3	0.16 ± 0.07	70	0.32 ± 0.16*	324
IgG4	0.04 ± 0.08	79	0.26 ± 0.24*	315

Data are means ±SD; *IgG,* total immunoglobulin; *in the column Non-deficient indicate significant differences between deficient and non-deficient immunoglobulin level ($p < 0.0001$) according to Mann–Whitney U test

There often was a coexistence of multi-IgG subclass deficiencies as well as deficiency of IgG subclasses accompanying deficiencies in the other major immunoglobulin classes, which was subject to further evaluation. Overall, 196 (49.7%) out of the 394 children had some deficiency in IgG subclasses.

3.1 Deficiency in IgG Subclass in Children with Respiratory Tract Infections Deficient in IgA, IgM, and CD19+ Cells

Out of the 33 children with IgA deficiency, 33.3% (11 cases) had decreases in IgG1, 12.1% (4 cases) in IgG2, 27.5% (9 cases) in IgG3, and 39.4% (13 cases) in IgG4 (Fig. 2). The corresponding results on IgG subclass deficiency among the 29 children with IgM deficiency were shown in Fig. 3. Here, the largest subgroup of 34.5%

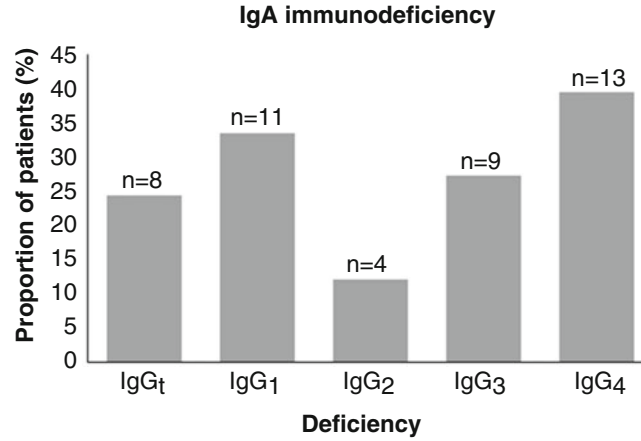

Fig. 2 Proportion of patients having deficiencies in IgG subclasses among the 33 children deficient in IgA

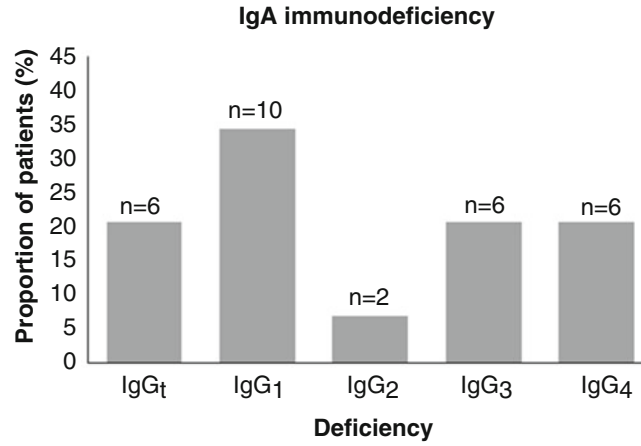

Fig. 3 Proportion of patients having deficiencies in IgG subclasses among the 29 children deficient in IgM

(10 cases) was with IgG1 deficiency, followed by 6.9% (2 cases) with IgG2, and 20.7% (6 cases) with IgG3 and IgG4 deficiencies each. Generally, proportion of children deficient in IgG subclasses was higher in IgM abnormality (9.6%) than in that in IgA abnormality (5.2%) ($p < 0.05$).

Figure 4 shows the proportion of IgG subclass deficiencies among the 16 children deficient in CD19+ cells. There were a relatively large number of children with deficiencies in all IgG subclasses, from 31.3% (5 cases) for IgG3 to 43.8% (7 cases) for IgG4.

The percentage of children with deficiency in total IgG was similar in IgA, IgM, and CD19+ deficient classes above outlined and amounted to about 19–23%.

Differences among the children counts, representing deficiency in individual IgG subclasses failed to reach statistical significance in the groups deficient in IgA, IgM, and CD 19+ cells ($p > 0.05$; Chi^2).

3.2 IgG Subclass Deficiency in Children with Non-deficient Levels of IgA, IgM, and CD19+ Suffering from Respiratory Tract Infections

More than one half of the children with RTI had the level of major immunoglobulin classes above the lower cut-off limit, although they may have been short of the normal level of IgG subclasses. A proportion of non-IgA deficient children but deficient in IgG subclasses is shown in Fig. 5. This proportion ranged from 26.9% for IgG1 to

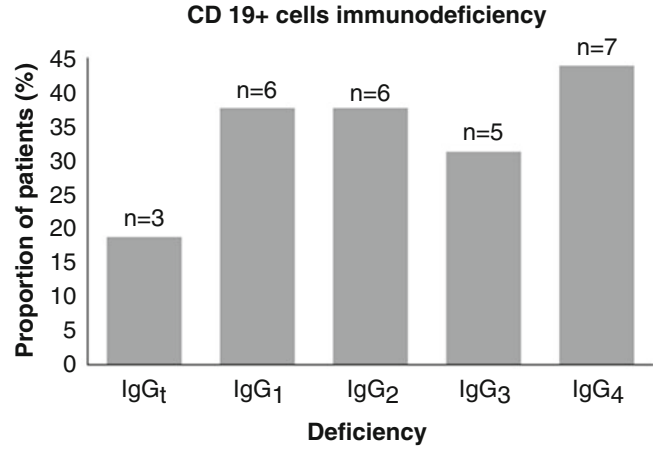

Fig. 4 Proportion of patients having deficiencies in IgG subclasses among the 16 children deficient in IgM

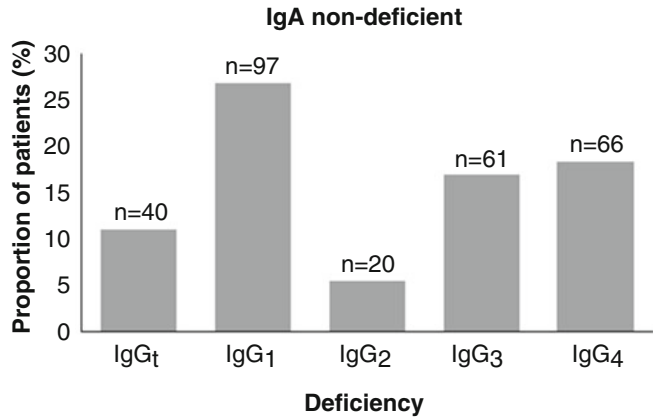

Fig. 5 Proportion of patients having deficiencies in IgG subclasses among the 33 non-IgA deficient children

5.5% for IgG2 and was smaller across all IgG subclasses compared to the IgA-deficient children, as shown in Fig. 2. Similar proportions of multi-IgG subclass deficiency were noticed in the non-IgM deficient children (Fig. 6); these proportions also were lower across all IgG subclasses compared to those in children with IgM deficiency, as shown in Fig. 3. Likewise, the proportion of IgG subclass deficiency was clearly lower in children who lacked deficiency in CD19+ cells (Fig.7) compared to those who were CD19+ cell deficient, as shown in Fig. 4. The difference was particularly distinct for IgG2 and IgG 4, whose proportions were severalfold lower in CD19+ non-deficient children. There were significant differences among the children counts, representing deficiency in individual IgG subclasses in the groups non-deficient in IgA, IgM, and CD19+ cells ($p < 0.05$; Chi^2), as opposed to those with insufficiency of major immunoglobulin classes outlined in the preceding subsection.

4 Discussion

The interdependence of immunoglobulin deficiency, underlying recurrent respiratory infections in children, has been rather rarely tackled in medical research. In this study, we found that deficiency of the IgG immunoglobulin class and its subclasses is clearly predominant and may be present in case of both deficient and non-deficient other immunoglobulin classes, as

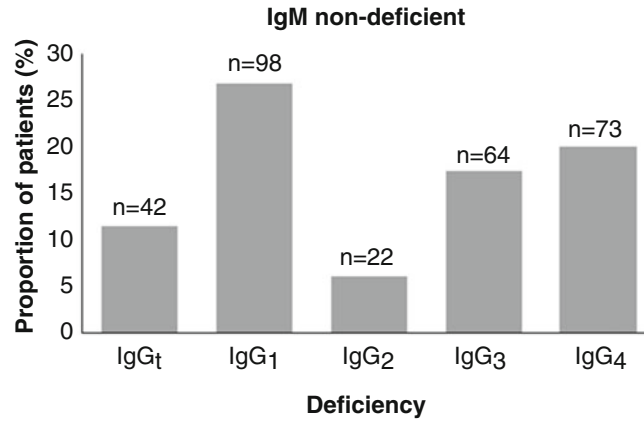

Fig. 6 Proportion of patients having deficiencies in IgG subclasses among the 33 non-IgM deficient children

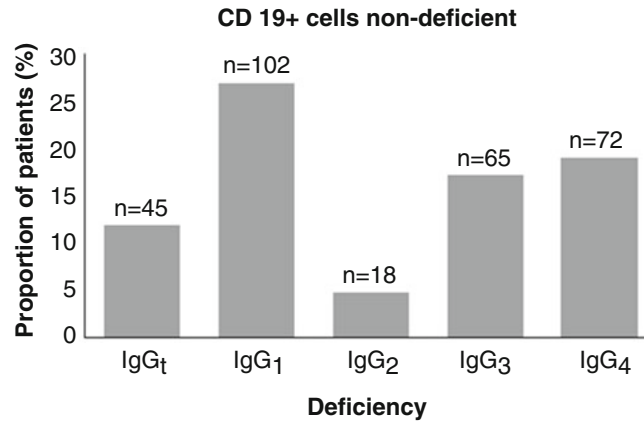

Fig. 7 Proportion of patients having deficiencies in IgG subclasses among the 33 non-CD19+ cells deficient children

well as CD19+ cells. These cells, belonging to B lineage cells in humans, are essential, inter alia, for the development and survival of the peripheral immune system in children. Dysfunction of CD19 + cells underlies immunodeficiency disorders characterized by diminished antibody production in response to infection. The present findings also demonstrate that IgG1 was the most often deficient immunoglobulin, whereas IgG2 most rarely deficient among IgG subclasses. We further found that a proportion of children deficient in IgG subclasses was higher in case of accompanying IgM abnormality than IgA abnormality. Of note, deficiency of IgG subclasses was clearly more expressed when it accompanied a deficiency in another major class of immunoglobulin, which suggests that insufficiency of immunity entails a mutually potentiating mechanism. Finally, the study confirms the presence of a substantial overlap of deficiencies of various types of major immunoglobulin classes, as well as the coexistence of multi-IgG subclass deficiencies during recurrent respiratory infections.

Bjorkander et al. (1988) have reported that IgA deficiency is usually accompanied by IgG2 and IgG4 deficiencies. The present study expands those findings by showing deficiency of total IgG, IgG3, and IgG4 in IgA deficient children. In case of selective IgM deficiency, a rare immunopathology, IgG subclass deficiency has been reported only in a few cases. Deficiency of IgG has so far been found unrestricted to a particular subclass, resulting in a variety of multi-immunodeficiency. That has also been confirmed for the association of IgG subclass deficiency with selective IgA deficiency (Yel et al. 2009; Ideura et al. 2008). In a study on serum immunoglobulins and lower respiratory tract infections in children with Down syndrome, Deepa et al. (2012) have shown a relationship between increased frequency of infections, on the one side, and reduced IgM and elevated IgG and IgA, on the other side. An understanding of production and mutual dependency of various immunoglobulin components remains an area of limited knowledge. Nonetheless, all the findings above outlined translate into the biological plausibility of a systemic impairment of the immune system maturity and function in children suffering from recurrent respiratory infections. The respiratory tract is the most common site of clinical manifestation of various immune deficiencies in children. Infectious and noninfectious respiratory complications determine the prognosis for such patients. The diagnostics directed at unraveling primary immune deficiencies could reduce morbidity and streamline the effectiveness of therapy, which would benefit the patient.

We conclude that respiratory tract infections in children are often underlain by a convergence of IgG with both IgA and IgM abnormal states. The meaning of this convergence for the infection course and thus functional respiratory changes remains to be explored in further studies.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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