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Time from venipuncture to cell isolation: Impact on granulocyte-reactive antibody testing

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ABSTRACT

Background and objectives: Classical neutrophil-reactive antibody testing depends on the quick isolation of neutrophils from freshly taken whole blood. To allow a better logistic preparation before testing, the influence of time interval between venipuncture and cell isolation has been evaluated in this study.
Materials and methods: Neutrophils and whole leukocytes were isolated from EDTA whole blood immediately (T0) as well as 4, 8 and 24 h after blood donation (T4, T8 and T24). These cells were tested against reference sera containing antibodies against HNA-1b, −2, −3a and HLA class I using granulocyte aggregation test (GAT), microscopic granulocyte immunofluorescence test (GIFT) and flow-cytometric white blood cell immunofluorescence test (Flow-GIFT/WIFT).
Results: GAT was the most error-prone test displaying overall weaker aggregation strengths already at T4 (overall accuracy OA = 0.72, κ = 0.58). GIFT results showed good agreement at T4 (OA = 0.86, κ = 0.79) and remained stable until T8, while test results were slightly impaired at T24 (OA = 0.71, κ = 0.55). Flow-GIFT/WIFT was identified as the most robust screening method, remaining stable even at T24. Calculated ratios (sample/negative control) decreased non-significantly and remained highly above the cut-off in all samples.
Conclusion: Acceptable time limits for cell isolation are different for each screening method investigated. For GAT, cell isolation should be performed within 4 h, while GIFT tolerates a neutrophil isolation delay of 8 h. Flow-GIFT/WIFT isolation can be performed even after 24 h without impairment of the results. Using the latter test as a stand-alone pre-screening test, whole blood can be used from donors who are not directly accessible.

1. Introduction

Granulocyte-reactive antibodies are directed against human neutrophil antigens (HNA) and play an important role in a variety of medical conditions, such as febrile transfusion reactions, transfusion-related acute lung injury (TRALI), autoimmune neutropenia (AIN), neonatal immune neutropenia (NIN) or refractoriness to granulocyte transfusions [1,2]. Since the introduction of granulocyte immunology routine testing several decades ago, the detection of granulocyte-reactive antibodies still remains a challenging issue. Besides the use of classical analysis methods, granulocyte serology is subject to ongoing improvements, including recombinant proteins coated on latex particles or expressed by transfected cell lines [3–9]. Nevertheless, using native leukocytes as target for granulocyte-reactive antibodies is still the most common and reliable approach. Classic screening methods like the granulocyte aggregation test (GAT) and the microscopic granulocyte immunofluorescence test (GIFT) are performed with a panel of freshly isolated cells from healthy volunteers of known HNA phenotype [10,11]. Within the last decade the flow cytometric white blood cell immunofluorescence test (Flow-GIFT/WIFT, also known as Flow-GIFT) was introduced. It has the advantage of simultaneously detecting granulocyte-, monocyte- and lymphocyte-reactive antibodies in serum samples [12–14].

Several factors influence the sensitivity of these tests. The availability of healthy blood donors, the possibility to assemble a cell panel with complementary HNA profile, and the quick processing of fresh

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cells are regarded essential for successful granulocyte immunology testing. Since antibodies against human leukocyte antigens (HLA) are also known to bind on neutrophils and are much more common than antibodies against HNA, the interpretation of results requires a lot of expertise. Thus, HNA-diagnostics is restricted to specialized laboratories with direct access to HNA- and HLA-typed blood donors.

Due to granulocytes having a short lifespan and being highly fragile [10,15,16], it is generally recommended to perform cell isolation and antibody testing immediately after blood donation [15,17]. Therefore, laboratories performing granulocyte serology require direct access to donors with specific HNA phenotypes. Moreover, in evaluating possible NIN, it is highly informative to test maternal serum against paternal granulocytes, which is not possible in the majority of cases due to the local distance between patients and the facility. Shipping blood samples without perturbing test results would markedly facilitate the logistic challenges of granulocyte serology.

To the best of our knowledge, no thorough evaluation has ever been performed to determine the maximum age of granulocytes usable for immunological clinical routine testing. To evaluate the stability of granulocytes usable for routine testing without impairment of the obtained results, this study investigates the time interval from venipuncture to cell isolation and its impact on granulocyte-reactive antibody testing.

2. Materials and methods

This study was performed at the Medical University of Vienna at the laboratory for granulocyte immunology. All sera were alloantibodies which originate from blood donor alloantibody testing (kindly provided by Dr. A. Reil of the DRK Blood Service West Hagen). Sera and cells used in this study were reference samples used in clinical routine testing.

Reference sera were selected regarding their compatibility with the HNA phenotype of the cells. Overall, depending on the method, approx. 30 different combinations of isolated cells and serum were tested using three different methods described below

2.1. Sera and cells

Eight sera containing alloantibodies against HNA-1b, −2, −3a and HLA-A1,-A2 and -A3 were used (anti-HNA-1b/-HLA class I n = 1, anti-HNA-2 n = 1, anti-HNA-3a n = 3, anti-HLA-A1 n = 1, anti-HLA-A2 n = 1, anti-HLA-A3 n = 1). Three sera of blood group AB (AB-Sera) without antibodies against leukocytes served as negative controls.

The assembled cell panel included donors previously typed for HNA by sequence-specific priming PCR (HNA Ready Gene, inno-train, Kronberg, Germany) and by serologic testing. The panel covered all antigens necessary to react with the applied antisera: HNA-1a, −1b, −2, −3a, −3b, −4a, −4b, −5a, and -5b, as well as HLA-A1, −A2 and -A3. Concerning HNA-3a, homo- and heterozygous cells were included in this study to obtain dose-related reaction strengths with respective antisera.

2.2. Cell isolation

Cells were isolated from 4 × 6 ml EDTA whole blood at different times: directly after blood donation as well as 4, 8 or 24 h later (denoted as time point (T) 0, T4, T8 and T24, respectively). For T4, T8 and T24, blood was stored at room temperature until cell isolation. Leukocyte-rich plasma was obtained by dextran sedimentation using 5% dextran 500 (Carl Roth, Karlsruhe, Germany) solution in DPBS (Dulbecco's phosphate-buffered saline) at 37 °C. For GAT and GIFT, granulocytes were separated by density gradient centrifugation using Ficoll-Paque™ PLUS (GE Healthcare, Uppsala, Sweden). Erythrocytes were depleted by ammonium chloride lysis. To prevent non-specific antibody-binding, 1% paraformaldehyde (PFA) was used to fix granulocytes for microscopic GIFT by incubation for 5 min at room temperature following two washing steps with DPBS [10]. For Flow-GIFT/WIFT, density gradient centrifugation and fixation were omitted. To obtain cell number and granulocyte purity, isolated cells were counted on a Coulter® Ac T diff™ analyzer (Beckman Coulter). For all methods, cells were prepared to have a final concentration of 5×10^3 white blood cells/µl before usage.

2.3. Granulocyte-aggregation testing

GAT was performed in paraffin-oiled Terasaki plates incubating 2 µl of granulocytes with 5 µl serum for 2 h at 37 °C. Each sample was checked for aggregate formation using an inverted microscope. Aggregate strengths were defined as negative (−), weakly positive (+), clearly positive (++) and strongly positive (+++).

2.4. Granulocyte immunofluorescence testing

In GIFT, 40 µl of fixed granulocytes were incubated with 40 µl of serum. After washing with DPBS, bound antibodies were detected using 40 µl of a FITC-labeled rabbit anti human IgG antibody (Dako, Glostrup Denmark) and evaluated via fluorescence microscopy. Fluorescence intensity was coded as negative (−), weakly positive (+), clearly positive (++) and strongly positive (+++). Mixed pattern reactions (typical for anti-HNA-2) were evaluated by regarding only the positive granulocyte population.

2.5. Flow cytometric white blood cell immunofluorescence testing

For Flow-GIFT/WIFT, 50 µl of serum was incubated with 50 µl of leukocyte suspension for 30 min at 37 °C. After two washing steps with DPBS/0.2% bovine serum albumin (BSA) membrane-bound antibodies were labeled with 50 µl of Alexa Fluor® 488-conjugated Goat Anti-Human IgG Fab Fragment (Jackson ImmunoResearch, New Market, England) while simultaneously adding 5 µl 7-AAD (BD Bioscience, San Jose, CA) for 30 min in the dark at room temperature following one additional washing step. Before measurement, cells were fixed with 200 µl Cellfix™ (Becton Dickinson, Heidelberg, Germany). Flow cytometry was performed on a FACS Canto II and analyzed with FACS Diva software (BD Biosciences). Median fluorescence intensity (MFI) of neutrophils and lymphocytes was detected. Samples were defined as positive if the fluorescence level was at least triple that of the median of the negative control samples. Cell viability measurements with 7-amino-actinomycin-D (7-AAD) were gated on neutrophils as these are the most fragile cells of all leukocytes.

2.6. Statistics

For all assays resulting in ordinal data (GAT and GIFT), the inter-rater reliability was tested using Cohen's kappa. When applicable, Fleiss' Kappa for multiple raters was calculated. Agreement was interpreted using the standard kappa agreement thresholds [18,19]: $\kappa \leq 0.20$: slight agreement, $\kappa > 0.20$; ≤ 0.40: fair agreement, $\kappa > 0.40$; ≤ 0.60: moderate agreement, $\kappa > 0.60$; ≤ 0.80: substantial agreement, $\kappa > 0.80$: (almost) perfect agreement. Since data was ordinal, weighted kappa was computed using a linear (therefore equally spaced) weight for κ. Confidence intervals were calculated for 95% of the distributions.

For all assays resulting in metric data (Flow-GIFT/WIFT, cell viability using 7-AAD), sample distribution was evaluated in a preliminary step using both the Shapiro-Wilk normality test as well as the Kolmogorov-Smirnov-Test. Non-parametric tests were used when samples were not normally distributed. Sample comparisons were performed using the Wilcoxon signed rank test. When analyzing values were tied, continuity correction was applied.

For all statistical tests a significance level of $p < 0.05$ was

considered statistically significant. Alpha adjustment for multiple comparisons was performed using the Bonferroni correction method. Statistical analyses were performed using R software [20] in the version 3.4.3 as well as the packages *BDSA* [21] in the version 1.2.0, *dplyr* [22] in the version 0.7.2, *ggplot2* in the version 2.2.1, *irr* [23] in the version 0.84, *obs.agree* [24] in the version 1.0, *psych* [25] in the version 1.7.5, *PASWR* [26] in the version 1.1.

3. Results

Granulocyte purity after cell isolation from whole blood was measured to perform GAT (n = 6 for every time point) and GIFT (n = 7 for every time point). For T0, T4 and T8, the relative granulocyte fraction was 83.7, 85.4 and 85.2%, and decreased to 50.8% at T24. Since Flow-GIFT/WIFT (n = 7 for every time point) was performed with whole leukocytes, thus, granulocyte purity was not considered relevant for this test.

3.1. Results of granulocyte aggregation testing depend strongly on time interval from venipuncture

When comparing GAT at T0 and T4, overall accuracy was found to be 0.72 (95%-CI: 0.46–0.90). Kappa statistics showed a substantial agreement between those time points (weighted kappa 0.66). The number of clearly positive and strongly positive samples was significantly reduced (balanced accuracy: 0.59 and 0.50, respectively). Despite that, in all cases negative results stayed negative and positive results (weakly, clearly and strongly positive) stayed positive. The comparison between T0 to T8 displayed similar results. In two cases an apparent, minor increase in aggregation strength was observed, which was not considered clinically relevant.

Major differences were detected when comparing GAT results T0 to T24. At this time point, the assay was not able to detect any clearly or strongly positive reaction. Therefore, Fleiss' Kappa revealed no significant agreement between samples with a positive or strong positive reaction.

In summary, increased cell age had a major influence on GAT results (Fig. 1, Table 1).

3.2. Granulocyte immunofluorescence test stability with cell age up to eight hours

When performing GIFT at T0 and T4, overall accuracy was 0.86 (95%-CI: 0.64–0.97). For negative and weakly positive samples, perfect agreement was found. In some cases, the assay was not able to differentiate between clearly positive and strongly positive results. The overall Kappa statistics showed an almost perfect inter-test agreement (weighted kappa 0.86, lower limit: 0.75, upper limit: 0.98). Results were similar when comparing T0 and T8. In three cases an apparent, minor increase in fluorescence was observed, which was not considered clinically relevant.

Table 1
Time Course of GAT-Results. Time course (T0-T24) of semiquantitative GAT results with 3 negative controls and 3 sera containing antibodies against HNA-3a are shown. Panel cell A was heterozygous for HNA-3a, cell B and C were homozygous for HNA-3a. At T4, most samples showed a weaker aggregation potential compared with T0. At T24, all except 2 samples became negative.

GAT time course					
Cell	Sample	T0	T4	T8	T24
A	AB serum 1–3	–	–	–	–
A	anti-HNA-3a	+	+	+	–
A	anti-HNA-3a	+ +	+	+	–
A	anti-HNA-3a	+	+	+	–
B	AB serum 1–3	–	–	–	–
B	anti-HNA-3a	+ +	+	+	–
B	anti-HNA-3a	+ + +	+ +	+	+
B	anti-HNA-3a	+ +	+ +	+	–
C	AB serum 1–3	–	–	–	–
C	anti-HNA-3a	+	+	+	–
C	anti-HNA-3a	+ + +	+	+ +	–
C	anti-HNA-3a	+ +	+	+ +	+

Between T0 and T24, kappa statistics was at the edge of showing a substantial agreement (weighted kappa 0.602) with an overall accuracy of 0.71 (95%-CI 0.48–0.89). Having said that, three samples being tested as clearly positive or strongly positive at T0 appeared negative at T24 (Table 2).

In conclusion, GIFT did not show an impairment as pronounced as GAT when using cells isolated at later time points. When comparing all time points together, Fleiss' Kappa showed a significant overall agreement between samples with a kappa of 0.70.

3.3. Decreased cell viability, but almost unaltered flow cytometric white blood cell immunofluorescence test results

Granulocyte viability analysis using 7-AAD displayed a significant reduction between cells at different time points. The number of 7-AAD-positive granulocytes was approximately 3.4% at T0 and increased to 5.5% and 10.5% at T4 and T24, respectively (Fig. 2). No significant difference in cell viability was found between T4 and T8.

Flow-GIFT/WIFT results measured as MFI were not significantly different between the different time points (Fig. 3). It was easily possible to distinguish between negative and positive samples at all time points. When analyzing granulocyte MFI, the raw values of negative controls displayed only minor changes and a (non-significant) trend to increase within 24 h (Fig. 4A), while SSC displayed a (non-significant) trend to decrease with time (adjusted R^2 of approx. 0.025, Supplementary Figs. 1 and 2). When analyzing the median differences

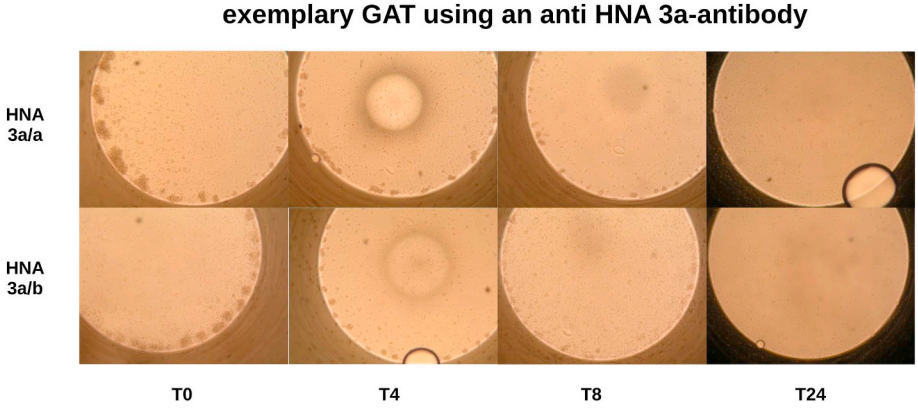

Fig. 1. Time Course of Granulocyte Aggregation Testing (GAT). Anti-HNA-3a was incubated with HNA-3a/a cells (upper row) as well as HNA-3a/b cells (lower row), which were isolated at different time points after blood donation (T0-T24). Anti-HNA-3a typically reacts strongly with 3a/a cells and weaker with 3a/b cells (dosage effect). Aggregation strengths decrease the longer cell isolation was delayed.

Table 2
Time Course of GIFT-Results. Time course (T0-T24) of semiquantitative GIFT results with 3 negative controls and 4 sera containing antibodies against HNA and HLA are shown. The cell panel carried all HNA and HLA necessary to react with selected sera. Results were categorized by microscopy. Results were stable until T8. At T24, most reactions decreased with the consequence that three cell/sample-combinations yielded negative results.

GIFT Time Course					
Cell	Sample	T0	T4	T8	T24
A	AB serum 1–3	–	–	–	–
A	anti-HNA-1b/-HLA class I	+ +	+ + +	+ +	+
A	anti-HNA-2	+ +	+ +	+ +	+
A	anti-HNA-3a	+	+	+	+
A	anti-HLA-A3	+	+	+	–
B	AB serum 1–3	–	–	–	–
B	anti-HNA-1b/-HLA class I	+ +	+ + +	+ +	+ +
B	anti-HNA-2	+ +	+ +	+ +	–
B	anti-HNA-3a	+	+	+	+
B	anti-HLA-A2	+	+	+	–
C	AB serum 1–3	–	–	–	–
C	anti-HNA-1b/-HLA class I	+ +	+ + +	+ +	+
C	anti-HNA-2	+ +	+ +	+ +	+ +
C	anti-HNA-3a	+	+	+	+
C	anti-HLA-A1	+	+	+	+

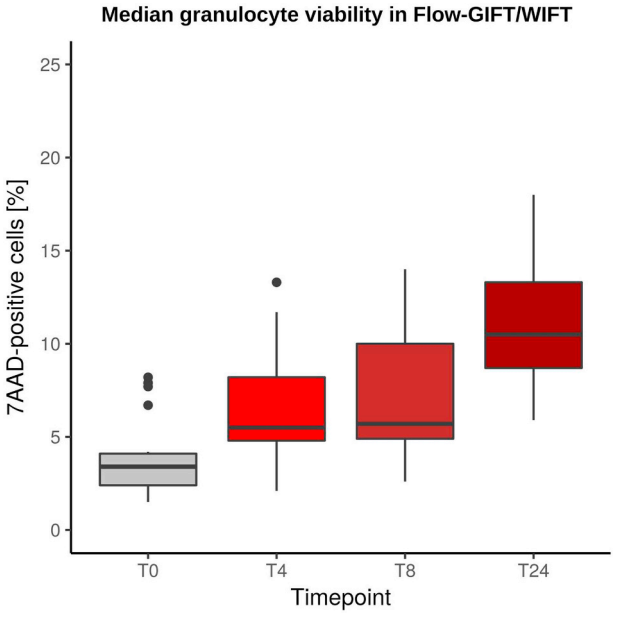

Fig. 2. Median Granulocyte Viability in Flow-GIFT/WIFT. Granulocyte viability was measured with every cell-sample-combination at all time points (T0-T24) using 7-AAD. The later cell isolation was performed, the more 7-AAD-positive (and therefore dead) neutrophils were found.

between positive and negative samples (Fig. 4B), the ratio was reduced from 17.5 (T0) to 11.2 (T24). In our laboratory we add an additional interpretation category between positive and negative results (gray area, ratios 2–3: results unclear, samples need to be further investigated). At T8 and T24, results changed from weakly positive to the gray area in two and one sample, respectively. The same is true when analyzing lymphocyte MFI (Fig. 4C,D).

Although some of the differences were found to be statistically significant, they are not considered to be of clinical relevance since the generally used cut-off between positive and negative samples is a gray area between ratio 2 and 3. Therefore in conclusion, the age of the cells did not lead to an impaired data analysis when performing Flow-GIFT/WIFT.

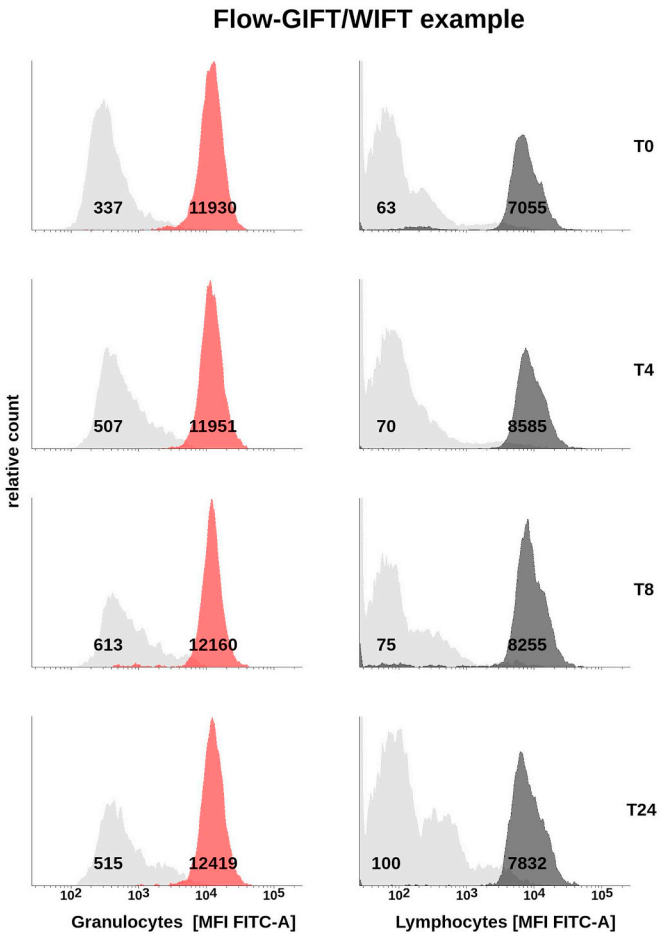

Fig. 3. Flow-GIFT/WIFT example. Time course of a sample containing anti-HNA-1b + anti-HLA antibodies (coloured histograms) compared to a negative control (gray histograms). MFI (values) in the granulocyte gate (left) as well as the lymphocyte gate (right) stayed almost stable from T0 to T24 while MFI of the negative control (AB serum) slightly increased over time.

4. Discussion

It is recommended that granulocyte-reactive antibody testing should be performed with freshly isolated cells [10,15–17]. Despite that, a thorough evaluation of the acceptable time limits of cell isolation for valid GAT, GIFT and Flow-GIFT/WIFT results has, to the best of our knowledge, never been published. The aim of this study was to shed some light in the intra-test reproducibility when dealing with older cell samples (Table 3).

Our data could show that the GAT is the most error-prone when being performed with older cells. Results from 24 h old samples displayed major differences to results with fresh cells and should therefore not be used. When comparing freshly isolated cells to those aged for 4 or 8 h, we could find several differences, although overall agreement was still strong. We conclude from this data that, although not being identical, cells as old as several hours can be used for granulocyte aggregation testing without major changes in the results. This notion is supported by the fact that although they lost some of their aggregation strength, in all cases the positive samples stayed positive even after 8 h.

Nevertheless, it cannot be ruled out that antibodies with weak aggregation potential could become undetectable using older cells, especially when using heterozygous cells for specific antigens. In our study GAT was focused on the detection of anti HNA-3a antibodies, as these are known to displaying a strong aggregation potential and as they are most relevant regarding TRALI.

In this study GIFT was demonstrated to be less prone to age-

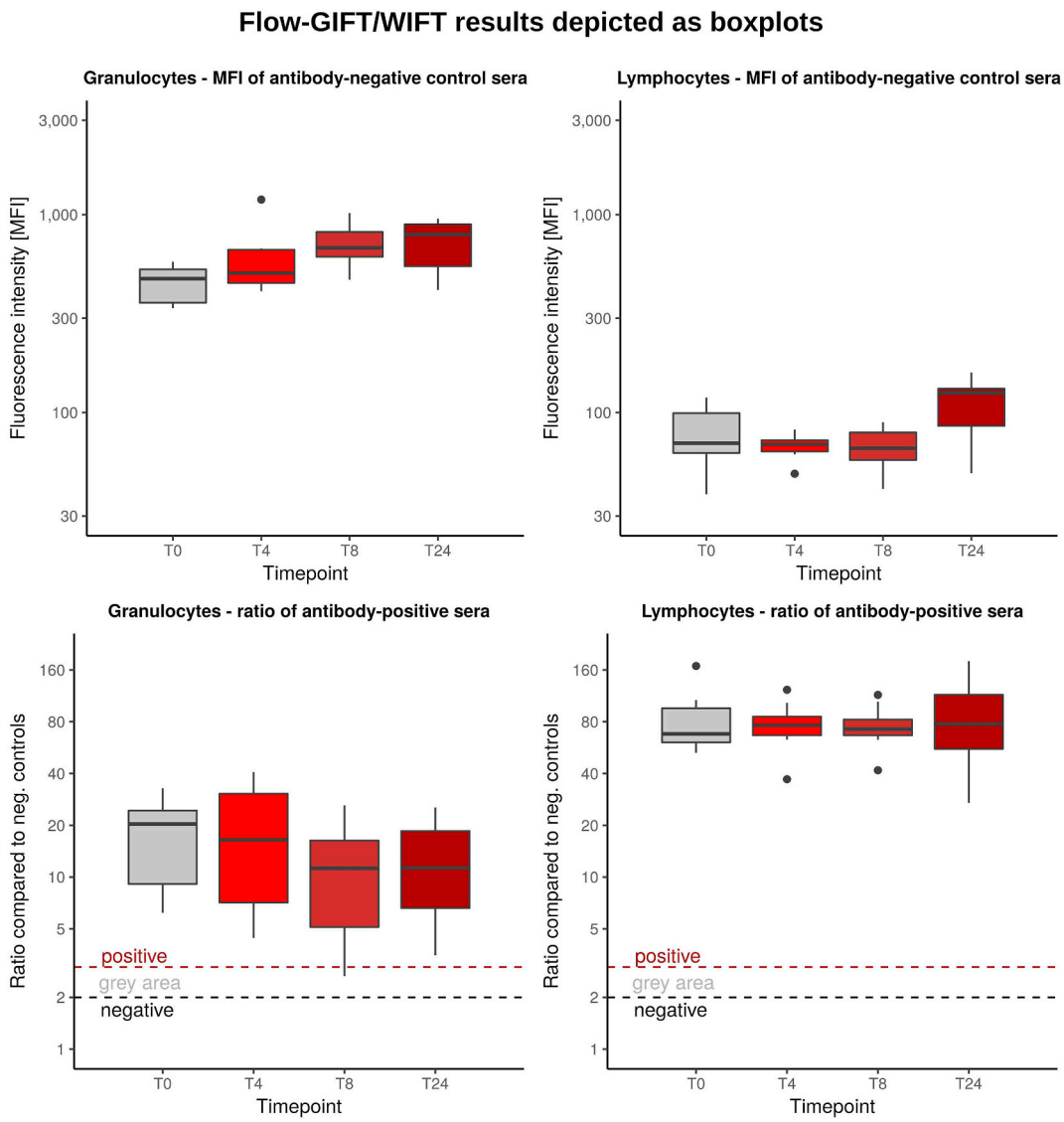

Fig. 4. Flow-GIFT/WIFT Results depicted as Boxplots. Median Fluorescence intensities (MFI) of the negative controls are shown for the granulocyte (A) and lymphocyte gate (B). MFI of the granulocyte gate increased at every time point while lymphocyte-MFI were almost stable until T8. Ratios of antibody-containing samples (MFI sample/MFI negative controls, n = 7 for every time point) are shown for the granulocyte (C) and the lymphocyte gate (D). While ratios decreased non-significantly considering the granulocyte gate, lymphocyte-ratios remained stable until T24.

dependent artifacts than GAT. Even with cells isolated 8 h after blood donation, weak antibodies could be identified. Performing the GIFT with cells isolated 24 h after blood donation resulted in weaker fluorescence signals than with freshly isolated cells. The reason for the decrease of fluorescence intensity might be lower yield of neutrophils in the isolate. Neutrophil isolation 24 h after blood donation was associated with a higher contamination with lymphocytes and monocytes (49.2%), which do not carry HNA-1 and HNA-2 on their surface. Therefore neutrophil-specific antibodies were more difficult to be detected.

Interestingly, Flow-GIFT/WIFT was the most stable method, yielding comparable results even with cells isolated 24 h after blood donation. Although fluorescence intensities slightly decreased when cells were older, all antibody-containing sera were easily detected with ratios highly above the cut-off value. Moreover, neutrophil viability was still about 85%, even 24 h after blood donation.

The possibility to perform the Flow-GIFT/WIFT with leukocytes isolated from whole blood as late as 24 h alleviates several logistic challenges. For example, crossmatches before granulocyte transfusion could be performed more independently from the blood withdrawal of the donor. Donor whole blood could be shipped to a specialized laboratory to perform the analysis and therefore enables external blood services to obtain crossmatch results. Furthermore, Flow-GIFT/WIFT has the potential to be used as a stand-alone pre-screening test: leukocytes can be isolated faster than granulocytes for GAT or GIFT, and interpretation of those results is easier and less error-prone than microscopic analyses. In the case of positively-screened samples, neutrophils can be isolated in a second step from freshly acquired samples to perform the GAT (which is the most successful test to detect anti-HNA-3a and -3b) and the monoclonal antibody-specific immobilization of granulocyte antigens assay to verify other HNA specificities [27]. If it is not possible to obtain fresh blood samples, it is recommended that cells for GAT should be isolated within 4 h and for GIFT within 8 h of blood donation.

For this study all analyses were performed by the same analyst to minimize intra-observer variability. To additionally reduce subjectivity in sample evaluation for the tests performed using a microscope (GAT and GIFT), all results were assessed by four-eye-principle. Furthermore, all samples and cells used in this study were well-characterized due to their frequent application in routine granulocyte diagnostics. The main

Table 3
Comparison of GAT, GIFT and Flow-GIFT/WIFT. All three tests were compared for detection method, objectivity, sensitivity, needed expertise, the need for cell isolation, required hands-on time, acquisition and running costs as well as stability (defined as the ability to obtain accurate results when using older cell panels).

	GAT		GIFT		Flow-GIFT/WIFT	
detection method	***microscopy***	aggregate formation	***fluorescence-microscopy***	single cell fluorescence	***flow-cytometry***	fluorescence intensity values
sensitivity	***limited***	only antibodies with aggregating potential are detectable	***good***	granulocyte-reactive antibodies are detected	***very good***	granulocyte- and lymphocyte-reactive antibodies are detected
objectivity	***limited***	semi-quantitative, microscopical analysis of aggregates	***limited***	semi-quantitative, microscopical analysis of cells	***high***	quantitative analysis
expertise needed	***medium***	differentiating type of aggregate formation	***high***	detecting membrane fluorescence and granularity	***low***	calculating ratios (can be performed automatically)
need for cell isolation	***high***	neutrophils	***high***	neutrophils	***medium***	whole leukocytes
hands-on time cost	***low***	incubation, microscopy	***medium***	fixation, incubation and washing steps, microscopy	***high***	incubation and washing steps, fixation, instrument start up, acquisition and shut down
costs	***low***	inverted light microscope	***medium***	fluorescence microscope, secondary antibody	***high***	flow cytometer, secondary antibody
stability	***poor***	optimal: ≤ 4 hrs possible: up to 8 hrs	***medium***	optimal: up to 8 hrs possible: up to 24 hrs	***good***	possible: up to 24 hrs, maybe longer (not tested)

limitation of this study is the small sample size, which is due to the fact that alloantibodies against HNA are difficult to obtain in sufficient quantities. According to estimates, only 0.5% of women with a history of pregnancy are immunized against HNA [28]. This is why only specialized laboratories dispose of a variety of sera containing HNA antibodies. As the Flow-GIFT/WIFT has a great potential to cope with older cells, further studies with older cells (> 24 h) may be considered.

This study was set up for quality assurance of the technically demanding field of granulocyte serology. The evaluation of the maximum time interval between venipuncture and cell isolation may allow a better logistic preparation before testing and the possibility to ship whole blood from other facilities to specialized granulocyte laboratories.

5. Conclusion

Neutrophil-reactive antibody screening with cell-based methods depends on the rapid processing of whole blood, but the acceptable time limits for cell isolation differ distinctly between the utilized methods. The Flow-GIFT/WIFT proved to be the most stable screening method working with leukocytes isolated up to 24 h after blood donation.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinbiochem.2018.10.017.

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