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Anti-polymorphonuclear neutrophil antibodies in patients with leukopenia or neutropenia

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SUMMARY

Introduction: Immune humoral neutropenia (Np) could be the consequence of anti-polymorphonuclear neutrophil (PMN) antibodies, circulating immune complexes (CIC) and/or antibodies against myeloid precursors. Granulocyte immunofluorescence test (GIFT) and a leukoagglutination technique (LAGT) assays are recommended for its diagnosis. *Methods*: Fifty adult patients with secondary Np were screened for anti-PMN. GIFT by flow cytometry from viable PMN and LAGT were employed. In addition, CIC levels, low expression of $CD16_b$ ($CD16_b^{low}$), PMN phenotype and sera tumor necrosis factor-alpha (TNF-α) were also evaluated. *Results*: Direct IgG-PMN binding (dir-GIFT) was positive in 16% of the patients. Antibodies against autologous PMN were detected in 32% of the samples by indirect (ind)-GIFT and demonstrated in 70% of the sera by both ind-GIFT and/or LAGT. Predominance of human neutrophil alloantigen (HNA)-1b and HNA-2 expression was confirmed. $CD16_b^{low}$ was detected in 16% of the patient's PMN and TNF-α in 68% of sera patients. *Conclusion*: Our results suggest that diagnosis of immune Np in the laboratory may be improved by focusing on patient's PMN together with the assessment of cellular markers.

INTRODUCTION

Leukopenia (Lp) is characterized by a decrease in the absolute WBC count below 4×10^9 WBC/l. Lp may or may not be associated with neutropenia (Np) (Haddy, Rana & Castro, 1999). According to the polymorphonuclear neutrophil (PMN) cell count, Np can be classified as: severe, with PMN cell counts below 0.5×10^9PMN/l, moderate, with a PMN count ranging from 0.5 to 1.0×10^9PMN/l, or mild, with a PMN count ranging from 1.0 to 1.5×10^9 PMN/l (Bain, Seed & Godsland, 1984; Weingarten, Pottick-Schwartz & Brauner, 1993; Haddy, Rana & Castro, 1999; Dale *et al.*, 2006). PMN are critical effector cells in the innate immune defence against bacterial and fungal infection (Newburger, 2006). Agents such as Parvovirus B19 (Lehmann, von Landenberg & Modrow, 2003), human immunodeficiency virus (Riera *et al.*, 1992; Babadoko, Aminu & Suleiman, 2008) and cytomegalovirus (Samant *et al.*, 2007) can also be involved in the development of Np (Palmblad & von dem Borne, 2002; Starkebaum, 2002). Otherwise, some drugs can also induce Np (Berliner, Horwitz & Loughran, 2004). Anti-PMN antibodies have been detected in sera of patients with Np, in patients with febrile transfusion reactions, in women after pregnancy and in polytransfused patients, suggesting their role in the pathophysiology of certain types of clinical disorders (Lalezari, 1984). Lalezari described the first PMN-specific antigen located in the FcγRIII ($CD16_b$) (Lalezari & Radel, 1974; Huizinga *et al.*, 1990a,b; Salmon, Edberg & Kimberly, 1990). The new nomenclature defines the human neutrophil alloantigen (HNA) system, including five antigen groups: HNA-1, HNA-2 (CD177), HNA-3, HNA-4 and HNA-5 (Bux, 2008). In line with this, PMN may elicit autoimmune or alloimmune Np.

Depending on its etiology, immune Np is classified as primary or secondary. Secondary immune Np has been associated with systemic auto-immune diseases such as rheumatoid arthritis (RA), Felty's syndrome (FS), the closely related to FS large granular lymphocyte syndrome, and Sjogren's syndrome with antithyroid treatment or with viral infections (Capsoni, Sarzi-Puttini & Zanell, 2005).

Polymorphonuclear neutrophils express constitutively two low-affinity Fc gamma receptors, Fc (FcγR): FcγRII (CD32), which is a transmembrane molecule and FcγRIII ($CD16_b$), which is linked via a glycosylphosphatidylinositol (GPI) anchor to the membrane (Edberg *et al.*, 1989). In addition, FcγRI (CD64) is also essential in many effector functions of the immune system. FcγRI is upregulated during infections (Wagner *et al.*, 2003a) through mediators of systemic inflammation. Circulating immune complexes (CICs) constitute a heterogeneous group of FcγR ligands that differ not only in their ability to trigger distinct cellular responses but also in the mechanisms by which they induce cell activation (Huizinga *et al.*, 1988; Hundt & Schmidt, 1992). CICs have also been demonstrated in Np patients (Cappio *et al.*, 1981; Fejes *et al.*, 1983; Berliner, Horwitz & Loughran, 2004). It is difficult to determine the role of anti-PMN antibodies in the genesis of Np, as this entity may also be associated with low expression of $CD16_b$ ($CD16_b^{low}$), such as in paroxysmal nocturnal haemoglobinuria (PNH), a defect in GPI anchorage (Nishimura, Murakami & Kinoshita, 1999), 'null' PMN $CD16_b$ expression (Huizinga *et al.*, 1990a,b), or PMN apoptosis (Dransfield *et al.*, 1994; Aref *et al.*, 2004). PMN apoptosis is modulated by a wide diversity of circulating cytokines and/or monokines such as tumor necrosis factor-alpha (TNF-α) that rapidly accelerate their apoptosis (Papadaki, Coulocheri & Eliopoulos, 2000). Moreover, CICs may also mask the interpretation of anti-PMN assays.

Two anti-PMN assays are recommended for diagnosis of auto-immune Np (Bux & Chapman, 1997): granulocyte immunofluorescence test (GIFT) (Verheugt *et al.*, 1977) and a leukoagglutination technique (LAGT) (Lalezari, 1984). Based on reports from the literature, GIFT is sensitive, reproducible and specific but not useful in discriminating between negative and weakly positive results. Achieving a high accuracy (100%) is unlikely because of variable levels of specifically or nonspecifically bound IgG that may be attached *in vivo* to the PMN surface (Verheugt *et al.*, 1977).

In this study, we evaluated PMN-reactive IgG by flow cytometry using a modified GIFT (Riera *et al.*, 2006) and LAGT (Riera *et al.*, 1990). In addition, the PMN phenotype from patients and donors, $CD16_b^{low}$, CIC and TNF-α level were evaluated to improve the laboratory diagnosis of immune Np. We present the results of studies performed in samples from 50 patients submitted to our laboratory for evaluation of anti-PMN antibodies and analyze the results in the context of Lp/Np diagnosis.

MATERIALS AND METHODS

Patients

Adult patients enrolled (50 patients aged between 21 and 76, comprising 40 women and 10 men) had not been especially selected for this study and include all those submitted to the laboratory for anti-PMN antibody screening. They were referred by the clinicians with the following diagnosis: 18 Lp (36%), 25 Np (50%), 5 patients having both Lp and Np (10%) and 2 with Lp and anaemia, (4%). Twenty-two of them (44%) did not have other associated pathology. In addition to cytopenia (Lp and/or Np), 28/50 (56%) had either auto-immune, infectious, haematological, or oncological diseases or were without any other comorbid diseases. No active infections were detected at the time of the study. Patients signed informed consent and the Institutional Ethics Review Committee approved the study.

Serum samples and controls

Ten ml of blood were drawn into a dry tube, evaluated the same day and stored at −70 °C. Serum from a healthy individual was used as reference throughout the study (Ref-S). Human gamma globulin (Cohn fraction, No. G2388; Cohn fraction II, III; SIGMA, St Louis, MO, USA; 6 mg/ml) aggregated by heating (aggreg-γ) at 63 °C for 20 min was used as positive control for GIFT.

Blood samples and cell preparation

Ten ml of blood was collected on 2.5% w/v ethylene diamine tetra-acetic acid. Platelets were removed by low-speed centrifugation (10 min, 90 *g*) and leukocytes were obtained by dextran sedimentation (Riera *et al.*, 2006). Hence, all the assays were carried out from total leukocytes.

Determination of human neutrophil alloantigen phenotype

Polymorphonuclear neutrophil phenotype (Bux, 2008) was determined by using human monoclonal antibodies against HNA-1a (CLB-gran/11,5D7 Accuratechemical.com); CD177/HNA-2a (MEM-166, BD Pharmigen, San Diego, CA, USA) and HNA-1b (GRM1, kindly provided by Federico Garrido, MD, Virgen de las Nieves Hospital, Granada, Spain). Cells were stained for 30 min at room temperature and washed twice. Then, antimouse IgG R-Phycoerythrin (PE), rabbit $F(ab')_2$ (Dako, Carpinteria, CA, USA) was added, incubated for 30 min at room temperature, washed twice and resuspended in IsoFlow (FACS Flow, Becton and Dickinson, Mountain View, CA, USA). Stained cells were analyzed in a FACScan cytometer using CellQuest software (Becton and Dickinson).

Granulocyte immunofluorescence test: direct and indirect test

Granulocyte immunofluorescence test was performed (Riera *et al.*, 2006) excluding nonviable cells, with 7-amino-actinomycin D (7AAD; A9400; Sigma) (Philpott *et al.*, 1996) and goat $F(ab')_2$ antihuman IgG (Fcγ)-PE (Beckman Coulter Company, Marseille Cedex 9, France). For direct (dir)-GIFT, 10 µl of antihuman Fcγ-PE [1/5 in phosphate-buffered saline (PBS)] and 5 µl of 7AAD (1/20 in PBS) were added to 0.25×10^6 unfixed leukocytes and incubated for 30 min at 4 °C in ice water (Maher & Hartman, 1993). Indirect (Ind)-GIFT was evaluated using 20% or 50% of each patient's serum and Ref-S, and either autologous PMN (Auto-ind-GIFT) or PMN from a normal donor (Allo-ind-GIFT). In addition, in 23 patients, undiluted sera were tested with HNA-1a+/HNA-1a+ and HNA-1b+/HNA-1b+ PMN. As positive control, aggreg-γ was added to cells. Thereafter, cells were analyzed as described above.

In vivo PMN-bound IgG was detected by dir-GIFT and results were expressed as a ratio (R) of dir-Median (Med) of fluorescence intensity as follows:

$$\text{dir-Med R} = \frac{\text{dir-Med-FI patient PMN}}{\text{dir-Med-FI donor PMN}}$$

IgG binding to donor or self PMN (Ind-GIFT) was evaluated *in vitro* using Auto- or allogeneic (Allo) PMN and results were expressed as:

$$\text{ind-Med R} = \frac{\text{ind-Med-FI unknown serum}}{\text{Ind-Med-FI Ref-S}}$$

According to this ratio, a shift to the right on the histogram from patient serum/donor or self PMN *vs.* normal reference serum/donor or self PMN would be associated with circulating IgG-anti-PMN antibodies (auto- or allo-antibodies).

Absence of in *vivo* IgG binding to the surface of the lymphocyte or monocyte was confirmed by analysis of the corresponding gates (Robinson *et al.*, 1987). No binding to autologous mononuclear cells was observed.

Cut-off value of Ind-Med ratios

Distribution was not considered normal when skewness or kurtosis was below −3 or above +3, and/or P was <0.05 in the Shapiro–Wilks test. As the results showed no normal distribution, the 95th percentile was used as a cut-off value for GIFT.

Leukoagglutination activity test

Leukoagglutination activity test was determined employing total leukocytes instead of purified PMN (Riera *et al.*, 1990). Results were scored as negative (−) or positive (1+, 2+, 3+, or 4+). A rabbit antihuman PMN prepared in our laboratory against a pool of 10 healthy donors PMN was used as a positive control.

Circulating immune complexes

Circulating immune complexes were evaluated by serum precipitation with 3.5% w/v polyethylene glycol (Digeon *et al.*, 1977).

Neutrophil subpopulation with low $CD16_b$ expression

$CD16_b^{low}$ was evaluated by direct staining with the following monoclonal antibodies CD49d-FITC (44H6, Immunotech, Beckman Coulter Company), CD16-PE (G38; Caltag-Invitrogen, California, CA, USA), CD45-TC (J33; Cytognos, Salamanca, Spain) or using the combination of CD16-FITC (3G8; Caltag-Invitrogen), CD56-PE (B159; BD) and CD3-PE-Cy5 (UCHT1; e-Bioscience, San Diego, CA, USA) and isotype-matched antibodies. Samples were acquired and analyzed using R3 strategy of region intersections (Riera *et al.*, 2003). Results were expressed as percentage (%) of $CD16_b^{low}$ cells obtained from $CD16_b$ histogram.

Paroxysmal nocturnal haemoglobinuria clone detection

The presence of PNH clone was studied by direct staining with CD59-FITC (MH clone MEM-43; Caltag), isotype CD55 (mouse IgG1) and CD55-PE (clone 67N-LO63; Caltag) on erythrocytes and PMN cells (Galassi *et al.*, 2001). In this evaluation, the $CD16_b$ expression was included as another GPI receptor marker.

Serum tumor necrosis factor alpha assay

Sera were heated at 56 °C for 30 min and stored at −70 °C. TNF-α was measured according to manufacturers' instructions by an ELISA commercial kit (e-Bioscience).

RESULTS

Haematological parameters of patients referred for anti-PMN evaluation

At the time of sample collection, haematological parameters were tested, WBC count was normal in 13/50 (26%) samples. The diagnosis for 74% (37/50) of the patients was Lp, and out of the 52% (26/50) of the patients for whom the diagnosis was Np: 1/26 was having exclusively Np and 25/26 were concurrently having both Lp and Np. Severe Np was found in 10/26 of the patients, moderate in 7/26 and mild in 9/26. Only two of these 26 patients had been referred for both Lp and Np: no relationship could be established between these cytopenias and gender or age of the patients.

Determination of HNA phenotype

Polymorphonuclear neutrophil phenotype from 26/50 randomly distributed patients and 124 healthy donors were performed. In agreement with a previous communication (de La Vega *et al.*, 2008) high prevalence of HNA-1b and HNA-2 was observed both in the patients and in healthy donors. Some of these phenotyped PMN were assessed in Ind-GIFT.

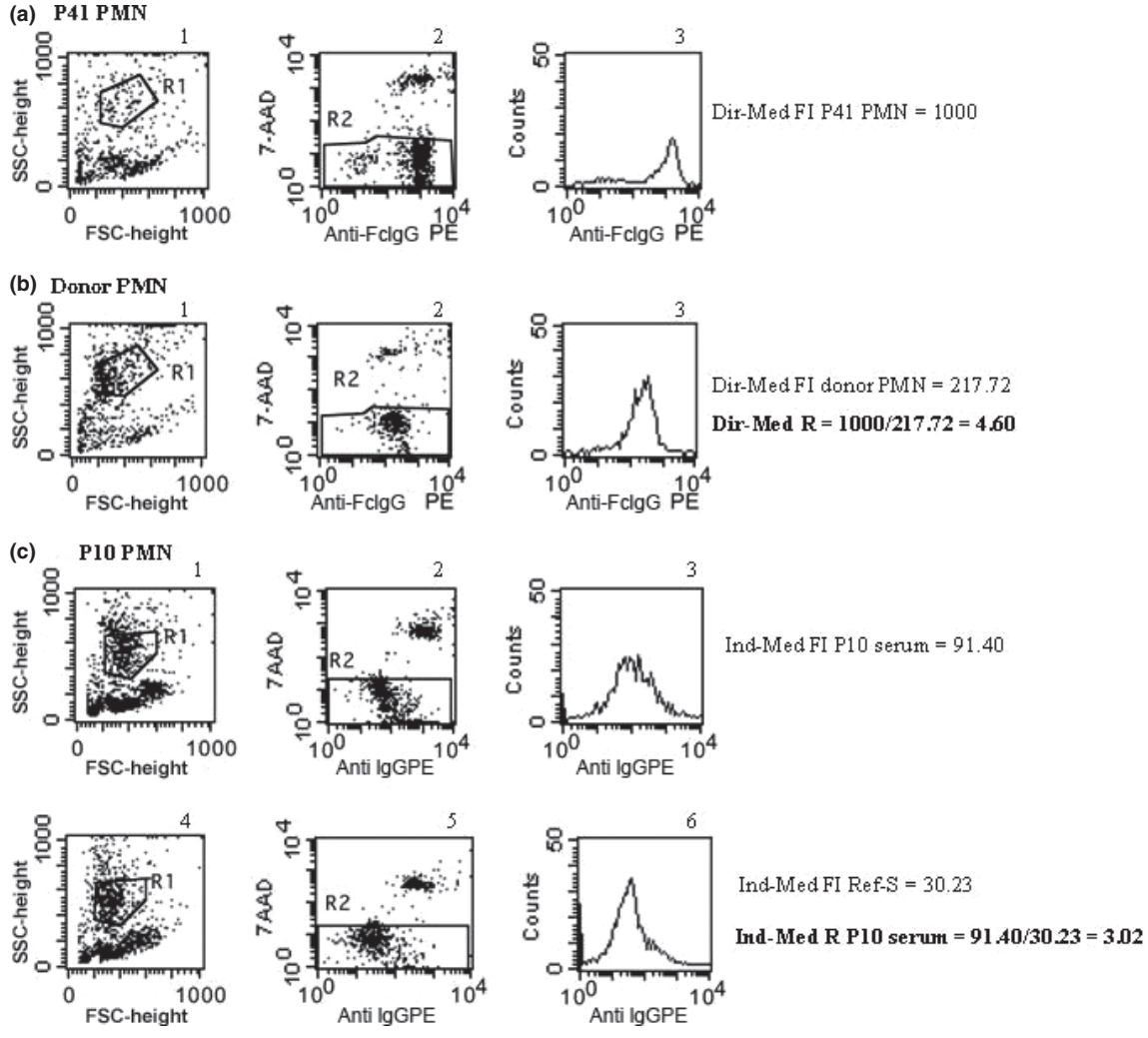

Figure 1. Flow cytometry analysis of dir-GIFT and auto- or allo-ind-GIFT in Np patients. Dir-GIFT analysis of PMN from P41 (a) and from one PMN donor out of 50 carried out (b) is depicted. A-1 and B-1: a PMN region R1 was drawn taking into account FSC *vs.* SSC. A-2 and B-2: R2 was carried out considering anti-Fc IgG PE and excluding 7AAD-stained cells gated on R1. A-3: direct median fluorescence intensity (dir-Med-FI) for P41 in a histogram of R2. B-3: dir-Med-FI for control PMN in a histogram of R2. The dir-Med-FI ratio (dir-Med R) calculated as described in Materials and methods is shown. (c) Ind-GIFT analysis of P10 serum or reference serum (Ref-S) and P10 PMN is shown in C-1 to C-6. C-1 and C-4: a PMN region R1 was drawn on the basis of FSC and SSC. C-2 and C-5: R2 was carried out considering anti-Fc-IgG PE and excluding 7AAD gated on R1. C3: ind-GIFT considering Med FI of P10 PMN and 50% serum (ind-Med-FI P10 serum). C-6: ind-GIFT considering Med FI of P10 PMN and 50% Ref-S (ind-Med-FI reference serum). Ind-Median-FI ratio for the P10 PMN sample was calculated by dividing ind-Med-FI P10 serum by ind-Med-FI Ref-S. ind, indirect; dir, direct; GIFT, granulocyte immunofluorescence test; Np, neutropenia; PMN, polymorphonuclear neutrophils; FSC, forward scatter; SSC, side scatter; 7AAD, 7-amino-actinomycin D; PE, R-Phycoerythrin.

GIFT, LAGT reactions and CIC detection for the study of Np patients

Analysis of dir-GIFT and ind-GIFT was carried out as it is shown in Figure 1. A positive dir-GIFT was observed in PMN from 8/50 patients (Figure 2a). Among these, 4/8 had been referred to the laboratory as having both Lp and Np associated with different conditions: anti-Parvovirus B19 antibodies and vascu-

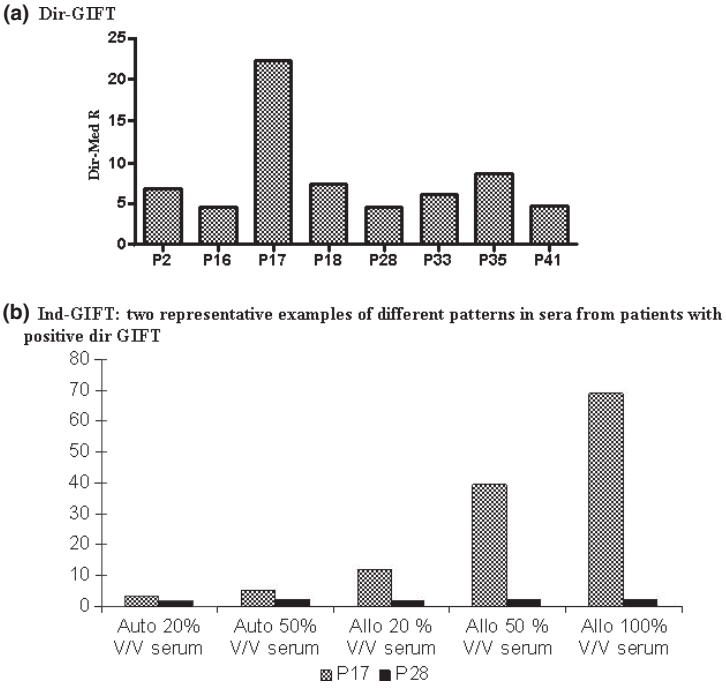

Figure 2. Dir- and Ind-GIFT in patients with Np. (a) Percentage of PMN bearing *in vivo* bound IgG (Dir-GIFT, %) in patients P2, P16, P17, P18, P28, P33, P35 and P41; (b) an example of ind-GIFT in P17 (light grey bars) and P28 (dark grey bars). Auto-ind-GIFT was performed by reacting autologous PMN with either 20% or 50% patients serum. For Allo-ind-GIFT, patient's serum (20%, 50% or 100% final volume) was reacted with allogeneic PMN. ind, indirect; dir, direct; Np, neutropenia; PMN; polymorphonuclear neutrophils; GIFT, granulocyte immunofluorescence test.

litis (P2), systemic lupus erythematosus (P16), RA (P18) and positive hepatitis C virus serology (P17). The other four patients were referred as Lp cutaneous allergy (P28); Np (P33); Lp (P35) and antibodies associated with hypothyroidism (P41).

Nevertheless, PMN from patients with high dir-GIFT showed different patterns when evaluated by Auto ind-GIFT using 20% and/or 50% V/V sera (Reference values <1.85 and <2.07, n = 50 respectively). In addition, reactivity of these sera against Allo-ind-GIFT (Reference values 20%, 50% and 100% v/v sera < 1.69; n = 50, <1.87; n = 53 and <1.89, n = 23 respectively) was different than that with Auto-ind-GIFT as it is shown with P17 where a higher Allo- than Auto-ind GIFT could be detected, while in P28 it did not (Figure 2b).

Reactivity with allogeneic (Allo) PMN was not related to previous PMN alloantigens exposure, as patients showing positive included both those who had been exposed and those who had not been exposed. Taken together, Auto-anti-PMN antibodies were found in 16/50 samples (32%). Nine of these samples (56%) were only LAGT positive against self-PMN: four of them belonged to patients with auto-immune diseases and two with infectious diseases. Afterwards, we evaluated sera from patients with known donor HNA-PMN through ind-GIFT and we found Auto-HNA-specific antibodies in one patient. Regarding CIC determination, sera from 5/48 patients were positive, P17 being

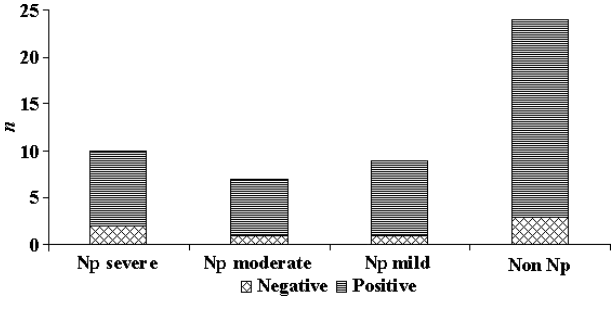

Figure 3. Anti-PMN antibodies in Np and non-Np patients. The number (n) of patients with severe (PMN count $<0.5 \times 10^9$ PMN/l), moderate (PMN count: $0.5–1.0 \times 10^9$ PMN/l) or mild (PMN count: $1.0–1.5 \times 10^9$ PMN/l) Np and non-Np patients (PMN count above 1.5×10^9 PMN/l) is shown. Dark grey area: patients with positive anti-PMN antibodies (dir-GIFT, ind-GIFT or LAGT) in each group. Light shaded area: patients with negative anti-PMN results. ind, indirect; dir, direct; Np, neutropenia; PMN; polymorphonuclear neutrophils; GIFT, granulocyte immunofluorescence test; LAGT, leukoagglutination technique.

one of them. In this context, Np in this patient may be associated with CIC. At the point of study, Auto-anti-PMN antibodies, either by dir- or ind-GIFT and/or LAGT, were detected in 35/50 sera from patients who were non-Np ($n = 24$) as well as those who had Np ($n = 26$). Allo-anti-PMNs were found in 32/50 by ind-GIFT and/or LAGT. Among Np patients 8/10 were severe, 6/7 moderate and 8/9 mild (Figure 3). In non-Np individuals, 21 were positive for anti-PMN antibodies and 11/21 were diagnosed with Lp.

Neutrophils with low $CD16_b$ expression and serum TNF-α

A defect in membrane GPI anchorage determines lower surface expression of molecules that are dependent on its link. Because Np has been associated with the presence of PNH (Nishimura, Murakami & Kinoshita, 1999; Abe *et al.*, 2006), we evaluated CD55 and CD59 expression in both erythrocytes and PMN together with $CD16_b$ expression to rule out PMN apoptosis. Apoptotic PMN, measured as $CD16_b^{low}$ (reference cut-off value, 1.81%; $n = 81$), were found in 8/45 patients without PNH clone. Among these, 7/8 showed immune humoral factors detected by GIFT and/or LAGT.

TNF-α has been suggested to be an underlying cause for low-grade chronic inflammation, which in turn has been really the cause of chronic idiopathic Np (Aref *et al.*, 2004) and, furthermore, as it is an apoptotic stimulus from PMN inducing $CD16_b^{low}$ on PMN, we measured its serum levels. Increased levels of TNF-α were detected in 68% (24/35) of the patient's sera (11.4 × 10 pg/ml, cut-off value 95 percentile, for the normal reference value). Among them, 50% of the patients were not associated with other pathology, while 33% were associated. TNF-α values of 6/8 patients who had higher percentages of $CD16_b^{low}$ PMN were examined, and in four of them they were elevated (12.3–18.5 pg/ml) (Figure 4).

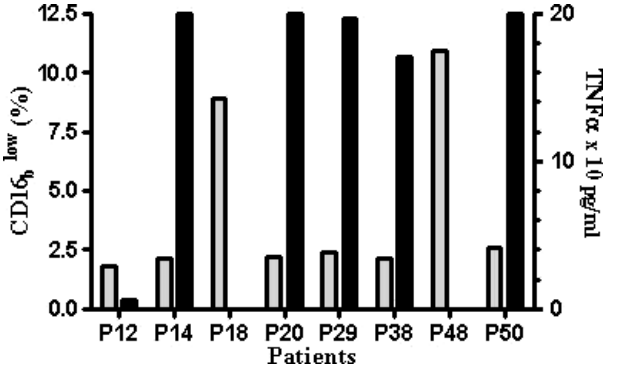

Figure 4. $CD16_b^{low}$ PMN and serum TNF-α levels in patients with apoptotic PMN. Percentage of $CD16_b^{low}$ PMN (light grey bars) and serum TNF-α levels (×10 pg/ml, normal range cut off value, 11.4 × 10 pg/ml) (dark grey bars) are shown for eight patients with apoptotic PMN (PMN $CD16_b^{low}$ expression, 1.81%). TNF-α was assayed by enzyme-linked immunorsorbent assay and $CD16_b^{low}$ expression by flow cytometry as described in Materials and methods. ind, indirect; dir, direct; PMN; polymorphonuclear neutrophils; TNF, tumor necrosis factor.

DISCUSSION

Immune humoral Np could be the consequence of anti-PMN, CIC and /or antibodies against myeloid precursors (Lamy, 2001; Boxer & Dale, 2002; Palmblad & von dem Borne, 2002; Papadaki *et al.*, 2003). Currently, there are no international gold standards available as reference parameters for the assessment of anti-PMN. As anti-PMN antibody testing poses some problems (Stroncek, 2004) the use of a neutrophil panel has been recommended (Bux, 2008) in order to obtain reliable results. In this study, we have used a combination of different laboratory tests (LAGT, dir GIFT and ind-GIFT) to detect anti-PMN antibodies (Bux & Chapman, 1997), in an attempt to provide information that may help in evaluating their relative impact. Additionally, we included evaluation of several surfaces markers and mediator of cellular responses such as FcγRIII /$CD16_b$ and TNF-α to better understand the genesis of Np.

As we had no access to a PMN panel, at the moment of each evaluation, we focused our attention on dir-GIFT (IgG bound *in vivo* to the patient's PMN) and Auto-ind-GIFT (reaction of the patient's sera with patient's PMN) but at least PMN cells from one healthy donor was also assayed (Allo-ind-GIFT). The same approach was followed for LAGT. Patients were included in this study at random and not dependent on the diagnosis they were referred, i.e. whether the patients had preliminary diagnosis of Lp

or Np associated with or without other pathologies. On the day of sample collections, 52% (26/50) had Np (severe, moderate or mild). We detected Auto-anti-PMN antibodies by dir-GIFT in 16% of the cell samples. Taking these results together, auto anti-PMN antibodies (GIFT and LAGT) were found in 70% of the patients (35/50) and CIC in 10% (5/48) of the sera. However, neither reactive nor agglutinating anti-PMN factors, and/or negative CIC could be detected in sera from other 8/50 patients. As at the time of study, one-half of the patients (21/24) did not have Np but had anti-PMN antibodies, it is probable that these patients were starting to develop Np. Interestingly, with the exception of one patient with RA, patients who had Np in association with other pathologies (infection or auto-immune diseases) had at least one GIFT or LAGT positive anti-PMN result. The differences observed between GIFT and LAGT may be explained by the fact that only IgG antibodies are evaluated by GIFT, while in LAGT, IgA and IgM isotypes may be also involved in PMN agglutination (Verheugt *et al.*, 1977) as well as CIC levels. Thus, GIFT, LAGT and CIC determinations are useful for detection of humoral factors involved in Np. However, these assays do not ensure the specificity of the antibodies from sera patients. In this context, the evaluation of PMN phenotypes from patients and healthy donors at the moment of the study, will allow further studies in order to better research the specificity antibodies, as we did by Allo-ind-GIFT with known PMN from healthy donors. In line with this, we found high prevalence of HNA-1 and HNA-2 among patients and healthy donors, which is in accordance with the frequency from Caucasian population (Hessner *et al.*, 1996; Stroncek, 2004; de La Vega *et al.*, 2008), and, furthermore, we were able to detect by Allo-ind-GIFT Auto-anti-HNA-1a with undiluted serum in the outcome of anti-PMN determination.

It is well known that low expression of $CD16_b$, can be the result of PMN apoptosis leading to Np. Besides, isoantibodies against $CD16_b$ may also downregulate its expression (Lamy, 2001). Therefore, we excluded a general failure of $CD16_b$ GPI anchorage to PMN membrane by checking CD55 and CD59 expression on PMN and erythrocytes. The PNH clone was not found in these Np individuals (Huizinga *et al.*, 1990a). Furthermore, we were able to identify a null PMN phenotype through the evaluation of $CD16_b$, albeit this receptor appears not to be critical for a normal effective PMN function (Huizinga *et al.*, 1990b; de Haas *et al.*, 1995; Wagner & Hansch, 2004b). Another factor that can lead to increased $CD16_b^{low}$ in PMN is TNF-α, a cytokine that is released along infectious processes. Although, in this study, we detected four patients with high TNF-α values and increased $CD16_b^{low}$ expression, no relationship could be established with infectious diseases. Although CD64 expression was not evaluated in this study and as it might be useful in monitoring these patients, we are including it in our current studies. Besides, we did not find correlation between TNF-α sera levels and the number of circulating PMN. Hence, in spite of previous reports (Papadaki, Coulocheri & Eliopoulos, 2000), ours results do not suggest any association between TNF-α and Lp/Np.

In summary, simultaneous application of dir-GIFT, ind-GIFT and LAGT as well as CIC determination lead to a more accurate detection of the humoral factors involved in the development of immune Np, however, laboratory diagnosis of this disease in adults can be improved by focusing on patient's PMN and assessment of cellular markers that may shed light on the genesis of Np.

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REFERENCES

Abe R., Shichishima T., Ogawa K., Saitoh W. & Maruyama Y. (2006) Neutropenia due to parvovirus B19 infections in patients with paroxysmal nocturnal hemoglobinuria. Blood transfusion and natural infection cases. Acta Haematologica 116, 245–248.

Aref S., El Refaei M.F., Sakrana M. & El-Nemre H. (2004) Enhanced neutrophil apoptosis in neutropenic patients with hepatosplenic schistosomiasis: evidence of serum Fas ligand. Hematology 9, 71–78.

Babadoko A.A., Aminu S.M. & Suleiman A.N. (2008) Neutropenia and human immunodeficiency virus-1 infection: analysis of 43 cases. Nigerian Journal of Medicine 17, 57–60.

Bain B., Seed M. & Godsland I. (1984) Normal values for peripheral blood white cell counts in women of four different ethnic origins. Journal of Clinical Pathology 37, 188–193.

Berliner N., Horwitz M. & Loughran T. (2004) Congenital and acquired neutropenia, Hematology; American Society of Hematology. Education Program, 63–79 (Review. Conference in San Diego, California, 4–7 December).

Boxer L. & Dale D.C. (2002) Neutropenia: causes and consequences. Seminars in Hematology 39, 75–81.

Bux J. (2008) Human neutrophil alloantigens. Vox Sanguinis 94, 277–285.

Bux J. & Chapman J. (1997) Report on the second international granulocyte serology workshop. Transfusion 37, 977–983.

Cappio F.C., Camussi G., Novarino A., Campana D., Masera C., Infelise V. & Gavosto F. (1981) Immune-complexes (IC) in idiopathic neutropenia. Scandinavian Journal of Haematology 27, 311–322.

Capsoni F., Sarzi-Puttini P. & Zanell A. (2005) Primary and secondary autoimmune neutropenia. Arthritis Research & Therapy 7, 208–214.

Dale D.C., Bolyard A.A., Schwinzer B., Pracht G., Bonilla M.A., Boxer M.H., Freedman J., Donadieu G., Kannourakis B.P., Alter B., Cham J., Winkelstein S.E., Kinsey C., Zeidler S.E. & Welke C. (2006) The severe chronic neutropenia international registry: 10-year follow-up report. Supportive Cancer Therapy 3, 220–231.

Digeon M., Laver M., Riza J. & Bach F.J. (1977) Detection of circulating immune complexes in human sera by simplified assays with polyethylene glycol. Journal of Immunological Methods 16, 165–183.

Dransfield I., Buckle A.M., Savill J.S., McDowal A., Haslett C. & Hogg N. (1994) Neutrophil apoptosis is associated with a reduction in CD16 (Fc gamma RIII) expression. Journal of Immunology 153, 1254–1263.

Edberg J.C., Redecha P.B., Salmon J.E. & Kimberly R.P. (1989) Human Fc gamma RIII (CD16). Isoforms with distinct allelic expression, extracellular domains, and membrane linkages on polymorphonuclear and natural killer cells. Journal of Immunology 143, 1642–1649.

Fejes M., Riera N., Palermo M., Mondini N.G., Rudoy S. & de Bracco M.M. (1983) Properties of polymorphonuclear leukocytes and serum factors in patients with chronic idiopathic neutropenia. Sangre 28, 563–569.

Galassi N., Canalejo K., Riera N., Bengió R. & Aixalá M. (2001) Flow cytometric analysis of an expansion paroxysmal nocturnal hemoglobinuria (PNH) clone in a patient with bone marrow failure. American Journal of Hematology 67, 277–278.

de Haas M., Kleijer M., van Zwieten R., Roos D. & von dem Borne E.G.K. (1995) Neutrophil Fc gamma RIIIb deficiency, nature, and clinical consequences: a study of 21 individuals from 14 families. Blood 86, 2403–2413.

Haddy T.B., Rana S.R. & Castro O. (1999) Benign ethnic neutropenia: what is a normal absolute neutrophil count? The Journal of Laboratory and Clinical Medicine 133, 15–22.

Hessner M.J., Curtis B.R., Endean D.J. & Aster R.H. (1996) Determination of neutrophil antigen gene frequencies in five ethnic groups by polymerase chain reaction with sequence-specific primers. Transfusion 36, 895–899.

Huizinga T.W., van der Schoot C.E., Jost C., Klaassen R., Kleijer Ml., von dem Borne A.E.G.K., Roos D. & Tetteroo P.A.T. (1988) The PI-linked receptor FcRIII is released on stimulation of neutrophils. Nature 333, 667–669.

Huizinga T.W., Kleijer M., Tetteroo P.A., Roos D. & von dem Borne A.E. (1990a) Biallelic neutrophil Na-antigen system is associated with a polymorphism on the phospho-inositol-linked Fc gamma receptor III (CD16). Blood 75, 213–217.

Huizinga T.W., Kuijpers R.W., Kleijer M., Schulpen T.W.J., Cuypers H.T.M., Roos D. & von dem Borne A.E.G.K. (1990b) Maternal genomic neutrophil FcRIII deficiency leading to neonatal isoimmune neutropenia. Blood 76, 1927–1932.

Hundt M. & Schmidt R.E. (1992) The glycosylphosphatidylinositol-linked Fc gamma receptor III represents the dominant receptor structure for immune complex activation of neutrophils. European Journal of Immunology 22, 811–816.

de La Vega E.D.C., Nogues N., Fernández Montoya A., Oyonarte S., Solis E. & Muñiz-Díaz E. (2008) HNA-1a, HNA-1b and HNA-1c gene frequencies in Argentineans. Tissue Antigens 71, 475–477.

Lalezari P. (1984) Alloimmune neonatal neutropenia and neutrophil-specific antigens. Vox Sanguinis 46, 415–417.

Lalezari P. & Radel E. (1974) Neutrophil-specific antigens: immunology and clinical significance. Seminars in Hematology 11, 281–290.

Lamy T. (2001) Autoimmune neutropenias. La Revue du Practicien 51, 1552–1557.

Lehmann H.W., von Landenberg P. & Modrow S. (2003) Parvovirus B19 infection and autoimmune disease. Autoimmune Reviews 2, 218–223.

Maher G.M. & Hartman K.R. (1993) Detection of antineutrophil autoantibodies by flow cytometry: use of unfixed neutrophils as antigenic targets. Journal of Clinical Laboratory Analysis 7, 334–340.

Newburger P.E. (2006) Disorders of neutrophil number and function, Hematology; American Society of Hematology. Education Program, 104–110 (Review. Conference in Orlando, Florida, 9–12 December).

Nishimura J., Murakami Y. & Kinoshita T. (1999) Paroxysmal nocturnal hemoglobinuria: an acquired genetic disease. American Journal of Hematology 62, 175–182.

Palmblad J.E. & von dem Borne A.E. (2002) Idiopathic, immune, infectious, and idiosyncratic neutropenias. Seminars in Hematology 39, 113–120.

Papadaki H.A., Coulocheri S. & Eliopoulos G.D. (2000) Patients with chronic idiopathic neutropenia of adults have increased serum concentrations of inflammatory cytokines and chemokines. American Journal of Hematology 65, 271–277.

Papadaki H.A., Eliopoulos A.G., Kosteas T., Gemetzi C., Damianaki A., Koutala H., Bux J. & Eliopoulos G.D. (2003) Impaired granulocytopoiesis in patients with chronic idiopathic neutropenia is associated with increased apoptosis of bone marrow myeloid progenitor cells. Blood 101, 2591–2600.

Philpott N.J., Turner A.J., Scopes J., Westby M., Marsh J.C.W., Gordon-Smith E.C., Dalgleish A.G. & Gibson F.M. (1996) The use of 7-amino actinomycin D in identifying apoptosis: simplicity of use and broad spectrum of application compared with other techniques. Blood 87, 2244–2251.

Riera N.E., Galassi N., Anselmo A., Aixalá M., Bouza M.B. & de Bracco M.M. (1990) Leukoagglutinins in patients with hemophilia. Immunology Letters 24, 107–111.

Riera N.E., Galassi N., de la Barrera S., Rickard E., Muchinick G., Pérez Bianco R. & de Bracco M.M. (1992) Anti-leukocyte antibodies as a consequence of HIV infection in HIV+ individuals. Immunology Letters 33, 99–104.

Riera N., Canalejo K., Aixalá M., Rosso M., Gaddi E., de Bracco M.M. & Galassi N. (2003) Detection of CD16low neutrophil subpopulations. Cytometry Part B. Clinical Cytometry 51, 45–46.

Riera N.E., Kantor G.L., Khoury M., Parias-Nucci R., Rapetti M.C., Aixalá M., Goldsztein S., Flores G. & de Bracco M.M. (2006) Autoimmune-alloimmune neonatal neutropenia. Serum reactive IgG and neutrophil-specific phenotype detected by flow cytometry. Medicina 66, 405–414.

Robinson J.P., Duque R.E., Boxer L.A., Ward P.A. & Hudson J.L. (1987) Measurement of antineutrophil antibodies by flow cytometry: simultaneous detection of antibodies against monocytes and lymphocytes. Diagnostic and Clinical Immunology 5, 163–170.

Salmon J.E., Edberg J.C. & Kimberly R.P. (1990) Fc gamma receptor III on human neutrophils. Allelic variants have functionally distinct capacities. The Journal of Clinical Investigation 85, 1287–1295.

Samant J.S., Namgoong S.H., Parveen T. & Katner H.P. (2007) Cytomegalovirus vasculitis and mucormycosis coinfection in late-stage HIV/AIDS. The American Journal of the Medical Sciences 333, 122–124.

Starkebaum G. (2002) Chronic neutropenia associated with autoimmune disease. Seminars in Hematology 39, 121–127.

Stroncek D. (2004) Granulocyte antigens and antibody detection. Vox Sanguinis 87, 91–94.

Verheugt F.W., von dem Borne A.E., Décary F. & Engelfriet C.P. (1977) The detection of granulocyte alloantibodies with an indirect immunofluorescence test. British Journal of Haematology 36, 533–544.

Wagner C. & Hansch G.M. (2004b) Genetic deficiency of CD16, the low-affinity receptor for immunoglobulin G, has no impact on the functional capacity of polymorphonuclear neutrophils. European Journal of Clinical Investigation 34, 149–155.

Wagner C., Deppisch R., Denefleh B., Hug F., Andrassya K. & Hänsch G.M. (2003a) Expression patterns of the lipopolysaccharide receptor CD14, and the FCgamma receptros CD16 and CD64 on polymorphonuclear neutrophils: data from patients with severe bacterial infections and lipopolysaccharide-exposed cells. Shock 19, 5–12.

Weingarten M.A., Pottick-Schwartz E.A. & Brauner A. (1993) The epidemiology of benign leukopenia in Yemenite Jews. Israel Journal of Medical Sciences 29, 297–299.