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Breast Implant- Associated Anaplastic Large Cell Lymphoma: CD30 Modulation Observed with Flow Cytometry

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ABSTRACT

Background & Objectives: *Anaplastic large cell lymphoma that occurs after breast prosthetic implantation (BIA-ALCL) is a rare neoplasm for which early diagnosis is necessary because surgery can be the definitive therapy, with recovery from the disease.*

There are centres in the world that make a diagnosis only with pathological anatomy studies, but not with flow cytometric analyses and/or molecular tests. The flow cytometry easily allows for the detection, in particular, of small clones. The integration of data from various disciplines enables to detection of this pathology.

To prevent neoplastic cell loss, and ensure sample quality, it's necessary to process the samples within hours. Our previous work analysed the viability and CD30 and CD45 expression on fixed cells in a simulated matrix.

Methods: *We assessed the viability and the intensity of CD30 and CD45 expression during the time in fixed and unfixed neoplastic cells of a BIA-ALCL case.*

Results: *The fixative preserved neoplastic cells in periprosthetic fluid without altering CD30 expression, allowing for accurate analysis and quantification. We consistently observed strong CD30 intensity in fixed cancerous cells. In contrast, samples stored without fixative showed decreased CD30 expression already in cells in the pre-apoptotic stage.*

Interpretation & Conclusions: *We believe it is important to evaluate CD30 expression with the awareness of differentiating between multiple neoplastic clones in the sample and the modulation of CD30 based on cellular stress. This would ensure a corrected identification and quantification of neoplastic cells, optimizing diagnosis through integration with pathological anatomy and molecular study.*

Keywords

BIA-ALCL, CD30, fixative, Flow cytometry, Viability.

Introduction

The 2022 fifth edition of the World Health Organization Classification of Haematolymphoid Tumours [1] and the International Consensus Classification [2] of myeloid and lymphoid neoplasms recognised the anaplastic large cell lymphoma associated with breast prosthetic implantation (BIA-ALCL) as a definitive entity of malignant T neoplasm of the mature T lymphocytes.

According to the latest research, this type of lymphoma appears to be primarily linked to textured prosthetic implants [3-6]. There is no difference in the incidence of BIA-ALCL after a prosthetic implant for aesthetic purposes or reconstruction after mastectomy for cancer treatment [7-9].

The prevailing theory suggests that the larger surface area of textured breast implants leads to a higher bacterial load (bacterial biofilm) [5,10-12]. The rarity of this condition can be attributed to genetic predisposition as a contributing factor [5,10,13]. Between 5 and 10 million women worldwide have breast implants, and over 1.5 million women receive them annually [3]. The incidence varies among study cohorts, with estimates ranging from 1 in 354 to 1 in 37.000 [8,14]. The National Comprehensive Cancer Network (NCCN) data suggests that bilateral cases could account for 2-4% of all BIA-ALCL diagnoses [7].

The efficient treatment for confirmed BIA-ALCL involves removing the prosthesis and performing a total capsulectomy [15]. The primary treatment for advanced stages (II, III, or IV) should include chemotherapy and radiotherapy according to the guidelines for systemic ALCL. Immunotherapy (Brentuximab-Vedotin) should be considered [7,16]. According to the latest international NCCN guidelines from 2024, diagnosis is based on cytofluorimetric, cytologic, immunohistochemical and possibly molecular analysis [17].

The lymphoma phenotype can be heterogeneous [18-21]. The neoplastic population has increased physical parameters, and the most frequent phenotype is CD2+ (sometimes weakly expressed), CD4+ (weak to moderate), CD30+ (bright), CD25+ (bright), HLA-DR+ (bright), CD45+ (weak or negative), surface CD3-, CD7-, CD5-, and CD8- [18,22].

Combining cytometric results with cytological and immunohistochemical examinations allows for a more accurate diagnosis of this type of lymphoma. This is particularly true when rare neoplastic cells are present in the periprosthetic fluid and when there are large activated cells that can simulate atypical lymphocytes in immunohistochemistry [11-13].

The literature reiterates the necessity to analyse fresh samples to obtain reliable cytometric results, and this concept is also crucial for diagnosing BIA-ALCL [23-26]. In routine clinical practice, the prompt sample analysis is sometimes not feasible. Although data in the literature report that different fixatives preserve cells for cytofluorimetric analyses, no specific data are reported on key markers of BIA-ALCL such as CD30, which expression might vary within the neoplastic cell population.

In our previous study, we observed that the fixative Transfix maintains CD30 expression in PHA-activated lymphocytes (phytohemagglutinin), with better MFI stability than CD45 [26-28].

The aim of this study was to verify whether CD30 expression of BIA-ALCL periprosthetic fluid cells varies not only because of tumour heterogeneity but also for pre-analytical handling and whether Transfix treatment could preserve CD30 intensity up to 96 hours from collection.

Methods

We analysed a periprosthetic fluid (tube anticoagulated with K3-EDTA) from a patient with BIA-ALCL. The total cell count was obtained by the XN-9000 haematology analyser (Sysmex, Japan), using the biological liquid mode of analysis.

After the diagnosis made by the Laboratory Medicin Unit, the sample was aliquoted for the experiment. Cell viability assessed by 7actinomicine D (7-AAD) and CD30 expressions were monitored in aliquots stored at +4 °C after 24 and 96 hours. A sample fixed with transfix reagent (Cytomark, United Kingdom, UK) [28,27] for 96 hours was evaluated and compared with the aliquot preserved without fixation for 96 hours. Staining was made by CD30-PE (clone HRS4), CD45-FITC (clone J33), and 7actinomicine D (7-AAD) (Beckman Coulter, USA). FCM analysis was performed with a DxFlex flow cytometer and data were analysed with Kaluza analysis software version 1.2.1 (Beckman Coulter, USA). Informed consent was taken from the patient.

Results

The total cell count obtained by the XN9000 series was 1123/µL. The microscopic cell count was the following: neutrophils 1%, lymphocytes 27%, monocytes-macrophages 11%, eosinophils 3%, and BIA-ALCL cells 58%. BIA-ALCL cells displayed varied sizes, often large, with pale or slightly basophilic cytoplasm, numerous small vacuoles, and infrequent nucleoli. Unusual mitoses and apoptotic cells were present (Figure 1).

Flow cytometric assay of the fresh sample demonstrated the following subpopulation of total lymphocytes: T lymphocytes 85%, T cytotoxic lymphocytes 68%, T helper lymphocytes 14%, B lymphocytes 0% and Natural Killer 15%. Neoplastic T cells with high albeit heterogeneous expression of CD30 were 56% of total leukocytes, with this phenotype: CD45+ weak, CD4+, CD15+, CD43+, CD2+ weak, CD3-, CD5-, CD7-, CD8-, CD56-, CD16-, CD19-. The other cell populations in the sample were lymphocytes (30%), monocyte-macrophages (13%) and neutrophils (1%).

As expected, more events were acquired in fresh (sample A, 33443 events) and 96-hour fixed sample (B, 36971 events) compared to the 96-old hours non-fixed sample (C, 18149 events). The percentage of live cells selected in the FSC/SSC plot (physical parameters) was higher in sample A (48.42%) and sample C (52.83%) compared to sample B (11.45%). The percentage of acquired events in the CD45 weak/SSC high gate, which delineates neoplastic cells and neutrophils, was 54.72% in sample A, 55.59% in sample C, and 15.46% in sample B. The minimal presence of neutrophils in the periprosthetic fluid (1%) led us to believe that the differences in these percentages were due to the viability of the cancer cells. Considering ungated events, we found consistent percentages of cells in the same area (53.58% for sample A, 56.82% for sample C, and 57.09% for sample B), indicating a similar proportion of cancerous cells in all three samples. Ultimately, in the analysis of live cells based on physical parameters, dead neoplastic cells were excluded.

Figure 2 shows the representations of the live cells in the plots for CD30 and CD45 at diagnosis, and after 96 hours at +4 °C with

and without fixative treatment. It is possible to appreciate the quality of the labelling and scattering of the inflammatory cells and neoplastic cells between the freshest sample and the 96-hour samples. In particular, the percentage of CD30 positive cells was 51% in the fresh sample, 48.35% in the 96-hour fixed sample and 14.4% in the 96-hour not fixed sample (Figure 2, right column). This percentage difference was due to the loss of CD30+ neoplastic cells during the time and also to the down expression of CD30 on pathological cells.

To better understand CD30 expression on neoplastic cells, we labelled the 24-hours-old and a 96-hours-old sample with the vitality marker 7AAD (Figure 3). We showed a progressive decrease in the CD30 expression in the neoplastic cells, from live cells (7AAD negative) to dead cells (7AAD positivity). Neoplastic cells with weak positivity for 7AAD already showed reduced CD30 expression as the death cells. The expression of CD45 also seems to decrease progressively with decreasing cell viability but is never lost.

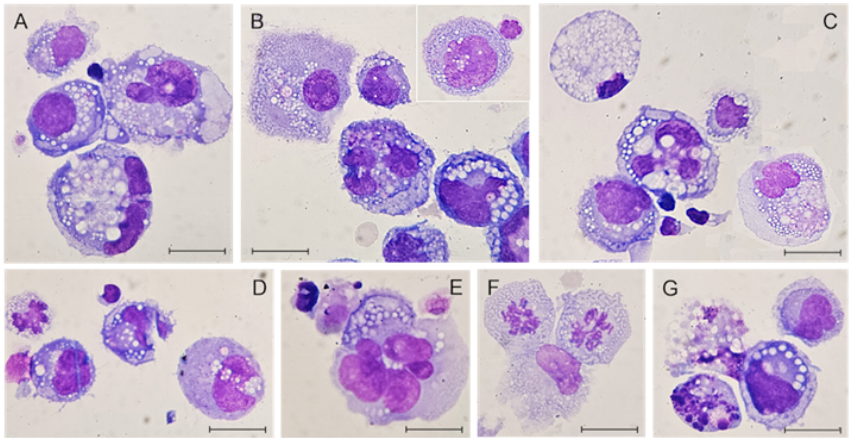

Figure 1: BIA-ALCL of periprosthetic effusion after May–Grünwald Giemsa staining (100× magnification).
The periprosthetic fluid contains atypical lymphoid cells of varying sizes. These cells have variably basophilic cytoplasm, containing small vacuoles, along with nucleoli. Some cells appear anaplastic, resembling Reed–Sternberg cells (E, in particular). Unusual mitoses (F) and apoptotic cells (G) are frequently observed. The typical cells of chronic inflammation are present, such as lymphocytes (A - D) and foamy macrophages (C). The scale bar is 20 µm.

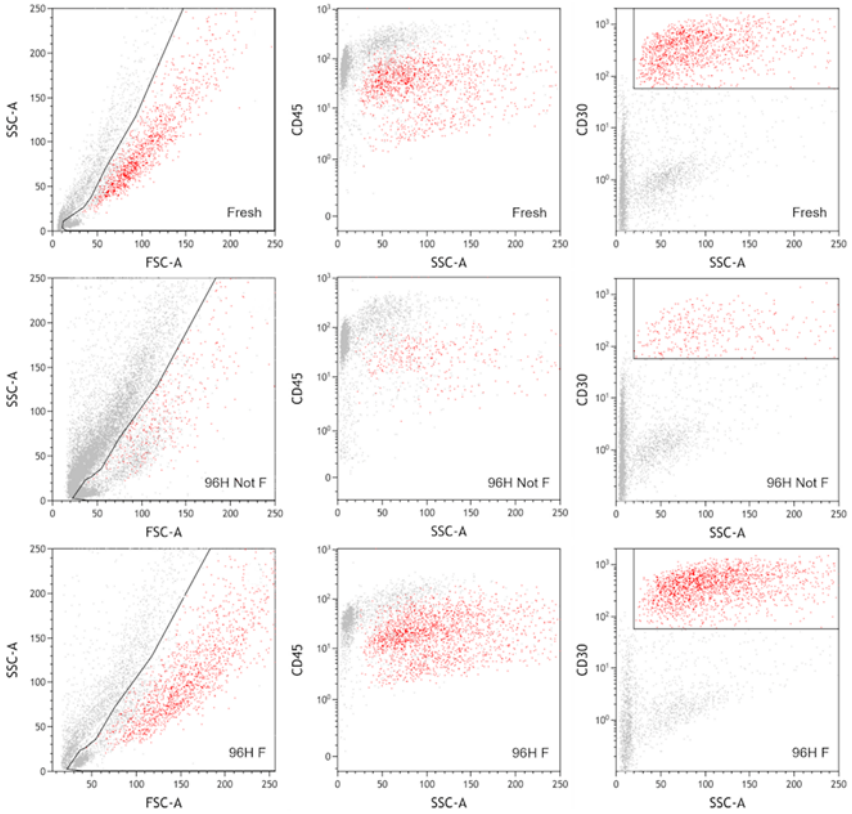

Figure 2: Plots representative of physical parameters, CD30 and CD45 expressions. There is a comparable quality of the labelling and scattering of the cells between the fresh sample and the 96-hour fixed sample compared to the 96-hour not fixed sample. Percentage of CD30+ cells: 51% in fresh sample, 48.35% in 96H F; 14.4% in 96H not F samples (fresh: fresh sample; 96H not F: 96-hour not fixed sample; 96H F: 96-hour fixed sample).

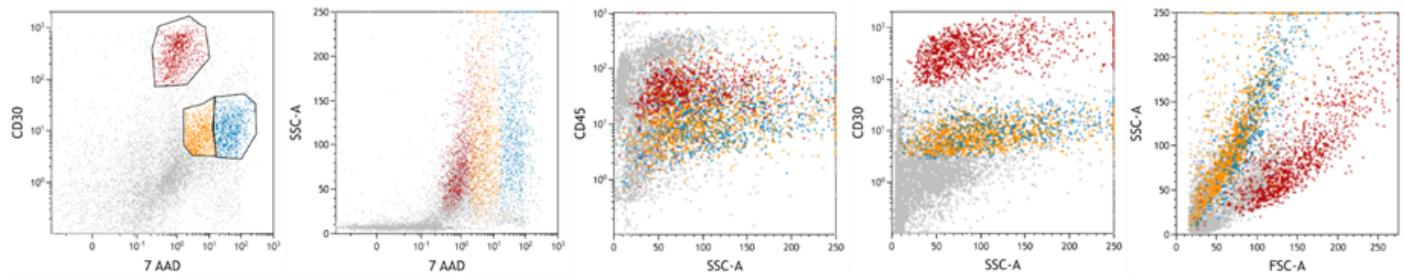

Figure 3: In the 24-hour-old sample, we observe the different expressions of CD30 on neoplastic cells related to cell viability. Red: cells with CD30 bright and 7AAD negativity (viable cells); ochre: CD30 with lower expression and weak positivity to 7AAD (dying cells); light blue: CD30 with lower expression and positivity to 7AAD (dead cells). CD45 also showed a higher intensity of expression on live neoplastic cells (7AAD negative).

As in our previous work [26] performed on a simulated periprosthetic fluid matrix, we tracked in the original periprosthetic fluid the median fluorescence intensity (MFI) over time to observe the expression of CD30 and CD45 in live neoplastic and inflammatory cells (lymphocytes and monocytes-macrophages) (Table 1).

Table 1: Evaluation of CD30 and CD45 MFI values on subpopulation cells in different conservation conditions.

Target population	CD30 MFI	CD45 MFI
BIA-ALCL cells		
At diagnosis	303.76	29.45
96 H fixed sample	371.86	17.28
96 H not fixed sample	169.68	23.35
Lymphocytes		
At diagnosis	0.69	71.03
96 H fixed sample	1.00	34.40
96 H not fixed sample	0.80	55.10
Monocytes- Macrophages		
At diagnosis	1.17	200.24
96 H fixed sample	1.66	92.38
96 H not fixed sample	1.30	172.17

MFI values were obtained from subpopulation cells under three different conditions. The MFI results for CD45 were notably reduced in the fixed 96-hour-old sample, regardless of the cell type. The MFI of CD30 showed a significant reduction in the 96-hour-old sample without fixative on neoplastic cells.

We observed similar variability in CD45 expression among the three populations studied. The 96-hour unfixed samples showed a reduction in intensity, although less than that observed in the fixed samples. Lymphocytes and monocyte-macrophages demonstrated an increasing trend in CD30 expression over time, particularly in the fixed samples. Even in the fixed neoplastic cells, an increase in the intensity of CD30 was observed. In contrast, the unfixed 96-hour sample showed a marked decrease in CD30 intensity suggesting a down expression of CD30.

Conclusions

BIA-ALCL is a very rare neoplasm that requires early diagnosis to ensure recovery from the disease with surgery and it is believed that there is an underestimation of this disease. Flow cytometry is an essential tool for diagnosing this neoplasm being CD30 expression a key marker.

Tumour cells are characteristically large, inclined to lower viability, which can affect diagnostic sensitivity and increase the likelihood of false-negative results. For this reason, it is recommended to process the samples within hours to prevent neoplastic cell loss. However prompt flow cytometry analysis is not always feasible in clinical labs for high workloads, samplings made at the end of the working day or of a working week (i.e. Friday late afternoon), and least but not last high distances between the surgical and the laboratory sites because of centralisation of cytometric labs. This demands sample treatment to prevent any potential alteration of key cellular markers. In our previous study, we used a flow cytometry dedicated fixative to guarantee viability and we demonstrated substantial stability of CD30 expression during the time on PBMCs naturally activated or activated with PHA [26,29].

In this work, we evaluated neoplastic cell viability, CD30 and CD45 expressions on periprosthetic neoplastic fluid, of a case of BIA-ALCL. We observed differences in the expression of CD30 and CD45 during the time, with and without fixative. We observed that, in unfixed samples, the percentage of CD30-positive neoplastic cells progressively declined over time with an associated reduction in MFI. This phenomenon is probably due to the cell's distress related to the high proliferative index characteristic of this pathology.

The fixed sample was well-preserved, both in terms of the absolute number of events acquired and the percentages of neoplastic cells which were similar to those of the fresher sample [28]. Moreover, the fixative guaranteed the CD30 expression stability. To our knowledge, this was not previously demonstrated.

On the other hand, the CD45 MFI demonstrated similar trends over time in all three populations studied, that is, a reduction in intensity in the unfixed sample but more pronounced in the fixed sample. This result leads us to hypothesize that in addition to its naturally reduced expression on older cells, there is also a possible different antigen-antibody interaction due to treatment with the fixative (formaldehyde). This seems to be independent of the cellular origin, neoplastic or inflammatory.

When we studied the samples with the 7AAD marker, we observed a decreased intensity of CD45 and even more CD30 expression on distressed neoplastic cells, which were 7AAD weakly

positive for their pre-apoptotic stage. This is consistent with the hypothesis of CD30 downregulation in the suffering cells and underscores the importance of correctly identifying pathological cells, especially as neoplastic cells experience more distress compared to accompanying inflammation cells in the absence of a fixative. These results contrast with what our group previously demonstrated: whereas CD30-positive lymphocytes activated naturally or by PHA do not exhibit selective death compared with the other leukocyte populations in the sample, lymphomatous lymphocytes exhibit increased cellular distress that leads them to progressively lose CD30 expression and then to apoptosis.

We believe that in flow cytometry, there is a risk of underestimating the percentage of neoplastic cells if the analysis depends on physical parameters to include only live cells, and/or on CD30 positivity, which can be very heterogenous in the sample, especially if the neoplastic quote is poorly represented.

In our case, in fact, we observed that the variability in cell vitality could account for the differing expressions of CD30 on neoplastic cells. Including a vitality marker for the cytometric analysis of BIA-ALCL cells would be beneficial, especially when the sample is not fresh.

The potential variation in CD30 expression on the cancerous cells can be observed also in fresh samples due to the long-term nature of the inflammatory process and the rapid growth of this disease. This could be explained by the presence of cells in various stages of the cell cycle. However, heterogeneous CD30 expression may depend not only on the degree of cell viability of the neoplastic clone but also on the presence of multiple BIA-ALCL clones with different CD30 expression, as demonstrated by Romero et al. [30]. In our opinion, distinguishing and understanding the cause of different CD30 expressions in the neoplastic specimen studied by flow cytometry could be helpful in more carefully identifying patients who may benefit from Brentuximab-Vedotin treatment.

The main limitation of our study is the evaluation of a single patient, but we must remember that this neoplasia is very rare and consequently very rare are materials for experiments. In our referral centre, less than 1 case per year has occurred. The strength of our study is the detailed cytofluorimetric analysis of any evaluated condition and the demonstration that the main BIA-ALCL marker, CD30, is preserved by formaldehyde-based preservative.

We believe that our evaluations can improve the processing of this kind of sample to optimize the flow cytometric analysis in the context of an integrated report with cytological, immunohistochemical and molecular studies.

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