High numbers of tumour infiltrating activated CTLs and frequent loss of HLA class I and II expression in aggressive B cell lymphomas of the brain and testis.


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Abstract

Loss of both HLA class I and class II expression in B cell lymphomas is a mechanism of escape from a cytotoxic T lymphocyte (CTL) immune response and will therefore give a strong selective survival advantage in tumours expressing strong immunogenic antigens. We investigated loss of HLA expression using specific antibodies on tissue sections from 254 B cell lymphomas originating from nodal and different extranodal sites in relation to numbers of tumour-infiltrating T cells.

Complete loss of HLA class I and II was observed in a minority of the nodal, stomach, and skin lymphomas but in the majority of the lymphomas originating from the testis and the CNS. Interestingly, relatively high percentages of activated CTLs were detected in both primary testicular and CNS lymphomas compared to lymphomas at the other sites, with highest percentages in the testis (p <0.0001).

We conclude that loss of both HLA class I and II expression occurs very frequently in lymphomas originating from the testis and the CNS as compared to nodal and some other extranodal sites. The presence of high percentages of activated CTLs in the testicular and CNS lymphomas suggests that loss of HLA expression provides a strong growth advantage for lymphoma cells in these immune-privileged sites.
Introduction

Diffuse large B cell lymphomas (DLBCLs) represent the most frequent type of the B cell non-Hodgkin lymphoma. Approximately 60% of these lymphomas arise primarily in lymph nodes and, of the extranodal sites, the gastrointestinal tract is the most common location. DLBCLs are thought to comprise several subtypes as they show a striking heterogeneity in clinical presentation, response to treatment and dissemination pattern.

Loss of HLA expression interferes with proper recognition by the cellular immune system and may provide DLBCLs with a mechanism to escape since B cell lymphoma cells are in principle excellent antigen presenting cells expressing both HLA class I and II molecules on their surface\(^1,2\). Loss of both HLA class I and II molecules has been reported in small numbers of DLBCLs\(^3-9\). In these studies, no separation was made between DLBCLs originating from lymph nodes and those from extranodal sites except for a group of aggressive lymphomas derived from the mediastinum\(^8-10\). We previously reported very frequent loss of both HLA class I and II expression due to extensive genetic alterations, including homozygous deletions of HLA class II genes, in DLBCLs primarily arising in the testis and the CNS\(^11\). Loss of HLA expression suggest that these lymphomas do express tumour-specific antigens and that a cellular anti-tumour immune response is generated with strong selection of HLA negative clones, even though both the testis and the CNS are regarded as immune privileged sites. The major effector cells of the specific cellular immune responses are activated cytotoxic T lymphocytes (CTLs) that can be detected by staining for granzyme B (GB) in combination with CD3, CD4 and CD8. Following recognition of antigen presented in the context of the proper HLA complex, GB accumulates in cytotoxic granules of CTLs. Subsequently, GB induces apoptosis via activation of the apoptosis signalling pathway after release into the target cell\(^12\).

In this study, we investigated whether tumour infiltrating activated CTLs were present in DLBCLs originating from the testis, CNS, stomach and skin, and compared this to the average number of activated cytotoxic T cells in primary nodal DLBCLs\(^13\). Moreover, we investigated the frequency of loss of HLA class I and II expression in DLBCLs originating from lymph nodes and the different extranodal sites and whether any association existed between loss of HLA class I or II expression and the number of the tumour-infiltrating activated CTLs in the testis and CNS.
Materials and Methods

Tumour samples

Formalin-fixed paraffin-embedded tissue blocks of 254 lymphomas matching the criteria for diffuse large B cell Lymphoma (DLBCLs) in the WHO classification, including all in the updated Kiel classification recognized large B cell lymphomas (centroblastic and centrocytic) with diffuse growth pattern, were collected. In all cases, a B cell origin was confirmed by immunohistochemical staining for CD20 or CD79a. Of these DLBCLs, 53 were primarily derived from lymph nodes, 74 were from the testis, 60 from the CNS, 22 from the stomach and 45 from the skin. Of the lymphomas derived from the skin, 19 were localized to the leg, while the other 26 were from either the trunk or the head. Neither DLBCLs presenting secondarily at these sites, nor DLBCLs from immune-compromised patients, were included in this study. In five CNS lymphomas from patients in whom there was doubt about their immune status, slides were stained for EBV and all were negative.

Lymphomas were retrieved from the tissue banks of the pathology departments of the Leiden University Medical Centre (Leiden, The Netherlands), the Free University Hospital (Amsterdam, The Netherlands), the Academic Hospital Groningen (Groningen, the Netherlands), the Leyenburg Hospital (The Hague, The Netherlands), Laboratorium voor de Volksgezondheid (Leeuwarden, The Netherlands) or obtained from the NHL Registry of the Comprehensive Cancer Centre West in The Netherlands between 1981 and 1989. Tissue blocks from 19 testicular lymphomas were collected by L. Looijenga from the Josephine Nefkens Institute (Rotterdam, The Netherlands). The Code for Proper Use of Human Tissue from the Federation of Medical Societies, published in 2002 and approved by Institutional Review Boards of the University Medical Centers in the Netherlands, was used.

Detection and interpretation of HLA class I and II expression

Sections were cut at 3µm thickness from tissue that had been fixed in neutral buffered formalin and embedded in paraffin wax, and immunohistochemical staining was performed according to standard procedures. Briefly, sections were dewaxed with xylene and endogenous peroxidase was blocked. After antigen retrieval with citrate buffer (10 mmol/l, pH 6.0 at 100 ºC for 10 min.), sections were incubated overnight with mouse monoclonal antibodies (MoAbs): HCA2 (anti-HLA-A; Dr. J. Neefjes, NKI, Amsterdam, The Netherlands), HC10 (anti-HLA-B/C; Dr. J. Neefjes), anti-HLA-DR (TAL.1B5, DAKO, Copenhagen, Denmark), anti-HLA-DR/DQ/DP (M0775, DAKO) and the primary rabbit polyclonal anti- B2M (A 072; DAKO). The staining results for all antibodies were scored
semi-quantitatively as follows: 1: < 5%; 2: 5-25%; 3: 26-50%; 4: 51-75%; 5: 76-100%. The first category (<5%) was intended to contain completely negative cases but we decided to use a safety threshold of <5% positive tumour cells as, in some cases with many reactive histiocytes, individual histiocytes were sometimes difficult to distinguish from neoplastic B cells. In each tumour, T cells, endothelial cells, macrophages and dendritic cells served as positive internal control for HLA class I and B2M expression, whereas macrophages and dendritic cells served as positive control for HLA class II expression. No distinction was made between weak or strong staining compared to the intermingled normal cells. Cases were considered not interpretable if reactive cells were not clearly positive.

Detection and quantification of activated CTLs

In 47 testicular, 29 CNS, 14 stomach and 10 skin (5 leg and 5 trunk/head) lymphomas, enough material was available to analyse the tumour-infiltrating T cells using the following antibodies: polyclonal anti-CD3 (A0452, DAKO) and MoAb anti-granzyme B (GB) (11F1, Sanbio, Uden, The Netherlands). Staining for CD3 and GB was performed after 1-hour incubation with the primary antibody in the DAKO Techmate™ plus (DAKO), according to standard manufacturial procedures. In addition, 12 CNS and 10 testicular lymphomas were stained for CD56 (MoAb 23C3, Sanbio) according to the same procedure after 1 h incubation. Quantification of the number of tumour-infiltrating CD3⁺ and GB⁺ lymphocytes was performed using a commercially available interactive video-overlay based measuring system (Q-PRODIT; Leica, Cambridge, UK) as has been described 18. For each tumour slide, 25-50 fields of view for CD3 and 45-55 fields for GB, were randomly selected using an automatic scanning stage, the size of one field being 95x127 µm. Only fields with solid tumour growth were included. Subsequently, the number of CD3⁺ and GB⁺ cells was calculated for 1 mm² of tumour. The percentage of activated CTLs in each case was expressed as a percentage of CD3⁺ lymphocytes (number of GB⁺ cells / number of CD3⁺ cells). The average numbers of infiltrating T cells in the several extranodal DLBCLs were subsequently compared to the average number in a group of 71 previously reported primary nodal DLBCLs 13.

Detection of PI-9

The same 47 testicular and 29 CNS DLBCLs were stained for PI-9 (clone PI9-17, VUMC Amsterdam, The Netherlands) after 1 h incubation as previously described 19. Staining intensity was enhanced using the catalyzed reported deposition (CARD) method (DAKO). Dendritic cells, which were previously shown to express PI-9, served as a positive internal control 19.
Phenotype analysis of tumour-infiltrating CTLs using double staining

In total, four CNS and six testicular lymphomas were double-stained for GB in combination with CD3, CD4 or CD8. These cases were selected on the basis of their broad range in HLA expression and numbers of CTLs (see Table 2). Tissue sections were simultaneously incubated overnight with anti-GB (1:50, Sanbio) in combination with either anti-CD3 (rabbit polyclonal, 1:200) (DAKO, Denmark), MoAb anti-CD4 (1:25, 1F6, Sanbio, The Netherlands) or MoAb anti-CD8 (1:200, 4B11, Sanbio) after antigen retrieval with either 10 mM citrate buffer, pH 6.0 (CD3 and CD8) or 1 mM TRIS/EDTA buffer, pH 9.5 (CD4). Sections were then incubated for 60 min. with A-546 goat α mouse IgG2a (anti-GB, 1:400) and respectively A-488 goat α rabbit IgG (anti-CD3; 1:100), A-488 goat α mouse IgG1 (anti-CD4; 1:200) or A-488 goat α mouse IgG2b (anti-CD8; 1:200) (Alexa, Molecular Probes, Leiden, The Netherlands).

Ten pictures of each slide were taken on a confocal laser scanning microscope (Zeiss LSM510) in a multi-track setting. Alexa-488 was excited at 488nm and detected using a 505-530 nm band-pass filter (channel 1). Alexa-546 was excited at 543 nm and detected using a 560-615 nm band-pass filter (channel 2). Each fluorochrome was given an artificial colour; Alexa-488 - green; Alexa-546 - red. The single and double positive cells were counted for each picture and the average percentage of activated cells (defined as GB+) that stained double positive was calculated for each case and each antibody.

Statistical analysis

Statistical analysis was performed using the SPSS software package (version 11.0, SPSS, Chicago, IL). The Pearson chi-square test was used to determine the significance of differences in HLA expression between the different groups of lymphomas. For statistical reasons, and because few tumours were scored in the 5-25%, 26-50% and 51-75% categories, we divided the tumours in a negative group if <5% of tumour cells showed staining and positive if ≥ 5% of tumour cells showed staining. The independent samples t-test was used to compare the means of tumour-infiltrating CD3+ and GB+ cells. Two-sided tests were used in all calculations. A p-value <0.05 was considered statistically significant.
Results

Loss of HLA class I expression is very common in DLBCLs originating from testis and CNS

Consistent with our previous results, complete loss of HLA-A expression was very prominent in primary lymphomas of the CNS and the testis with, respectively 36% (p<0.01) and 66% (p<0.0001) of the cases showing complete loss of expression compared to 16% of the nodal and 19% of the stomach DLBCLs (see Fig. 1a). The DLBCLs of the skin showed loss in 0% and 5% of the cases, respectively of the trunk and the leg. Complete loss of HLA-B/C and B2M expression was less frequent (see Figure 1a). The number of negative CNS DLBCLs differed significantly from the number of negative testicular DLBCLs for HCA2 (p< 0.001) and HC10 (p< 0.05) but not for B2M (p<0.5). In total 32 lymphomas showing concomitant loss of all class I molecules (p< 0.0001) (see Table 1).

Loss of HLA class II expression is very common in DLBCLs originating from testis and CNS and correlates with loss of HLA class I expression

The differences in loss of class II expression between the several groups were even more striking. The large majority of testicular (76%) and half of the CNS lymphomas showed loss of HLA-DR expression compared to 6% of the nodal lymphomas (see Figure 1b). Loss of all class II molecules (HLA-DR, DQ, and DP) was seen in 53% of the testicular and 40% of CNS lymphomas. The other groups of extranodal DLBCLs showed loss of class II expression in only very few cases. Loss of HLA-DR expression differed significantly between the testis and the CNS (p<0.005). Concomitant loss of HLA-DR and HLA-A expression was seen in 56 cases (p<0.0001) (see Table 1).
Figure 1: Loss of HLA class I (a) and II (b) expression versus site. The percentage of cases showing complete loss of HLA class I (a) and II (b) expression differed significantly between nodal, stomach and skin lymphomas, and testicular and CNS lymphomas (p < 0.0001, for each staining). Moreover, the number of testicular DLBCLs negative for HLA-A and HLA-B/C expression differs significantly from the number of negative CNS DLBCLs (respectively p<0.001 and p< 0.05). The same applies to loss of HLA-DR expression (p< 0.005).
**Activated CTLs are present in testicular and CNS DLBCLs**

The average number of CD3⁺ tumour-infiltrating lymphocytes in the testicular DLBCLs was comparable to the nodal cases but significantly higher than the average number in the CNS and the stomach lymphomas (see Figure 2a). The skin lymphomas however, showed the highest number of CD3⁺ T cells. The absolute number of infiltrating GB⁺ cells, on the other hand, was the highest in the testicular DLBCLs (see Figure 2b, testis versus nodal, p<0.0001). Moreover, the nodal DLBCLs showed a significantly lower percentage of activated cytotoxic T cells (mean 22%) compared to the average of the CNS (mean 44%, p<0.03) and especially the testicular DLBCLs (mean 64%, p<0.0001) (see Figure 2c). The percentages of activated CTLs in the skin (24%) and stomach lymphomas (27%) were comparable to the nodal cases.

**Higher numbers of infiltrating CTLs in HLA class I positive compared to class I negative testicular DLBCLs.**

The numbers of tumour-infiltrating activated CTLs were compared between cases with and without loss of HLA-A, HLA-B/C and HLA-DR expression. The testicular DLBCLs showed more infiltrating CD3⁺ and GB⁺ cells in the HLA-A positive compared to the negative cases (respectively p<0.01 and p<0.05) but the percentage of activated CTLs was equal (Figure 3). HLA-B/C and HLA-DR expression did not correlate with the number of infiltrating CD3⁺ T cells or GB⁺ cells (data not shown). In the CNS lymphomas no significant difference in infiltrating T cells was seen between the cases with or without HLA class I or II expression.

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Table 1: Correlation between loss of HLA class I and II expression. Loss of HLA-A expression correlated highly with loss of both HLA-B/C (a) and HLA-DR (b) expression.
Figure 2: High numbers of activated CTLs in DLBCLS of the testis compared to other sites. Averages of absolute numbers of infiltrating CD3⁺ cells (a) and GB⁺ cells (b) per mm² of tumour in 10 skin, 14 stomach, 29 CNS, 47 testicular DLBCLs, and 71 nodal DLBCLs. The testicular DLBCLs contained a significantly higher percentage of activated CTLs compared to both the nodal and all other extranodal lymphomas (p < 0.0001) (c).
Figure 3: Correlation between HLA-A expression and number of infiltrating CD3+ and GB+ cells. The testicular DLBCLs showed a significant difference in the average number of infiltrating CD3+ cells (p < 0.01) (a) and GB+ cells (p < 0.05) (b) but not in the percentage of activated CTLs (c) between HLA-A positive and negative cases. HLA-A expression in CNS DLBCLs was not associated with higher numbers of CTLs.
In testicular DLBCLs the majority of tumour-infiltrating CTLs express CD8 and a minority CD4.

Four CNS and six testicular lymphomas were analysed using immunofluorescence double staining for GB and various T cell markers. The percentage of infiltrating GB- cells that co-expressed CD8 ranged from 34-75% in the CNS to 45-92% in the testicular DLBCLs (Table 2, Figure 4) while the majority of CD8 negative CTLs showed co-expression with CD4. The percentage of activated CTLs that co-expressed CD3 ranged from 83 to 95%. To confirm that natural killer cells (NK) probably play a minor role in the anti-tumour response in these lymphomas, we performed a single staining for CD56 in 12 CNS and 10 testicular cases as we were not able to perform double staining for GB and CD56. Sporadic CD56- NK cells were detected in only 1 CNS lymphoma.

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Table 2: Most tumour infiltrating CTLs co-express CD8 and CD3. Cases of CNS (C) and testicular (T) DLBCL were analysed by fluorescence double immunostaining. Expression of HLA-A (A), HLA-B/C (B/C), B2M, HLA-DR (DR) and HLA-DR/P/Q (DR/P/Q, the number of infiltrating T cells, and the percentage of activated cytotoxic cells (GB+ cells) that showed co-expression with CD3 and CD4 or CD8 are given.

° 1 (negative): <5% positive tumour cells; 2 (positive): ≥5% positive tumour cells

NE: not evaluable

◊ Absolute numbers of CD3+ cells or GB+ cells per 1 mm² of tumour

■ Percentage of GB+ cells that co-express CD3, CD4 or CD8.
Figure 4: Activated CD8+ T-cells in testicular DLBCLs. A testicular DLBCL (T28) showing loss of HLA-A expression (A), tumour- infiltrating CD3+ cells (B) and GB+ cells (C). The infiltrating T-cells co-expressed CD8 (green) and GB (red) (D).

Discussion

In this study we confirm, in a large group of DLBCLs, our previous report on extensive loss of HLA class I and II expression in DLBCLs from the testis and the CNS 11 as compared to lymphomas originating from lymph nodes, stomach or skin. Primary DLBCLs of the stomach were included, as antigen-driven and T cell- mediated proliferation of tumour cells has been suggested in at least some of these lymphomas 20. Primary cutaneous DLBCLs were separately analysed for those arising on the trunk or the leg, as these two groups show significant differences in morphology and clinical outcome 21.

Besides extensive loss of HLA expression, we demonstrate here high percentages of tumour-infiltrating activated CTLs in DLBCLs originating from the testis (64%) or the CNS (44%). These percentages were significantly higher than the averages of 22-27% in the nodal, skin or stomach DLBCLs suggesting that the lymphomas in the testis and the CNS are subject to a higher selective pressure mediated by the immune system.

The CNS and testis are considered to be immune-privileged sites, with specific immune reactions and different microenvironments. One of these factors is the so-called blood-organ barrier 22. This blood-organ barrier is not however absolute as, in the normal human
testis small populations of lymphocytes expressing the CD8<sup>+</sup> or CD8<sup>−</sup>/cytotoxic phenotype are present<sup>21</sup>. Also, MHC-positive tumour cells inoculated into the cerebrum or the eye were eventually killed by cytotoxic CD8<sup>+</sup> T-cells<sup>,24,25</sup> whereas MHC-negative tumours were not<sup>26</sup>. To elicit an efficient anti-tumour immune response, presentation of tumour-specific antigens to CD4<sup>+</sup> T cells in the context of HLA class II molecules is required. HLA expressing neoplastic B cells could efficiently elicit specific anti-tumour immune responses by presenting their own idiotype<sup>1,27-30</sup> or tumour-specific antigens derived from hypermutated genes including Ig genes, as has been described in primary DLBCL of the CNS<sup>31</sup>. Subsequent killing of tumour cells by activated CD8<sup>+</sup> CTLs requires interaction with HLA class I molecules. Loss of HLA class I expression therefore provides the testicular and CNS DLBCLs with a mechanism to escape from CTL attack. HLA-negative tumours, however, are prone to NK cell attack<sup>32</sup>. However, in line with other reports<sup>33-35</sup>, this defense mechanism probably plays a minor role in these lymphomas, as only sporadic CD56<sup>+</sup> cells were detected in only one of the CNS DLBCLs investigated. In a minority of testicular tumours with high levels of infiltrating CTLs, no loss of HLA class I expression was observed. Previously, expression of PI-9, the granzyme B inhibitor, has been described in a large proportion of nodal DLBCLs as a mechanism to escape attack by CD8<sup>+</sup> CTLs<sup>13,19</sup>. In contrast, none of the 47 testicular or 23 CNS lymphomas including the class I positive tumours, showed expression of PI-9. Other genetic alterations might also play a role in immune escape of the HLA class I positive lymphomas such as defects in the apoptosis pathway<sup>36</sup>. Several authors also described deleterious mutations in CD95 in sporadic DLBCLs including some cases derived from the CNS<sup>31,37,38</sup>. Two major killing pathways are directly used by CD4<sup>+</sup> T-cells without intervention of CD8<sup>+</sup> effector cells. The first is CD95 mediated<sup>39,40</sup>, the second requires exocytosis of granzyme granules potentially allowing for lysis of all HLA-class II positive targets<sup>41-44</sup>. Also loss of class II expression probably provides DLBCLs with a survival advantage, as we found CD4<sup>+</sup> CTLs infiltrating in the lymphomas of the testis and the CNS. Several authors reported a correlation between loss of HLA-DR expression in lymphomas and a worse prognosis<sup>3-7,45,46</sup>. Moreover, in vitro studies in B cell malignancies showed direct apoptosis or enhanced susceptibility for CD95-induced apoptosis or antibody-dependent cellular cytotoxicity by anti-HLA-DR antibodies<sup>46-53</sup>. Recently, we reported homozygous deletions and hemizygous deletions in combination with mutations of the HLA-DR and DQ genes in a large proportion of testicular DLBCLs<sup>31,54,55</sup>, thereby providing evidence at the genetic level for the importance of HLA class II loss for lymphoma genesis.
One can only speculate about the reason why loss of HLA class I and II expression is such a dominant feature in primary DLBCLs of the testis and the CNS. In an immune-mediated inflammatory response the lymphoma cells probably initially function as antigen presenting cells but due to various genetic alterations they eventually become antigen independent and grow autonomously. Support for loss of HLA expression as a secondary event comes from our observations that some cases only show subpopulations of tumour cells with HLA loss. The high percentages of activated CTLs in the testicular and cerebral DLBCLs compared to the nodal DLBCLs suggests that the former are more immunogenic and elicit stronger CTL responses.

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52) Blancheteau V, Charron D, Mooney N. HLA class II signals sensitize B lymphocytes to apoptosis via Fas/CD95 by increasing FADD recruitment to activated Fas and activation of caspases. Hum Immunol. 2002;63:375-383.


