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The level of natural autoantibodies to IFN-gamma in varicella infection treated with antiviral drug Anaferon for children: A pilot study

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ABSTRACT

Natural circulating antibodies (NAbs) to endogenous regulators have shown to be potential biomarkers in medicine. Due to the lack of reliable assays, only few of them have been well studied. To employ NAbs as biomarkers, an evaluation of changes over the course of a treatment is required. This paper describes our work to analyze the dynamics of NAbs titer to interferon-gamma (IFN-γ) among healthy children of different age and in patients with varicella infection receiving an antiviral drug Anaferon for children (AC, the API are highly diluted antibodies to IFN-γ) in comparison with placebo, and to correlate the findings with the treatment results. IFN-γ plays an essential role during varicella infection, and this fact causes the consequent increase of NAbs to IFN-γ level. The mean anti-IFN-γ NAbs level in the healthy volunteer group was 101 × 10^3 U/ml (age: 0–15 years), which was significantly lower than the mean pre-treatment value in patients with varicella infection 167 × 10^3 U/ml (age: 3–17 years). In the AC group, the NAbs level observed on days 5 and 10 decreased significantly to a level of 154 × 10^3 U/ml, whereas in the placebo group it continued to rise in a time-dependent manner reaching 229 × 10^3 U/ml on day 10. Our findings suggest that treatment with AC is characterized by "normalization" of the anti-IFN-γ NAbs levels in patients with varicella infection.

1. Introduction

Natural circulating antibodies (NAbs) found in the body have been a subject of a number of studies [1,2]. Especially many investigators have focused on NAbs specific for cytokines – a group of intercellular signaling proteins that regulate cell growth and development [3,4]. Cytokines (*e.g.* interleukines, tumor necrosis factors, interferons) are essential mediators of inflammation; corresponding specific NAbs are therefore recognized as important biologically active regulators of *in vivo* immune responses [5,6]. Continuous immunological processes, which occur also in healthy individuals, are accompanied by the generation of these NAbs in the blood. Substantially high levels of anti-cytokine NAbs are produced in patients with various infections, autoimmune diseases and tumors [7–16].

A close relationship exists between the production of a specific cytokine and the generation of the corresponding NAbs during a disease [17]. For example, NAbs specific for the cytokine interferon-gamma (IFN-γ), which plays an important role in regulating viral replication, have been found in the sera of patients with various viral conditions [18,19], mycobacterial infections [20–26], as well as in the sera of healthy volunteers [17,18]. A number of studies have demonstrated the presence of anti-IFN-γ NAbs in immunocompetent individuals with intracellular infection while being healthy in all other respects [27–29]. It is assumed that these antibodies neither inhibit the antiviral and antiproliferative effects of IFN-γ nor prevent the binding of IFN-γ to its cell receptors. Yet they are able to inhibit IFN-γ-induced HLA-DR antigen expression on some cells. In animal models, anti-IFN-γ Nabs were shown to be able to play a role in immunoregulation and fine tuning of the amplitude and duration of the immune response [18].

The blood concentration of anti-IFN-γ NAbs rises during the acute phase of a viral disease and decreases as the recovery progresses [7]. The measured specific features of these antibodies depend on multiple factors such as the type of infection, the patient's age and immunological status, *etc.* In the acute phase, interferon-alpha and IFN-γ have proven to be detectable in high quantities in the sera of patients with varicella infection. IFN-γ has been shown to be released by T-cells isolated from individuals during primary *in vivo* sensitization and *in vitro* stimulation with varicella virus antigens [30,31]. Taking into

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account the fact that anti-IFN-γ NAbs may reflect IFN-gamma production [18], we assumed that the serum level of anti-IFN-γ NAbs would change along with disease progression. Therefore, our primary objective was to determine anti-IFN-γ NAbs levels at different disease stages of varicella infection. Furthermore, since NAbs levels were reported to be influenced by age [32,33], we examined them in three age groups of healthy volunteers: < 5 years, 6–11, and 11–15 years old.

Available evidence suggests that some ethnic groups are more liable to anti-IFN-γ NAbs generation (*e.g.*, anti-IFN-γ NAbs are highly prevalent and recognized to be responsible for disease severity in Asian patients with disseminated nontuberculous mycobacterial infection [21–232834]). Nevertheless, the presence of NAbs to IFN-γ should be considered in different infectious diseases, regardless of a patient's age and ethnicity [35], particularly when IFN-γ is used in the treatment [36]. We also hypothesized that, apart from therapeutic IFN-γ, any other antiviral treatment with an effect on the immune system should have some influence on the anti-IFN-γ NAbs level.

Anaferon for children (AC) is an antiviral drug belonging to released-active compounds group [37,38] and based on highly diluted antibodies specific to IFN-γ, whose antiviral activity against different viruses has been demonstrated in previous non-clinical studies and clinical trials (among the latest are [39,40]. AC has been shown to be able to regulate the functional activity and production of endogenous interferons and to modulate the immune response depending on the initial body condition [41–46]. The AC effect was shown to be specific, and mechanism of its action lies in the modification of IFN- γ conformational characteristics [37,45,47,48]. Our findings suggest that the efficacy of the treatment corresponds to a level of NAbs to IFN-γ in the patients' sera.

2. Materials and methods

2.1. Patients

The samples for analysis were collected during two trials. The first one was "A double-blind, randomized, parallel group placebo-controlled clinical trial of the efficacy and safety of Anaferon for children in the treatment of patients with varicella" facilitated by Bashkir State Medical University (authorized by ethics committee as of 06 September 2007). The second one was a trial entitled "Determination of reference levels of natural anti-interferon gamma Abs in healthy children" conducted at the Moscow Health Department's City Children's Hospital No. 133 (approved by the hospital's local ethics committee as of 08 September 2015).

The first trial included patients aged 1–18 years old with varicella on days 1–2 of the eruptions. The diagnosis was proved by clinical symptoms including temperature higher than 37.5 °C, malaise and lesions (spots, papules, vesicles, scarlatina-like and measels-like rash). The patients have not received any kind of antiviral, antibacterial or immunomodulating therapy before being included in the trial. Written informed consents obtained from the parents/guardians were required. The exclusion criteria included the following: the presence of conditions requiring the immunomodulating or antiviral therapy; polyvalent allergy in the patients' background; the lack of tolerability of the drug components and taking part in the other clinical trials within one month before.

In case of the healthy subjects, the same criteria excluding the disease conditions were employed; on the contrary, the children were enrolled in the trial with no apparent signs of any infection.

2.2. Regimen of therapy

Each tablet of AC contains microcrystalline cellulose (30 mg), magnesium stearate (3 mg), and lactose monohydrate (267 mg) saturated with an active pharmaceutical ingredient (API). API is a solution of affinity purified rabbit polyclonal antibodies to IFN-γ, which have previously undergone a process of gradual reduction of their initial concentration (2.5 mg/ml) *via* their multiple dilutions in accordance with a patented biotechnological platform [49]. The dilutions were performed in water-ethanol solutions with either 12, either 30 or 50 defined steps of centesimal (one centesimal: 1 part of the substance and 99 parts of the solvent) dilution. Thus, the initial substance was diluted at least 10^{24} times. The initial forms of the antibodies were produced in accordance with the current EU requirements for Good Manufacturing Practice for starting materials [50] by AB Biotechnology (Edinburgh, UK). The placebo tablets were identical to AC in composition, except of the API.

The participants of the first clinical trial were assigned randomly to receive AC (group 1) or placebo (group 2). In the whole trial there were 30 patients in AC group and 30 patients in the placebo group but due to the limitations of number of the ELISA kits for assessment of the NAbs level, sera from only 10 random patients in each group were chosen. One of the selected samples was unsuitable for testing. Either AC or placebo were administered according to the following regimen described in the product instruction: on day 1, five tablets were taken in the first 2 h (one tablet every 30 min), followed by three more tablets regularly spaced during the rest of the day. From day 2 to day 10, one tablet was administered three times daily. All tablets were provided by OOO "NPF "Materia Medica Holding".

2.3. ELISA for NAbs to human IFN-γ

The blood serum samples were analyzed using an ELISA kit for anti-human IFN-γ NAbs (U-CyTech Bioscience, cat. no. CT803) to measure the serum titer of NAbs to human IFN-γ in patients with varicella infection treated either with AC (n = 9) or placebo (n = 10) and in healthy volunteers (n = 32). A total of 89 human sera samples were analyzed. Natural autoantibody levels were measured three times in patients with varicella infection (at baseline, on day 5 and day 10 of treatment). Table 1 summarizes the characteristics of the patients for whom serum anti-IFN-γ NAbs measurements were performed.

The NAbs levels (IgG) were quantified in accordance with the manufacturer's instruction manual (U-CyTech Bioscience, cat. no. CT803, Utrecht, the Netherlands). Both free circulating anti-IFN-γ NAbs and 'hidden' redox-reactive anti-IFN-γ NAbs were quantified together. The latter autoantibodies need first to be converted to antibodies with a

Table 1
Characteristics of the patients with varicella infection and the healthy volunteers.

Disease	Treatment	Number of patient	male/femaleratio, %	Age*	Days after the first blood draw*	
Varicella	P	10	70/30	16.4 (1.5) [6.0–17.8]	5 (0) [5. 5]	10 (0) [10. 10]
	AC	9	55/45	9.2 (12.6) [3.8–17.4]	5 (0) [5. 5]	10 (0) [10. 10]
Healthy Volunteers	–	12	75/25	4.5 (1)[0–5]	NA	NA
		11	80/20	7.0 (0)[6–10]	NA	NA
		9	45/55	13.0 (2)[11–15]	NA	NA

P-placebo; AC – Anaferon for children.
* Presented as median, interquartile range, and maximum and minimum (where applicable).

strong binding activity to human IFN-γ by a procedure originally described by McIntyre et al. [51]. The procedure was based on treating serum or plasma with a strong redox reactive reagent thereby unmasking more than 90 % of all anti-IFN-γ NAbs (including immunocomplexes with IFN-γ), presenting in plasma or serum. After redox activation, anti-IFN-γ NAbs were detectable in 44,000-fold diluted serum or plasma samples.

2.4. Statistics

All statistical analyses were performed using R 3.2.1. Statistical software. Statistical analysis included the calculation of descriptive statistics, two-way ANOVA to determine differences in the anti-IFN-γ NAbs levels between the AC group and the placebo group before, during and after the treatment, and Dunnett's tests for comparing the NAbs values of the test samples with the values of healthy volunteers. For statistical analysis of the clinical signs, Mann-Whitney test was applied.

3. Results

3.1. Analysis of serum samples from patients with varicella infection and healthy volunteers for levels of anti-IFN-γ NAbs

Table 2 shows the mean anti-IFN-γ NAbs titers of varicella patients receiving either AC or placebo presented by the time of blood collection (before treatment, on day 5 and day 10 of treatment), and mean anti-IFN-γ NAbs titers calculated for healthy volunteers and presented by age group. The healthy controls consisted of children of different ages, which were assigned into three different age groups. For each group, the mean anti-IFN-γ NAbs value was calculated and it was found that the samples from elder children demonstrated a higher mean NAbs level (136x10^3 U/ml) as compared with younger individuals (0-5-year-olds: 70x10^3 U/ml and 6-10-year-olds: 97x10^3 U/ml) (Table 2).

Comparisons at different time points before and after the treatment revealed significant differences within and between the patient groups supporting the suggested correlation between the anti-human IFN-γ NAbs titer and stage of disease. Anti-IFN-γ NAbs concentrations were compared between the two patient groups (placebo and AC) by two-way ANOVA, with plate ID number used as a block factor. The analysis demonstrated statistically significant differences in the NAbs levels at each time point considered, both in the placebo and AC group, as compared with healthy volunteers ($p < 0.001$, Dunnett's contrasts). The study shows that AC and placebo had different effects on the NAbs dynamics in varicella patients. Also, statistically significant differences in the NAbs levels were identified for the AC and placebo groups in the analysis of the NAbs serum concentrations over time ($p < 0.05$, ANOVA). The mean anti-IFN-γ NAbs level in the healthy volunteer group was 97 × 10^3 U/ml (age: 0–15 years), which was significantly lower than the mean pre-treatment value in varicella patients, 167 × 10^3 U/ml (age: 3–17 years). In the AC group, the NAbs level observed on days 5 and 10 decreased significantly to a level of 154 × 10^3 U/ml, whereas in the placebo group it continued to rise in a time-dependent manner reaching 229 × 10^3 U/ml at the point of 10 days.

Interestingly, the improvement in the dynamics of clinical symptoms, such as occurrence of new lesions or general unwellness, was statistically higher in the AC group than in the placebo group, as observed on different days of treatment (Fig. 1A, B). Other variables (*e.g.*, skin itching, Fig. 1C) tended to improve in the AC group as well [insert Fig. 1].

4. Discussion

There is no reference for autoantibody concentration in the body liquids since people produce autoantibodies at different rates and in different amounts [52,53]. Indeed, in our study, anti-IFN-γ NAbs levels were not identical at baseline between the test drug and placebo groups. However, the analysis of changes in concentration over time revealed statistically significant differences between time points and by Sample-Time interaction. This indicates variations in the samples' behavior at different time points. In summary, treatment with AC was found to normalize the anti-IFN-γ NAbs levels, as compared to the placebo group. The mean anti-IFN-γ NAbs values tended to approach the values of age-matching healthy controls. These results correspond to the changes in clinical symptoms that must be an important factor while using NAbs as a biomarker of the therapy efficacy [54–56]. It is important to highlight that there was no possibility to randomize the patients in the groups of placebo and AC as they were randomized during the enrollment in the clinical trial and as all of the available sera were tested.

Sera from the older children contained a higher mean NAbs level relative to the younger ones. There are several publications indicating that NAbs of the IgM class mainly decrease or lose their effectiveness with age [57,58], while the titer of NAbs of the IgG class can increase with age [59,58], which correlates with our findings. There was only one publication where the subjects of study were babies 1–4 years old [60] and the authors did not compared the level of the NAbs with elder children. Here we had a unique opportunity to analyze the dynamics of the anti-IFN-γ NAbs in healthy children within ages ranging from newborns to eighteen-year-old. Since some experts believe that the development of the immune system is not completed before the children reach teenage years [61], increases in children's anti-IFN-γ NAbs levels observed with age seem plausible. This fact should be considered when generating age-matching groups of healthy controls. Yet, due to the difficulty to obtain sera samples from such young healthy individuals, the number of healthy sera samples was obviously limited. The small number of patients that were enrolled in our analysis was due

Table 2
Mean anti-IFN-γ NAbs values as concentrations normalized to dilution rates (expressed as U/mlx10^3).

Disease	Treatment	Number of patients	Age range	Time point of blood draw		
				Day 0 U/mlx$10^{3\#}$ [min-max]	Day 5	Day 10
Varicella	P	10	[6.0–17.8]	141 [79–233]	189^{+} [118-251]	229^{+} [101-365]
	AC	9	[3.8–17.4]	194^{+} [66-238]	176^{+} [123-365]	154$^{*,+}$ [97-207]
	Total	19	[3.8–17.8]	167 [66–238]	NA	NA
Healthy volunteers	No	12	[0–5]	70[35–180]	NA	NA
		11	[6–10]	97 [26–180]	NA	NA
		9	[11–15]	136[31–220]	NA	NA
		Total 32	[0–15]	101 [26–220]	NA	NA

P-placebo; AC – Anaferon for children.
$^{\#}$ Presented as group means multiplied by the dilution factor of 44,000x.
* - $p < 0.001$ (*vs* placebo), two-way ANOVA (concentration over time).
$^{+}$ - $p < 0.001$ (*vs* healthy volunteers), Dunnett's contrasts.

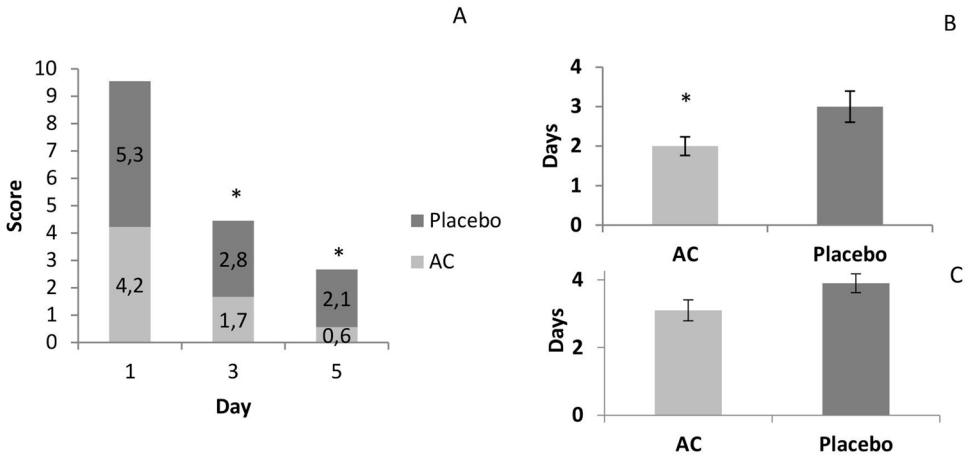

Fig. 1. A. Changes in malaise scores (the scale is equal to the sum of the scores for each day of the study, where 0 means good condition of a patient; 1 means subtle malaise; 2 means moderately severe malaise and 3 – severe malaise), of varicella patients treated with AC or placebo (*-p < 0.05, Mann-Whitney test). B. Presence of new eruptions (days, *-p < 0.05, Mann-Whitney test). C. The duration of skin itch (days, *-p = 0.11, Mann-Whitney test).

to the pilot nature of the study.

Interestingly, in contrast to our data, in another study by A. Caruso *et. al.*, no correlation was found between the anti-IFN-γ NAbs level in healthy individuals and their age, although they did report different NAbs level in the sera of healthy people across all age groups not depending on the age [62]. Since Caruso et al. only measured the free circulating anti-IFN-γ NAbs and did not take into account the type of the viral infection and the differences in immunological status [7], the absence of correlation is understandable. Chi et al. have shown that while IFN-γ is fundamental cytokine in controlling latent varicella virus infection, this cytokine is not essential for some other chronic viral infections such as hepatitis B and C, cytomegalovirus, herpes simplex virus, or Epstein-Barr virus [28]. Given the relationship between the production of a specific cytokine and its corresponding NAbs [17], it is conceivable that the quantity of IFN-γ should also have an impact on the anti-IFN-γ NAbs level. The findings of this study confirm higher serum levels of anti-human IFN-γ NAbs in varicella patients compared to healthy controls of the same age.

5. Conclusions

Natural autoantibodies are becoming increasingly common as biomarkers for diagnosis, stage determination of disease, or treatment efficacy evaluation (in case of appropriate mechanism of action) for a wide range of ailments. Our findings suggest that anti-IFN-γ NAbs can be used as one of the biomarkers for therapy efficacy for varicella (and presumably other viral infections) and also for estimation of the severity of disease. The study also exhibited the diversity in the anti-IFN-γ NAbs level among healthy children of different age groups. Nevertheless, this investigation was the first pilot study intending to evaluate the potential of anti-IFN-γ NAbs for being used as a biomarker. As the anti-IFN-γ NAbs level can vary from patient to patient, the further study of this parameter in a larger number of patients is absolutely required.

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Declaration of Competing Interest

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