Improved cytochemical method for detecting Jordans' bodies in neutral lipid storage diseases

We describe an improved cytochemical procedure for detecting the presence of cytoplasmic lipid droplets (Jordans' bodies) in blood cells from patients suffering from neutral lipid storage diseases (NLSDs). The method employs Oil red O (ORO), Nile red (NR) or, preferably, Bodipy (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene) staining, coupled with DAPI (2-(4-amidinophenyl)-6-indolecarbamidine) staining of nuclei, to visualise clearly by fluorescence microscopy the presence of abundant neutral lipids (triacylglycerols and cholesterol esters) in granulocytes and monocytes. Using these reagents, we easily identified Jordans' bodies in buffy coats from patients affected by Chanarin–Dorfman syndrome (NLSD with ichthyosis) and NLSD with progressive myopathy, a laboratory finding critical for the diagnosis of both inherited metabolic disorders. Due to their yellow-gold and green fluorescence arising selectively from neutral lipid binding and their water solubility that makes alcohol unnecessary for staining, NR and particularly Bodipy are superior to ORO for the specific detection of Jordans' bodies in leucocytes.

Neutral lipid storage diseases (NLSDs) are a clinically heterogeneous group of non-lysosomal inherited disorders characterized by a cytoplasmic accumulation of lipid droplets (LDs) in most tissues. Rather than being an inert lipid inclusion, an impression given prima facie by their simple morphological structure under the light microscope, LDs were recently shown to be distinct organelles consisting of a core of neutral lipids, predominantly triacylglycerols or cholesteryl esters, that are surrounded by a monolayer of phospholipids and associated proteins.[1 2] LDs take active part in lipid metabolism as well as in membrane trafficking and other cell funtions.[3]

Since the pioneering observation by Jordans of numerous LDs in the leucocytes of two Dutch brothers suffering from progressive muscular dystrophy,[4] several reports have described the presence of abundant triacylglycerol deposits in the non-adipose cells of patients manifesting a variety of defects. Clinical phenotypes include myopathy (skeletal and heart muscle), liver damage, ataxia, neurosensory hearing loss, ichthyosis, subcapsular cataracts, nystagmus, strabismus and, rarely, mental retardation. When non-bullous congenital ichthyosiform erythroderma, presenting as fine scaling on an erythematous skin, is the dominant feature of NLSD since birth, the disorder is commonly referred to as Chanarin–Dorfman syndrome (CDS).[5 6] Neutral lipid storage skeletal myopathy[4 7] and cardiomyopathy[8] are two clinical variants of NLSD in which no skin involvement is observed.

Since NLSD is a genetic and allelic heterogeneous autosomal recessive disorder, so far mutational analysis is of limited use in clinical practice for a correct diagnosis of the congenital metabolic syndrome. The presence of 2–6 May–Grünwald–Giemsa (MGG)-negative round vacuoles (cytoplasmic LDs)—called Jordans' bodies (JBs)—in otherwise normal neutrophils and eosinophils from peripheral blood is the most common laboratory finding in NLSDs and, together with normal serum carnitine levels, is of clinical value for a rational diagnostic approach to the disorder. However, since MGG-negative spots in granulocytes and monocytes may result either from cell inclusions not containing neutral lipids (NLs) or from staining artefacts, a more specific cytochemical procedure seems desirable for an unambiguous identification of JBs.

To obviate this problem and to facilitate accurate and quantitative detection of LDs in NLSD leucocytes, we suggest the use of lipophilic dyes coupled with chromatin staining for visualising and counting JBs in buffy coat cells under fluorescent light.

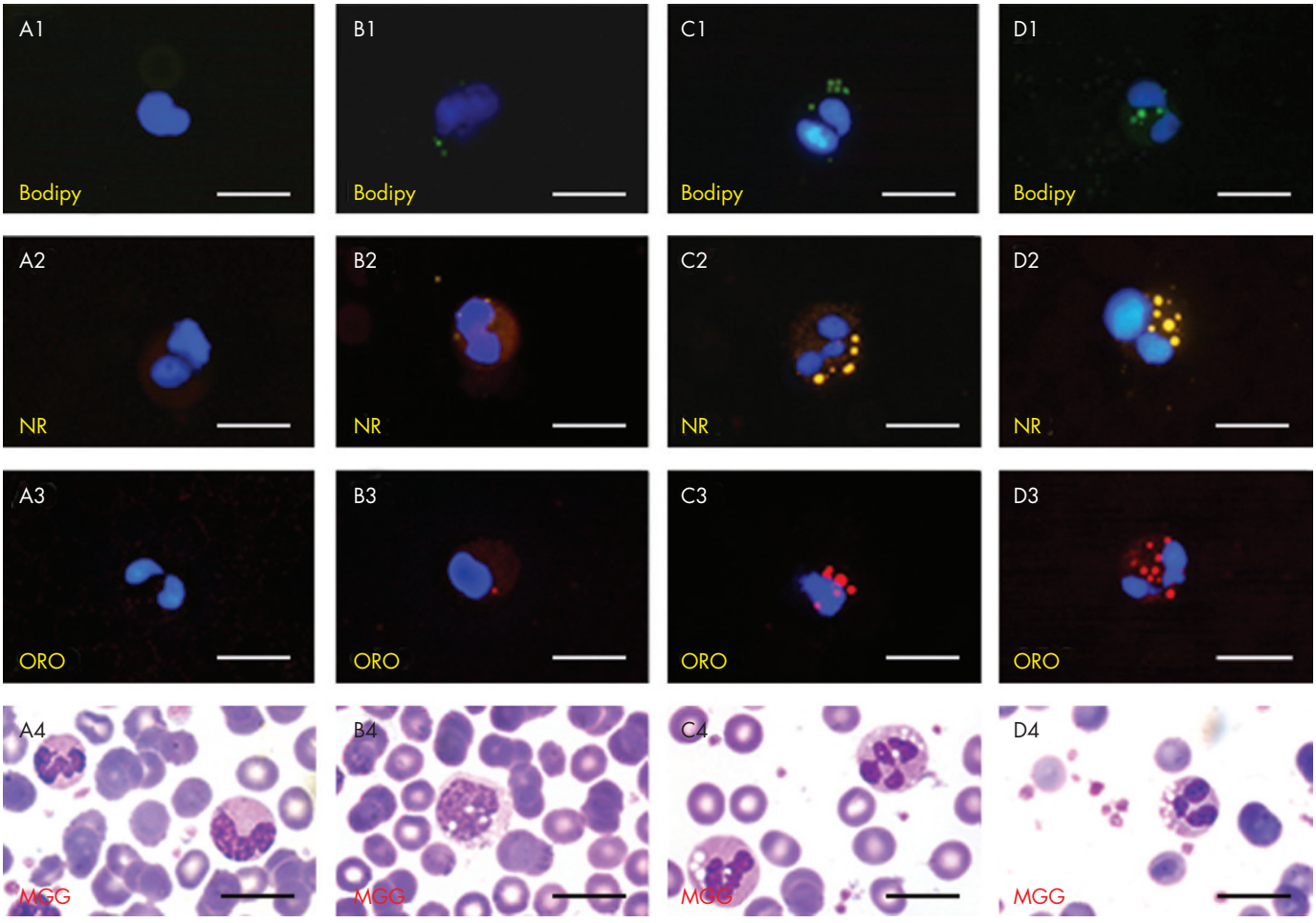

Figure 1 Microphotographs of Bodipy-, Nile red (NR)-, Oil red O (ORO)-, and May–Grünwald–Giemsa (MGG)-stained buffy coats: A1–A4, control subjects; B1–B4, carriers (ABHD5 mutation); C1–C4, patients affected by neutral lipid storage disease without ichthyosis; and D1–D4, Chanarin–Dorfman syndrome patients. Filters were I3 (excitation: 450–490 nm) for NR and Bodipy, TX2 (excitation: 540–580 nm) for ORO, and A4 (excitation: 340–380 nm) for 2-(4-amidinophenyl)-6-indolecarbamidine (DAPI). Bodipy, NR and ORO fluorescence images were merged with DAPI images by Adobe Photoshop software (v. 7). Scale bar: 10 μm.

Table 1 Average number, total perimeter and total area of LDs per cell as determined by Bodipy, Nile red and Oil red O staining of buffy coats from NLSD patients with and without ichthyosis. Values are given as the mean (SD).

	Bodipy (n = 98)	NR (n = 138)	ORO (n = 224)
Average LD number per cell*	4.9 (2.43)	6.7 (2.56)	11.2 (7.32)
Total LD perimeter (μ) per cell†	21.45 (9.36)	41.05 (14.73)	53.6 (36.09)
Total LD area (μ^2) per cell‡	8.4 (4.16)	18.6 (8.16)	23.25 (21.22)

LD, lipid droplet; NR, Nile red; ORO, Oil red O; SD, standard deviation; n, number of LDs.
*$p_{Bodipy/NR}<0.05$; $p_{Bodipy/ORO}<0.001$; $p_{NR/ORO}<0.05$.
†$p_{Bodipy/NR}<0.001$; $p_{Bodipy/ORO}<0.001$; $p_{NR/ORO}=0.158$.
‡$p_{Bodipy/NR}<0.001$; $p_{Bodipy/ORO}<0.001$; $p_{NR/ORO}=0.366$.

Materials and methods

Five patients for which a clinical and molecular (ABHD5 mutation detection) diagnosis of CDS had been unambiguously established and four patients affected by NLSD with skeletal myopathy and cardiomyopathy, together with their unaffected relatives, were included in this study. Informed consent was obtained from each patient and the control subjects.

Fresh EDTA-treated peripheral blood samples (6 ml) from patients, carriers and control subjects were centrifuged at 3300 *g* for 10 min. Buffy coats were carefully collected by gentle pipette suction, immediately smeared onto slide glasses, dried completely, and fixed with Biofix (Bio-Optica, Milan, Italy). Cells were sequentially washed with Dulbecco's phosphate-buffered saline (DPBS; Gibco, Grand Island, NY, USA), stained at room temperature for 20 min with Oil red O (ORO), Nile red (NR) or 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (Bodipy 493/503), and then with 2-(4-amidinophenyl)-6-indolecarbamidine (DAPI). ORO (Sigma-Aldrich, St Louis, MO, USA) staining was performed by a 0.2% (w/v) solution in 60% (v/v) isopropanol–water, twice filtered immediately before use. NR (Sigma-Aldrich) staining solution was freshly prepared in DPBS (1:100 v/v) from a saturated solution (1 mg/ml) in dimethylsulphoxide. Bodipy (Molecular Probes, Eugene, OR, USA), 1 mg/ml in absolute ethanol, was diluted 1:500 (v/v) in DPBS to a final concentration of 2 μg/ml. DAPI (Sigma-Aldrich) staining was performed using a 5 μg/ml water solution. After rinses with distilled water, cells were mounted on a glass slide with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and observed by a Leica MB5000B microscope equipped with a 100× Fluortar oil-immersion objective. Fluorescence images were captured using a Leica DFC480 R2 digital camera and a Leica Application Suite (LAS) software which allows the acquisition of digital images under defined set-up conditions. Set-up (exposure time, saturation, gamma and gain, respectively) was as follows: ORO, 4 s, 1.2, 1.43 and 1×; NR, 1 s, 1.1, 1.21 and 1.1×; Bodipy, 7 s, 1.1, 1.28 and 1.3×; and DAPI, 0.03–0.05 s, 1.1, 1.52 and 1×. LDs present in leucocytes were analysed for number and dimension using ImageJ 1.35 software (NIH, Bethesda, MD, USA). Traditional MGG staining was carried out according to the standard procedure.

Results and discussion

Using fluorescent dyes which specifically bind to the NL core of LDs, their presence and distribution have been investigated in many cells, both in culture and within tissue samples.[9–13] However, these selective dyes have not yet been used to detect LDs in leucocytes of NLSD patients, where they are identified as JBs.

To investigate how LDs differ in number and size among NLSD patients, carriers and healthy controls, we performed ORO, NR and Bodipy, as well as MGG staining of buffy coats from blood samples. Representative microphotographs of leucocytes (100×) obtained by traditional and NL-specific fluorescence staining are reported in fig 1. The presence of LDs is negligible in control subjects (A1–A4), whereas LDs of variable number and size are clearly detectable both in NLSD patients without ichthyosis (C1–C4) and in CDS patients (D1–D4). Interestingly, a few small LDs were also identified in the leucocytes of heterozygous (ABHD5 mutation) subjects (B1–B4), which is of diagnostic value for the carrier status.

While no NL-specific fluorescence could be detected in the stained leucocytes of control subjects, we were able to perform a quantitative analysis of the LD fluorescence signal from leucocytes of patients. Using an image analysis software (ImageJ), the mean number and dimension (perimeter; area) of LDs per cell were determined for each slide. Values reported in table 1 represent the means of 20 cells and were obtained from different microscopic fields chosen at random for image analysis. For each blood sample, the average number of LDs per cell is significantly different in Bodipy-, NR- and ORO-stained smears. Also, total LD perimeter and area per cell increase progressively from Bodipy to NR and ORO staining of buffy coat preparations. Earlier studies on LD staining with the same fluorescent dyes in cells other than leucocytes provide evidence for an explanation of these striking results. Fukumoto and Fujimoto[14] demonstrated that the presence of isopropanol in ORO staining solutions causes a deformation of adjacent LDs, eventually leading to their fusion. NR or Bodipy staining does not affect LD morphology to such an extent since these dyes are applied in alcohol-free aqueous solutions. Furthermore, while Bodipy binds only to the NL core of LDs, NR often causes diffuse background fluorescence. NR fluorescence arising from the dye–lipid interaction can be selectively measured using an excitation wavelength of 450–500 nm for NLs (yellow-gold emission) and 515–560 nm for polar lipids (red emission).[15 16] Since NR red fluorescence is very intense, there might be a possible red spill-over into the yellow-gold fluorescence as a consequence of leaking of stray red light through the filter barriers.[17 18] Together with our results, these observations point to Bodipy as the best tracer of LDs in leucocytes as well as in other cell types.

In conclusion, this study shows that a specific and quantitative detection of JBs in granulocytes and monocytes can be easily performed using lipophilic fluorescent dyes, leading to an improved cytochemical method for the laboratory diagnosis of NLSDs.

Take-home messages

- Neutral lipid storage diseases (NLSDs) are a clinically heterogeneous group of non-lysosomal inherited disorders characterised by a cytoplasmic accumulation of lipid droplets in most tissues.
- The presence of May–Grünwald–Giemsa-negative lipid droplets (Jordans' bodies) in otherwise normal neutrophils and esosinophils from peripheral blood is the most common laboratory finding in NLSDs.
- We describe an improved cytochemical method for detecting Jordans' bodies in buffy coats which employs lipophilic fluorescent dyes such as Oil Red O, Nile red and Bodipy, coupled with DAPI staining of nuclei.
- Our results show that a specific and quantitative detection of Jordans' bodies can be easily performed in human leucocytes using Nile red or, preferably, Bodipy, allowing an unambiguous laboratory diagnosis of NLSDs.

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PostScript

LETTERS

Albumin-bound paclitaxel (ABI-007; Abraxane) in the management of basal-like breast carcinoma

We read with great interest the paper by Banerjee *et al*[1] regarding the clinical outcome and response to chemotherapy in basal-like carcinoma of the breast. After extensive discussion of chemotherapeutics used in the management of basal-like carcinoma, they concluded that new treatment options should be investigated for patients with this subtype of breast cancer. A recent study by Pinilla *et al*[2] showed that caveolin-1 (CAV1) expression is associated with a basal-like phenotype in sporadic and hereditary breast cancers. They looked at CAV1 expression in 509 sporadic and 47 hereditary BRCA1-/BRCA2-associated carcinomas. A strong association was found between CAV1 expression and a basal-like phenotype. This phenotype was present in 52% of tumours that expressed CAV1, compared with only 9% of CAV1-negative carcinomas ($p<0.001$). Interestingly, Rouzier *et al*[3] showed that the basal-like and HER-2-positive subtypes of breast cancer are more sensitive to paclitaxel- and doxorubicin-containing preoperative chemotherapy than the luminal and normal-like cancers.

ABI-007 (Abraxane; American BioScience, Santa Monica, California, USA) is a new, biologically interactive, nanometer-sized albumin-bound paclitaxel particle initially developed to avoid the toxicities associated with polyethylated castor oil. It is the first of a new class of anticancer agents that incorporate albumin particle technology and exploit the unique properties of albumin, a natural carrier of lipophilic molecules in humans. After many phase I and II studies in metastatic breast cancer, Gradishar *et al*[4] in their phase III study compared albumin-bound nanoparticle paclitaxel, ABI-007, with polyethylated castor oil-based paclitaxel in women with metastatic breast cancer. This study showed greater efficacy and a favourable safety profile of ABI-007, although no subgroup analysis of molecular phenotypes for differential efficacy of the treatment was performed. After the incorporation of ABI-007 with albumin in circulating blood, the drug is preferentially transported from the blood to the tumour site in two ways. The first is through the leaky junction of endothelial cells that are highly pronounced around the tumour tissue by induction of angiogenesis, and the second, perhaps more prominent, way, is acting through receptor-mediated transcytosis of this albumin-bound ABI-007. This second mechanism is mediated by CAV1. Moreover, one study showed a 4.5-fold increase in paclitaxel transport across endothelial cells for ABI-007 compared with standard paclitaxel.[5]

In the light of the above information, we suggest that in patients with breast cancer with higher CAV1 expression, such as cancer with ABI-007 may be more effective and have a basal-like phenotype, more favourable safety profile.

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CD10 positivity in breast epithelial neoplasms

We read with great interest the study of Kalof *et al*[1] concerning immunostaining patterns of myoepithelial cells in breast lesions. The

Table 1 Comparison of CD10 immunohistochemical methods

Method	Kalof *et al*	Moritani *et al*	Our study
Antigen retrieval	Sodium citrate, pH 6.0 (Dako, Carpentaria, CA, USA)	Autoclaving at 121°C for 25 min	CCI (Tris/borate/EDTA), pH 8 (Ventana Medical Systems, Tucson, AZ, USA)
Antibody clone	Monoclonal, clone 56C6 (NCL-CD10-270; NovoCastra, Newcastle upon Tyne, UK)	NU-N1(Japanese Nichirei, Tokyo, Japan)	Monoclonal, clone 56C6 (Cell Marque, Hot Springs, AZ, USA)
Antibody dilution	1:80	1:50	Prediluted (dilution unknown)
Signal detection	Avidin-biotin-peroxidase method with diamino- benzidine (DAB)	Streptavidin-biotin-peroxidase method with DAB	Streptavidin-biotin-peroxidase method with DAB