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SHORT COMMUNICATION

Recombinant Human PH20: Baseline Analysis of the Reactive Antibody Prevalence in the General Population Using Healthy Subjects

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Abstract

Background Recombinant human PH20 (rHuPH20) is used to depolymerize hyaluronan in the subcutaneous space, increasing the dispersion and absorption of co-administered drugs. While ~ 5 to 10% of rHuPH20 treatment-naïve healthy volunteers have demonstrated rHuPH20-reactive antibodies, associations with age, sex, fertility, and immune disorders remain unknown.

Objectives Using demographically diverse healthy volunteers, we assessed the prevalence of rHuPH20-reactive antibodies in the general population and potential associations with fertility and autoimmunity diseases.

Methods In total, 896 subjects aged ≥ 12 years (767 adults; 129 children) without prior exposure to rHuPH20 were enrolled. A demographic and limited medical history review was performed, and K3-EDTA-anticoagulated plasma was analyzed for rHuPH20-reactive antibodies using a bridging immunoassay.

Results Adult and pediatric positivity rates for rHuPH20-reactive antibodies were 5.2% (40/767) and 1.6% (2/129), respectively. Titers ranged from 5 to 2560 (median 30). In five antibody-positive subjects from whom repeated samples were available, antibody titers remained unchanged or decreased fourfold over periods up to 590 days. The prevalence of rHuPH20-reactive antibodies significantly increased with age ($p = 0.0006$) and was significantly higher in males than in females ($p = 0.0010$). Men who had fathered children had a significantly increased prevalence of rHuPH20-reactive antibodies than men who had not ($p = 0.0036$), whereas the rate of childbearing was not significantly different between rHuPH20 antibody-positive and -negative women. The prevalence between racial/ethnic groups was not significantly different, nor was the presence/absence of an autoimmune disorder.

Conclusions Approximately 1/20 of the adult population had rHuPH20-reactive antibodies. The reason remains unknown; however, no evidence for a negative effect on fertility or association with autoimmune disease was demonstrated.

Key Points

Approximately 5% of the US adult population test positive for recombinant human PH20 (rHuPH20)-reactive antibodies in the absence of exposure to rHuPH20. The prevalence is higher in male than in female subjects.

Although the reason for this phenomenon remains unknown, no evidence for a negative effect on fertility in these individuals was found.

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1 Introduction

Recombinant human PH20 (rHuPH20) is a soluble recombinant form of human PH20 that can be used to depolymerize hyaluronan in the subcutaneous space [1–3]. rHuPH20 acts locally and transiently to degrade hyaluronan in the extracellular matrix within the subcutaneous space by cleaving the linkage between the two sugars that comprise hyaluronan acid (HA; *N*-acetylglucosamine and glucuronic acid) [1–3]. By degrading HA in the extracellular matrix at the local injection area, rHuPH20 enables increased subcutaneous bulk fluid flow and the dispersion and absorption of co-administered agents [3–6].

The endogenous counterpart of rHuPH20, PH20, is a hyaluronidase expressed at the apical head of male mature sperm, where a glycosylphosphatidylinositol (GPI) anchor tethers it to the cell membrane. The testis constitutes an immunologically privileged organ, and the breach of the blood–testis barrier can predispose an individual to autoimmunity against sperm antigens, of which several have been identified [7]. As a result, anti-sperm antibodies may develop; a well-documented example is found in men who have undergone vasectomy and have increased rates of anti-sperm antibody positivity [8]. In women, the underlying cause for sperm immunity is less well understood and, to our knowledge, no single antigen has been consistently identified as the cause of immunologic infertility in humans.

In clinical studies, approximately 5–10% of rHuPH20 treatment-naïve subjects and healthy volunteers demonstrated evidence of rHuPH20 reactive antibodies [9]. The reason is unknown, and sex or age associations with rHuPH20-reactive antibodies were not identified in a study of healthy plasma donors [9].

1.1 Objective

We conducted a healthy volunteer, blood sample collection, demographically representative study using a diverse population to investigate the prevalence of rHuPH20-reactive antibodies in the general population and the potential for association with fertility or autoimmunity disorders.

2 Methods

2.1 Samples from Healthy Subjects

Healthy subjects aged $\geq$ 12 years (children aged 12–17 years; adults $\geq$ 18 years) weighing at least 50 kg without prior exposure to rHuPH20 or significant comorbidities (e.g., cardiovascular, gastrointestinal, hepatic, neurological, psychiatric, endocrine, cancer, HIV infection, diabetes mellitus, or other major systemic disease) were enrolled.

The Institutional Review Board/Independent Ethics Committee reviewed and approved the protocol before subjects were enrolled. All patients provided written informed consent before study initiation. For pediatric subjects, informed consent was provided by a legal guardian, and assent by the study subject was required.

A complete demographic review and focused medical history was performed using separate questionnaires for male and female participants (Table 1 in the Electronic Supplementary Material [ESM]). Pediatric subjects received a shorter questionnaire focusing on autoimmune/inflammatory conditions only. At the clinic visit, vital signs and a blood sample were taken to determine hematocrit; blood ($\sim$ 40 ml for adults and $\leq$ 20 ml for children) was then drawn into K3-EDTA blood collection tubes and processed to plasma.

To ensure demographically representative enrollment, the US Census (2010) was used to calculate desired enrollment numbers within each age/ethnic/sex subgroup, and enrollment stopped within each subgroup once 100% enrollment was achieved.

2.2 Electrochemiluminescent Bridging Assay Procedure

Per the US FDA draft guidance for immunogenicity testing, the presence of anti-rHuPH20-binding antibodies was determined using a format-validated bridging electrochemiluminescent (ECL) immunoassay that has been described previously [9]. In this assay, a bi-valent binding event is required to bridge biotin-labeled rHuPH20 and Sulfo-TAG-labeled rHuPH20 to generate a signal. Briefly, after an overnight co-incubation of plasma sample diluted 1:5 with rHuPH20 conjugated to biotin and rHuPH20 conjugated to Sulfo-TAG (250 ng/ml each; Meso Scale Discovery, Rockville, MD, USA), the resulting immune complex was captured onto streptavidin-coated plates and detected in a SECTOR 2400 instrument using ECL Read buffer (all Meso Scale Discovery). Analysis for rHuPH20-reactive antibodies was performed using a three-tier testing strategy: (1) screening against a statistically established cut-point, (2) confirmation of specificity for rHuPH20 in putative positives, and (3) a titer determination for confirmed positives. In the first tier of testing, samples were evaluated for the presence or absence of an ECL signal above the screening cut-point to determine the presence of rHuPH20-reactive antibodies [10]. Then, to verify the specificity of rHuPH20 binding, 10 μg/ml of unlabeled rHuPH20 was added to separate competition reactions. The percent inhibition for each sample was calculated, and

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samples yielding inhibition greater than the specificity cut-point were considered to contain antibodies reactive to rHuPH20.

Lastly, samples that were confirmed positive for rHuPH20-reactive antibodies were then subjected to titer determination by dilution in 20% pooled plasma using the same experimental conditions just described. The pooled plasma was generated from 31 healthy human individuals who were screened in the assay and was considered rHuPH20-reactive antibody free. The inverse of the last sample dilution yielding an ECL value above the determined titration cut-point was reported as the titer value for that sample.

2.3 Statistical Analysis

Questionnaire responses were compiled by SeraTrials. All data were analyzed using JMP statistical software version 10.0.0 (SAS Institute). The prevalence of rHuPH20-reactive antibodies in various subpopulations was compared by contingency analysis using Fisher's exact test, followed by calculations of odds ratios with 95% confidence intervals in cases where all four quadrants in the analysis contained one or more subjects. Demographic analyses were conducted using the likelihood ratio Chi-squared test. Titer distributions in men and women, and the ages of antibody-positive and antibody-negative subjects, were compared using Wilcoxon's nonparametric rank-sum test.

3 Results

3.1 Subjects

A total of 896 evaluable subjects were recruited over 28 months. The presence or absence of rHuPH20-reactive antibodies, and titers if present, was determined in plasma from samples obtained from 767 adult subjects (381 men; 386 women) along with 129 subjects aged 12–17 (70 boys; 59 girls).

3.2 Recombinant Human PH20 (rHuPH20)-Reactive Antibodies

3.2.1 Age/Duration

Adult and pediatric antibody positivity rates of 5.2% ($n = 40/767$) and 1.6% ($n = 2/129$; both male) were confirmed, respectively. Titers ranged from 5 to 2560 (median 30). Antibody positivity increased with age through 59 years then slightly decreased in subjects aged ≥ 60 years (Fig. 1a), but the small number of older subjects renders the significance of this finding unclear.

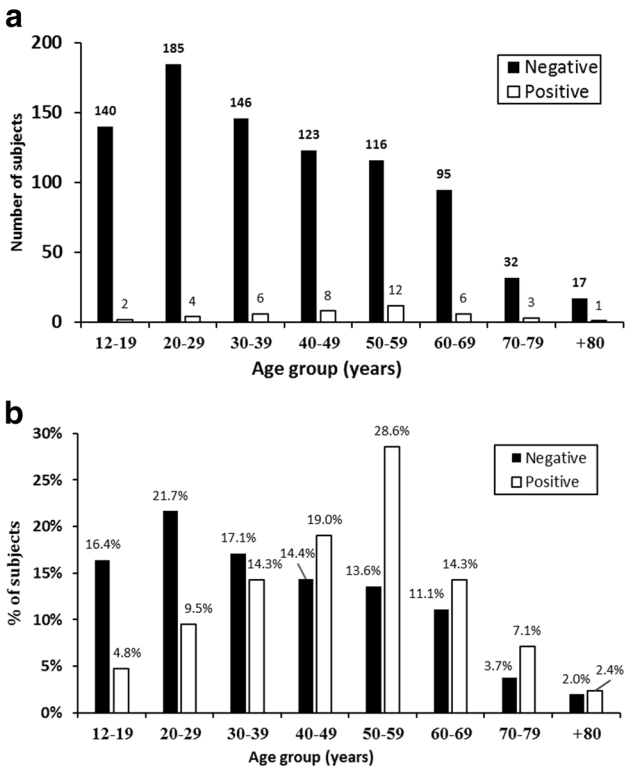

Fig. 1 Age distribution of recombinant human PH20 (rHuPH20)-negative and positive subjects by **a** number and **b** percentage of patients (percentages given atop bars are within negative and positive subject populations). Data are shown as frequency distributions across age within rHuPH20-reactive negative ($n = 854$) and positive ($n = 42$) subject populations. Wilcoxon's rank-sum test $p = 0.0006$

When the proportions of antibody-positive and antibody-negative individuals within each decade of age were constructed into age distributions (Fig. 1b), the distributions were significantly different ($p = 0.0006$), with a higher prevalence of antibody-positive subjects among older individuals. Five of the antibody-positive subjects, all adult Caucasian men, donated a second blood sample 152–246 days ($\sim$ 5 to 9 months) after the first. In addition, a third sample was collected from two subjects 588–590 days ($\sim$ 20 months) after the first sample. All five subjects remained positive for rHuPH20-reactive antibodies throughout, and the titers remained unchanged ($n = 1$), decreased by only one dilution ($n = 3$), or decreased by two dilutions ($n = 1$; Fig. 2).

3.2.2 Sex/Race

In both adult and in pooled adult and pediatric populations, antibody positivity was significantly more prevalent in male than in female subjects ($p \leq 0.0010$; Table 1). Despite this sex disparity, no significant difference in antibody titer magnitude between antibody-positive male and female subjects was detected ($p = 0.41$). In a subset of 657

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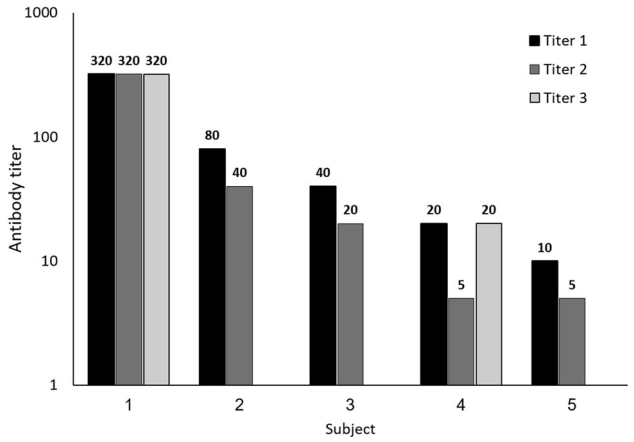

Fig. 2 Long-term assessment of recombinant human PH20 (rHuPH20)-reactive antibody titers using repeated sampling of five subjects. The second blood sample was obtained 152–246 days (~ 5 to 9 months) after the first. In addition, a third sample was collected from two subjects 588–590 days (~ 20 months) after the first sample

subjects aged 18–64 years, in which accrual across demographics was considered representative of the general population, 26/452 (5.8%) Caucasian subjects (not Hispanic/Latino), 5/100 (5.0%) Hispanic/Latino subjects, 2/84 (2.4%) African-American subjects, and 0/21 Asian subjects were confirmed positive for rHuPH20-reactive antibodies. The prevalence across groups of different race/ethnicity was not significantly different ($p = 0.24$).

3.2.3 Fertility/Pregnancy

Of the 123 men who reported having fathered children, 17 (13.8%) tested positive for rHuPH20-reactive antibodies compared with 258 never fathers, of whom 13 (5.0%) were antibody positive ($p = 0.0036$). The age distributions of these antibody-positive fathers and never fathers were not significantly different ($p = 0.8261$). Of the 34 men who reported having had a vasectomy, five (14.7%) tested positive for rHuPH20-reactive antibodies, whereas 25 of 347 (7.2%) non-vasectomized men tested positive ($p = 0.17$). Of the 381 participating men, two reported visiting a fertility specialist, and one of those reported being diagnosed with infertility; however, neither was positive for rHuPH20-reactive antibodies. None of the 30 antibody-positive adult male subjects reported having had testicular surgery, injury/trauma, inflammation, testicular mumps, or recurring urinary tract infections.

Eight women who tested positive for rHuPH20-reactive antibodies had borne at least one child, and none of them reported any miscarriages; the rate of childbearing was not significantly different between antibody-positive and -negative women ($p = 0.20$). One antibody-positive and three antibody-negative women reported a premature birth (<37 weeks of gestation); these rates were not significantly different ($p = 0.14$). None of the ten antibody-positive female subjects reported recurring urinary tract or other infections/inflammatory disease of the pelvic area. Of the 386 participating women, two had visited a fertility specialist, and four reported being diagnosed with infertility (including the two who had visited a fertility specialist); however, all were negative for rHuPH20-reactive antibodies.

3.2.4 Autoimmune or Inflammatory Conditions

An autoimmune or inflammatory disease was identified in 25 subjects (12 asthma; four vitamin B_{12} deficiency; three urticaria; two psoriasis; two celiac disease; one inflammatory bowel disease; one systemic lupus erythematosus; two

Table 1 Prevalence of recombinant human PH20-reactive antibodies in healthy subjects

Variable, n	Antibody +	Antibody −	OR (95% CI)	*p* value
Male	32	419	3.32 (1.61–6.84)	0.0007
Female	10	435		
Father of one or more child	17	106	3.13 (1.47–6.68)	0.0036
Never father	13	245		
Vasectomy	5	29	2.22 (0.79–6.24)	0.17
Non-vasectomy	25	322		
Mother of one or more child	8	207	3.27 (0.68–15.6)	0.20
Never mother	2	169		
Premature birth	1	3	9.81 (0.90–106.6)	0.14
Never premature birth	7	206		
Autoimmune/inflammatory conditions	2	23	1.80 (0.41–7.91)	≤ 0.33
No autoimmune/inflammatory conditions[a]	40	829		

CI confidence interval, *OR* odds ratio

[a]Two antibody-negative subjects chose not to respond to this question

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subjects reported two such conditions each). Two of these subjects (one with asthma and one with urticaria) were rHuPH20-reactive antibody positive, but there was no significant overall association between antibody presence and autoimmune/inflammatory disease ($p = 0.33$).

4 Discussion

In 2005, recombinant hyaluronidase injection (Hylenex® recombinant hyaluronidase human injection) was approved by the FDA as an adjuvant in subcutaneous fluid administration for hydration and to increase the dispersion and absorption of other injected drugs [1]. When co-administered subcutaneously with other therapeutics, sequentially or in a co-formulated product, rHuPH20 enables the therapeutic agents to more rapidly permeate the subcutaneous space by diffusion and/or convection, thereby gaining access to central circulation via the capillaries or lymphatics for small and large molecular therapies [9]. Studies have also demonstrated the ability of co-administered rHuPH20 to increase the absorption of subcutaneous lactated Ringer's solution [11] and to improve the pharmacokinetic and pharmacodynamic properties of insulin [12–16] and human immunoglobulins [17, 18]. Along with Hylenex® [1], three other treatments containing rHuPH20 are currently in use: HyQvia® (immune globulin 10%; USA and EU) [19], subcutaneous Herceptin® (trastuzumab; EU) [20], and subcutaneous MabThera® and Rituxan Hycela® (rituximab; EU and US, respectively) [21, 22].

Antibodies to rHuPH20 were previously assessed after subcutaneous rHuPH20 co-administration with human immunoglobulin, trastuzumab, rituximab, or insulin [9]. Among individual trials assessed by Rosengren et al. [9], the prevalence of pre-existing rHuPH20-reactive antibodies varied between 3 and 12%, except for the primary immune deficiency trials. The incidence of treatment-induced rHuPH20 antibodies ranged from 2 to 18%. Neutralizing antibodies were not observed in any case [9]. In this set of studies, no association between antibody positivity and either local or systemic adverse events were demonstrated. Pre-existing and treatment-induced antibody populations were of similar immunoglobulin isotypes and cross-reacted to endogenous PH20 to similar extents [9]. It was concluded that rHuPH20 induced only modest immunogenicity, with no demonstrable association with adverse events. However, the robust prevalence of pre-existing antibodies prompted further investigations, which are reported herein.

This study assessed the prevalence of rHuPH20-reactive antibodies in the general population and the potential for association with disorders of fertility or autoimmunity in a demographically representative population. Rates of antibody positivity were similar to those reported in Rosengren et al. [9], where a rate of 5.8% was reported in the normal population. In this study, adult and pediatric antibody positivity rates of 5.2 and 1.6% (two boys out of 129 pediatric subjects) were confirmed, respectively; 7.8% of adult men and 2.6% of adult women were antibody positive. Antibody-positive subjects were found to be, on average, significantly older than antibody-negative subjects, although there was considerable overlap in age between the two populations. Even though PH20 is present on the apical head of male sperm, male subjects had approximately threefold significantly higher rates of rHuPH20 antibody positivity than female subjects. Although some antisperm antibodies are associated with decreased fertility [7], no evidence of negative effects on fertility could be determined in rHuPH20-reactive antibody-positive subjects of either sex. In addition, no association was observed between antibody positivity and autoimmune/inflammatory conditions. Pre-existing antibodies to biotherapeutics before exposure have been reported previously [23, 24], and the reason is typically poorly understood, but the data continue to support the notion that pre-existing antibodies to rHuPH20 are not associated with any pathology.

Unlike a direct or sandwich format assay, the assay format used in this study requires a bi-valent/multivalent binding event to generate a signal. There are no known multivalent ligands for rHuPH20, and rHuPH20 does not have any soluble cellular receptors (unlike many therapeutic proteins or monoclonal antibodies). In Rosengren et al. [9], we described the isolation of rHuPH20-reactive antibodies from patients who screened positive in the same ECL bridging assay, and we performed extended characterization as well as isotype identification of these antibodies. This confirms that the signal generated in the bridging assay is due to the presence of an antibody since we were able to determine the human immunoglobulin (Ig) isotype. These analyses were conducted with plasma from individuals with treatment-emergent responses and from healthy subjects with pre-existing reactivity (figure 5 in Rosengren et al. [9]). This directly demonstrates that the ECL bridging assay method detects rHuPH20-reactive antibodies in treatment-naïve individuals.

4.1 Study Limitations

We intended to enroll 1000 demographically diverse subjects (800 adults and 200 children). However, because of difficulties in recruiting certain demographic/age groups, such as children and subjects of advanced age, enrollment was terminated and the analysis limited to the samples obtained from August 2012 through December 2014. In addition, the racial/ethnic composition of participating donors was not well balanced in all age groups. Therefore,

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an analysis based on race/ethnicity was limited to subjects aged 18–64 years where accrual across all demographics was close to 100% within each ethnic/sex subgroup based on the reported US Census in 2010.

5 Conclusions

In this study, ~ 5% of the adult population had anti-rHuPH20-reactive antibodies, found more often in the elderly and men. While the reason for this remains unknown, no evidence of a negative effect on fertility in either sex or association with autoimmune/inflammatory conditions was demonstrated.

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Compliance with Ethical Standards

The study was approved by the independent ethics committee at each participating institution, and 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 International Conference on Harmonisation E6 requirements for Good Clinical Practice and with the ethical principles outlined in the Declaration of Helsinki. All patients provided written informed consent before study initiation. For pediatric subjects, informed consent was provided by a legal guardian, and assent by the study subject was required.

Conflict of interest Jennifer Souratha is a current employee of, and Dave Conway, Barry Sugarman, Sanna Rosengren, and Douglas Muchmore are former employees of, Halozyme Therapeutics, Inc.

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Supplemental Table 1. Individual Questionnaires for Adult Male and Female Participants.

Male
Age
Race/Ethnicity
Have you had children?
How many children have you had?
Have you ever visited an infertility specialist clinic?
Have you ever been diagnosed with infertility or not being able to have children?
Have you ever had a vasectomy?
Have you ever had surgery to your testicles?
Have you ever been diagnosed with a testicle that did not descend by itself?
Have you ever had injury or trauma to your testicles?
Have you ever had inflammation of the testicles?
Have you ever had a urinary tract infection?
Have you ever had mumps that affected your testicles?
Has the donor ever been diagnosed with any of the listed autoimmune or inflammatory conditions?

Female
Age
Race/Ethnicity
Have you ever visited an infertility specialist?
Have you ever been diagnosed with infertility or not being able to have children?
Have you had children?
How many children have you had?
How many live births have you had?
How many miscarriages have you had?
Were any of your children born before 37 weeks of pregnancy?
Have you ever had more than one urinary tract infection?
Has the donor ever been diagnosed with any of the listed autoimmune or inflammatory conditions?