Expression of human leukocyte antigens in diffuse large B cell lymphomas

Riemersma, Sietske Annette

Chapter 2

Extensive Genetic Alterations of the HLA Region Including Homozygous Deletions of HLA Class II genes in B-cell Lymphomas Arising in Immune Privileged Sites.

Sietske A. Riemersma, Ekaterina S. Jordanova, Roelandt F.J. Schop, Katja Philippo, Leendert H.J. Looijenga, Ed Schuuring, and Philip M. Kluin.

Blood 2000; 96 (10): 3569-3577

Abstract

In B-cell lymphomas, loss of human leukocyte antigen (HLA) class I and II molecules might contribute to immune escape from CD8+ and CD4+ cytotoxic T-cells, especially since B-cells can present their own idiotype. Loss of HLA expression and the possible underlying genomic alterations were studied in 28 testicular, 11 central nervous system (CNS) and 21 nodal diffuse large B-cell lymphomas (DLBCL), the first two sites considered as immune privileged sites. The analysis included immunohistochemistry, loss of heterozygosity (LOH) analysis and fluorescent in situ hybridization (FISH) on interphase cells and isolated DNA fibers. Total loss of HLA-A expression was found in 60% of the extranodal and 10% of the nodal cases ($p<0.01$), whereas loss of HLA-DR expression was found in 56% and 5%, respectively ($p<0.01$). This was accompanied by extensive LOH within the HLA region in the extranodal DLBCL. In 3 cases retention of heterozygosity for D6S1666 in the class II region suggested a homozygous deletion. This was confirmed by interphase FISH showing homozygous deletions in the class II genes in 11 of the 18 extranodal but in none of the 7 nodal DLBCL ($p<0.001$). Mapping by fiber FISH showed variable deletions always including HLA-DQ and HLA-DR genes. Hemizygous deletions and mitotic recombinations often involving all HLA genes, were found in 13 of 18 extranodal and 2 of 7 nodal lymphomas. In conclusion, a structural loss of HLA class I and II expression might help the B-cell lymphoma cells to escape from immune attack.

Introduction

Approximately 40% of all Non Hodgkin's lymphomas (NHL) are large B-cell lymphomas (DLBCL), and from these approximately 40% present at extranodal sites, most commonly the gastrointestinal tract. Both the origin and homing of the tumour cells play an important role in the distribution at specific nodal and extranodal sites.[1] Few DLBCL present in the testis or the central nervous system (CNS), which together with the eye and ovary, are considered as immune-privileged sites.[2,3] Strikingly, a link between the testis, the CNS and the eye is supported by the observation that testicular DLBCL cells preferentially disseminate to the contralateral testis and the CNS[4] and that at least 20% of the primary CNS lymphomas disseminate to the eye.[5,6]. This suggests that, in addition to specific homing mechanisms, tumour cells may be locally selected by topographical differences in immune attack.

The existence of an in vivo immune response against human cancer cells has been demonstrated for a variety of human tumours, especially virally mediated tumours. In other neoplasias, tumour specific antigens are supposed to be a target of cytotoxic cells. In B-cell lymphomas, the immunoglobulin idiotype has raised much interest as a unique target for immunotherapy.[7-9]

So far most attention has been paid to the role of CD8+ cytotoxic T-cells and in consequence to loss of human leukocyte antigen (HLA) class I expression on tumour cells as a route for immune escape.[10,11] However, recent observations indicate that HLA class II and CD4 play an important role as well. Firstly, HLA class II molecules on the tumour cells may be a direct target for CD4+ cytotoxic T-cells.[12-14] Secondly, to mount a sufficient immune response, CD8+ cytotoxic T-cells need assistance by antigen presenting cells (APC) and CD4+ helper T-cells.[15] Since, like their normal counterparts, neoplastic B-cells potentially express these molecules as well as CD40 and costimulatory molecules, they might have a dual role as APC's and target-cells for cytotoxic T-cells.[16] In consequence, loss of HLA molecules on neoplastic B-cells likely contributes to the natural poor in vivo anti-tumour immune response and the failure of T-cell directed immunotherapy.[10,11] Interestingly, in a subset of aggressive B-cell lymphomas (which mainly represent DLBCL) loss of HLA class I and class II expression has been described, and this loss correlated with extranodal disease[17] and poor survival.[18-20]. Similarly, in a preliminary analysis of 258 DLBCL, we found a relatively frequent loss of class II expression in primary extranodal DLBCL. Seven DLBCL of the testis, considered as an immune privileged site, showed a coordinate loss of class I and class II expression (unpublished results). At immune-privileged sites, several factors mediate immune escape,[2,3] however, the privilege is not absolute[21] implying that loss of

HLA expression on the tumour cells might additionally help them to escape from killing by cytotoxic T-cells.

The molecular mechanisms leading to loss of HLA class I molecules in carcinomas and melanoma include defects in expression of β2-microglobulin (β2M)22 and TAP23 but also genetic aberrations in the HLA class I genes at 6p21.10,24 In contrast, little is known about the mechanisms leading to loss of HLA class II expression in B-cell lymphomas. The coordinate loss of class I and II expression in the testicular lymphomas prompted us to search for a common mechanism in a large series of these tumours. Here we report on the analysis of 60 DLBCL, including 28 primary testicular, 11 primary CNS and 21 primary nodal cases. A very frequent loss of expression of HLA class I and II was found in both types of extranodal lymphomas but not in nodal DLBCL. Molecular studies revealed very frequent homozygous deletions of the HLA-DR and -DQ region as well as larger hemizygous deletions and mitotic recombinations in the HLA class I and III region.

Materials and Methods

Tumour Samples.

Formalin-fixed paraffin-embedded tissue blocks of 60 lymphomas with the histopathologic diagnosis of diffuse large B-cell lymphoma (Revised European-American Lymphoma classification)[25] were collected. The B-cell origin was confirmed by immunohistological staining for CD19, CD 20, CD22 or CD79a. From these DLBCL, 21 were of primary nodal, 28 of primary testicular and 11 of primary CNS origin. Tissue blocks from 12 of the 60 cases were obtained from the tissue bank of the Pathology Department at the Leiden University Medical Center ([LUMC] Leiden, The Netherlands) while 12 testicular DLBCL were collected by Dr. L. Looijenga from the Josephine Nefkens Institute (Rotterdam, The Netherlands). The other 35 cases were obtained from the NHL Registry of the Comprehensive Cancer Center West in the Netherlands between 1981 and 1989[26].

Immunohistochemistry.

Immunohistochemical staining was performed on freshly cut 3 μM thick buffered formalin-fixed paraffin-embedded tissue sections according to standard procedures.[27] Slides were incubated overnight with mouse monoclonal antibodies (MoAbs): HCA2 (anti-HLA-A) (Dr. J. Neefjes, NKI, Amsterdam, The Netherlands), anti-HLA-DR (cloneTAL.1B5, DAKO, Copenhagen, Denmark), the primary rabbit polyclonal anti-β_2M (A 072, DAKO) and the rabbit MoAb anti-TAP1[28]. Frozen sections were fixed in acetone, washed in phosphate-buffered saline (PBS) and subsequently incubated with the mouse MoAb W6/32, a pan-class

I MoAb, SPV-L3 directed against DQ,[29] and B8.11.2 directed against DR[30] for 1 hour. For immunodetection the same protocol was used as for the paraffin sections. In each tumour T-cells, endothelial cells, macrophages and dendritic cells served as a positive control for class I expression while macrophages and dendritic cells served as a positive control for class II expression. Tumour cells were only scored negative if absolutely no staining was present as compared to a strong staining of internal control cells. If some staining was present but reactive cells stained much stronger tumour cells were scored weakly positive.

Microdissection and DNA Extraction.

DNA was extracted according to the protocol described by Isola et al.[31] with some adjustments. Paraffin-embedded tissue was cut in 10 μm sections and hematoxylin and eosin-stained. Before the normal dehydration steps, the staining procedure was interrupted for microdissection. To enrich for tumour cells, selected areas containing over 70% tumour cells were microdissected using a needle under direct light microscopic visualization. Normal control tissue was obtained using the same procedure. DNA was extracted by incubation for 72 hours at 56^{0}C in 1 mL of isolation buffer (100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, pH 8, 0.5% SDS). 30 μL aliquot of proteinase K was added and this step was repeated 24 and 48 hours later. DNA was isolated using phenol-chloroform-isoamyl alcohol, 20 μg/mL glycogen and 250 μL 7.5 M ammonium acetate and precipitated with 1 mL of 100% ethanol. DNA was dissolved in Tris-EDTA (10 mM Tris, 0.1 mM EDTA, pH 7.6) and 1 μL was used as a template for PCR.

LOH Analysis.

DNA from normal and tumour microdissected material was analyzed for LOH by PCR amplification. For most microsatellite markers the primers have been described before[32] except for C125, MICA, TY2A, BAT2, C47 and X87344 which were retrieved from submitted sequences (GenBank). The sequences of the latter markers are as follows:

C125.R:	5'AAGTCAAGCATATCTGCCATTTGG	C125.F:	5'CCCCAAACCCTGAAACTTG
MICA.R:	5'GGTGCTTCAGAGTCATTGG	MICA.F:	5'CTTTTTTTCAGGGAAAGTGC
TY2A.R:	5'TCAAACCAATCAGGGTGGC	TY2A.F:	5'AGAAGCAGTATACAGGGGC
BAT2.R:	5'AAGGGCTTTAGGAGGTCTG	BAT.F:	5'CCAGCCTGGATAACAGAAC
C47.R:	5'TCCTCCAGGTTCATCCATG	C47.F:	5'GTCTGTCCTGCATCAAATGG
X87344.R:	5'CTCTAACTCCTTTCATGCTGC	X87344.F:	5'CAAGCAGAGGAACAAAGTCA

Standard PCR amplifications[33] were carried out in a 12 μL reaction volume containing 1 μL purified template DNA, 6 pmol of each primer, 2mM dNTP-C, 0.1 mg/mL BSA, *Taq*

polymerase buffer (10 mM Tris-HCl, 1.5 mM MgCl, 50 mM KCl, 0.01% (w/v) gelatine, 0.1% Triton), 0.06 units Super*Taq* polymerase (Sphaero Q, HT Biotechnology, Cambridge, UK) and 1 μCi [α-^{32}P]-CTP (Amersham, Buckinghamshire, UK). Samples were denatured for 5 minutes and amplified for 33 cycles consisting of 1 minute denaturation at 94^{0}C, 2 minutes primer annealing at 55^{0}C, 1 minute elongation at 72^{0}C followed by a final extension of 6 minutes at 72^{0}C. The amplification reactions were carried out in 96-wells microtiter plates (DYNAX DPC, Breda, The Netherlands) using a thermal cycler (MJ Research, Watertown, MA). Radiolabeled products were denatured in formamide loading dye and analyzed on 6% polyacrylamide gels. Dried gels were autoradiographed at room temperature for 18-24 hours. The Molecular Dynamics Phosporimager 445SI (Molecular Dynamics, Sunnyvale, CA) was used for quantification of the PCR products. An allelic imbalance factor was calculated by the quotient of the ratios of the peak heights from normal and tumour DNA. A calculated factor >1.7 was considered as allelic imbalance while a factor <1.7 was regarded as retention. Loci showing microsatellite instability were not scored as allelic imbalance. Each PCR reaction was performed at least twice.

Interphase FISH.

From the 60 cases used in immunohistochemical and LOH studies, 14 testicular, 4 CNS and 7 nodal DLBCL with available frozen tissue material were analyzed by interphase FISH as previously described.[34] The α-satellite centromeric 6-probe (D6Z1, Oncor, Gaitersburg, MD) was biotin-16dUTP-labelled. PAC 223H1 was isolated from the RCPI-1 Human PAC Library of the Roswell Park Cancer Institute (Dr. J. den Dunnen, Genome Technology Center, LUMC, Leiden) using a TAP1 cDNA probe.[10] Based on known DNA sequences PACs 93N13 and 172K2 were directly obtained from the RCPI 1-library (GenBank accession numbers: PAC 93N13, Z84489; PAC 172K2, Z84814). Cosmids 619pWE1, DV19 and U16 were kindly provided by Dr. H. Inoko (Tokai University School of Medicine, Kanagawa, Japan), Dr. J. Trowsdale (University of Cambridge, Cambridge, UK) and Dr. G. Blanck (University of South Florida, Tampa, Fl) respectively. Cosmid c109K2118 derived from the ICRF flow-sorted chromosome 6 library was obtained from the Resource Center/Primary Database of the German Human Genome Project (RZPD, Berlin, Germany) and cosmid M31A from the American Tissue Culture Center (ATCC). All cosmid and PAC probes were labelled with digoxenin-12-dUTP (ROCHE, Basel, Switzerland) by standard nick translation. Hybridization was performed as previously described.[35] A 5 μl hybridization mixture containing 3 ng/μl of the centromere 6 probe combined with 3 ng/μl of each cosmid or PAC probe, 1.5 μg human Cot-1 DNA and 3.5 μL hybridization mix (50% formamide, 10% dextransulphate, 50 mM sodiumphosphate, pH 7.0, 2 x Sodium Chloride/Sodium Citrate (SSC)) was denatured for 8 minutes at 80^{0}C and

then pre-annealed for 30 min at 37^{0}C. After denaturation for 3 min at 80°C, nuclei were hybridized overnight at 37^{0}C in a moist chamber containing 60% formamide in 2 x SSC. Immunodetection was performed as previously described.[35]

In 10 tonsils of healthy individuals, for each probe combination the signal ratio in 200 nuclei was determined; the cut-off level for homozygous or hemizygous loss was set at the average of the controls plus 3 times the standard deviation (SD). Hemizygous deletions were defined by the presence of a lower number of locus specific PAC or cosmid signals relative to the number of centromere 6 signals (usually one locus specific signal and 2 centromeric signals). Homozygous deletions were defined as the complete absence of PAC or cosmid signals in cells with one or more preserved centromere 6 signals. Thus, in all cases technical artefacts were as much as possible excluded by inclusion of the centromere 6 probe in each hybridization experiment, by simultaneous hybridization with other probes for the HLA region and by the analysis of 10 normal controls. Additionally, in all cases, cell counts were performed by 2 independent investigators (SAR and ESJ).

Fiber FISH.

DNA fibers were prepared according to the halo technique.[36] The probe set used to study the genomic abnormalities in extranodal lymphomas consisted of PAC clones 223H1, 93N13 and 172K2 and cosmid clones DV19 and U16 (see under interphase FISH). Additional PAC clones 214F11, 122L3, 60C22 and 71I17 were isolated from the RCPI-1 Human PAC Library using respectively a TAP1 cDNA probe (see under interphase FISH) or PCR generated probes for unique sequences (GenBank) in the vicinity of microsatellite marker C47 and the genes TNXB and Hsp70. A normal "barcode" was generated on DNA fiber preparations obtained from normal peripheral blood leukocytes (PBL). The hybridization solution consisted of 30% formamide, 10% dextransulphate, 50 mM sodiumphosphate, pH7.0, 2xSCC, 3 ng/μL of each probe, and a 50-fold excess of human Cot-1 DNA. Hybridization and immunodetection were performed as previously described.[36]

Fluorescence microscopy.

Slides were analyzed with a Leica DM-RXA fluorescence microscope (Leica, Wetzlar, Germany). Images were captured using a COHU 4910 series monochrome CCD camera (COHU, San Diego, CA) attached to the fluorescence microscope equipped with a PL Fluotar 100x, NA 1.30 to 0.60 objective and I3 and N2.1 filters (Leica) and Leica QFISH software (Leica Imaging Systems, Cambridge, UK). Images were processed with Paintshop Pro and Corel Draw 8.0.

Statistical analysis.

The Fisher's exact test was used for determining the significance of differences in immunohistochemistry and LOH data between extranodal and nodal DLBCL cases. Statistical analysis for interphase FISH was performed using the Mann-Whitney test for independent samples. Two-sided tests were used in all calculations. A *P* value of <0.05 was considered statistically significant for both tests.

Results

Loss of HLA class I and II expression in DLBCL of the testis and CNS

Tissue sections of 21 nodal, 28 testicular and 11 CNS DLBCL were stained for HLA-A and HLA-DR expression (see Figure 1). Loss of HLA-A expression was observed in 61% and 55% of the primary testicular and CNS lymphomas, respectively, against 10% of the primary nodal ones (difference between extranodal and nodal DLBCL significant, $p<0.01$; Table 1). A similar trend was observed for W6/32, a well established pan-class I antibody, which could only be applied on 25 cases of which frozen material was available. The TAP1 and β2M proteins are essential for respectively transport and stabilization of HLA class I molecules, and loss of expression of either one results in very low or undetectable levels of class I expression.[22,23] In 7 of 12 cases with total loss of HLA class I expression as assessed for W6/32, absence of β_2M or TAP1 expression provided an explanation for the loss of class I expression.

Significant differences between extranodal and nodal lymphomas were also found for expression of HLA class II molecules, with 61% and 46% of the testicular and CNS lymphomas completely lacking HLA-DR expression compared to only 5% of the nodal DLBCL (difference between extranodal and nodal DLBCL significant; $p<0.05$; Table 1). Similar results were obtained with antibodies recognizing HLA-DR and HLA-DQ on frozen tissue sections (Table 2). Interestingly, 15 of the 23 cases analyzed on paraffin sections showed a coordinate loss of both class I and class II expression.

Increased Loss of Heterozygosity at 6p21 in DLBCL of the testis and CNS.

To investigate whether genetic alterations of the HLA-region at 6p21.3 contributed to loss of class I or class II expression, the same 60 cases were studied by LOH analysis. Nineteen microsatellite markers on chromosome 6 including 12 markers in the HLA region were used (Figure 2A). In contrast to allelic imbalances at 6q[37] which were equally frequent in nodal and extranodal DLBCL of our series, the testicular and the CNS lymphomas frequently showed allelic imbalance in the HLA region. The differences between these extranodal and

nodal DLBCL were statistically significant for C125, TY2A, BAT2 and X8344 (Figure 2A). However, the individual markers are closely linked and therefore, allelic imbalances for adjacent markers are not independent from each other.[38] Furthermore, homozygous deletions will counterbalance allelic imbalance and thus will give rise to discontinuous patterns of allelic imbalance if a set of adjacent markers are used.[39]

Three testicular lymphomas (T18, T20, T25) showed retention of heterozygosity at marker D6S1666 but allelic imbalance at the flanking markers C47 and X87344 (Figure 2B). This suggested the presence of a homozygous deletion at D6S1666 in the class II region, with the remaining signal resulting from contaminating normal cells in the tumour sample.

	Extranodal DLBCL									Total extranodal vs. nodal DLBCL
	Testicular % of cases (n=28)			CNS % of cases (n=11)			Nodal DLBCL % of cases			
Antibody	-	+/-	+	-	+/-	+	-	+/-	+	P
HLA-A	61	29	11	55	18	27	10	33	57	0.008
β2M*	23	46	31	18	27	55	11	5	84	0.7
TAP*	26	33	41	30	40	30	0	9	91	0.02
HLA-DR	61	29	11	46	18	36	5	10	85	0.004
W6/32*	62	0	38	75	0	25	25	13	62	0.4

Table 1. HLA class I and class II expression.
-: No detectable staining of tumour cells compared to staining of internal control cells (respectively T lymphocytes, macrophages and dendritic cells for HLA class I, β2M and TAP and macrophages and dendritic cells for class II) within each tumour;
+/-: Weaker or heterogeneous staining of tumour cells compared to internal control cells.
*: Frozen tissue sections of 13 testicular, 4 CNS and 8 nodal DCLC were stained for W6/32. One testicular and one CNS lymphoma were not interpretable for TAP. Two testicular and 3 nodal lymphomas were not interpretable for β2M.
The P-value was determined using a two-sided Fisher's exact test comparing the total loss of expression in extranodal (testis and CNS) and nodal DLBCL.

Interphase Fluorescence In Situ Hybridization shows frequent homozygous deletions in the class II region and more extensive hemizygous deletions in DLBCL of the testis and CNS.

To evaluate homozygous deletion of HLA class II genes, we applied two-colour FISH on isolated nuclei of all cases with available frozen tissue material, i.e. 14 testicular, 4 CNS and 7 nodal lymphomas. Six digoxigenin-labelled PAC and cosmid clones specific for the HLA-region, each in combination with a biotin-labelled centromere 6 probe were used (Table 2; Figure 3A). The panel consisted of 3 PAC clones specific for the class II region, including PAC 93N13 containing marker D6S1666 and covering the DQA1 and DRB1 genes. For each probe combination, the cut-off level was determined as the average plus 3 times the SD as assessed on 10 normal tonsils (see Table 2, co 1-10). For the detection of homozygous deletions (loss of all HLA allele specific signals in combination with retention of chromosome 6 centromeric signals), this cut-off level varied between 0% and 5.8%. To exclude the possibility that hybridization artefacts accounted for loss of signals, we fixed the cut off level for homozygous deletion at >6% cells. Using this threshold, the homozygous deletion was confirmed in all 3 cases, 64.5% (T18), 59,5% (T20) and 60,5% (T25) of the cells showing loss of both allelic signals for PAC 93N13 (Table 2; Figures 3B, 3C, and 3D). In fact, PAC 93N13 was homozygously lost in 8 of 14 testicular (T2, T11, T14, T16, T18, T19, T20, T25) and 2 of 4 CNS lymphomas (C1, C9) but in none of the nodal cases (difference between extranodal and nodal DLBCL significant; $p<0.001$; Table 2). Similarly, PAC 172K2 covering the HLA-DRA gene was homozygously lost in respectively 7 of 14 testicular DLBCL (T2, T11, T13, T14, T16, T18, T25) and in the same 2 CNS lymphomas (C1, C9) but in none of 7 nodal lymphomas (difference between extranodal and nodal DLBCL significant, $p<0.001$; Table 2). Two lymphomas (T18 and C9) showed extension of the homozygous deletion to 223H1, a PAC covering TAP1 (Table 2). In case C9 an similar fraction, i.e. 30%, 44% and 52% of the cells showed a homozygous deletion for probes 223H1, 93N13 and 172K2, whereas in T18 223H1 was deleted only in a small fraction (7%) of the cells.

The percentage of cells with homozygous deletion varied between 7.0% and 71.5%. The low percentages of cells with homozygous deletion might have been caused by either the presence of many reactive cells in the frozen tissue sample or by the presence of tumour heterogeneity. The first could be the case in T16, T19 and C1 in which maximally 32.5% of all cells showed any abnormality (loss of one or 2 spots; see Table 2). Tumour

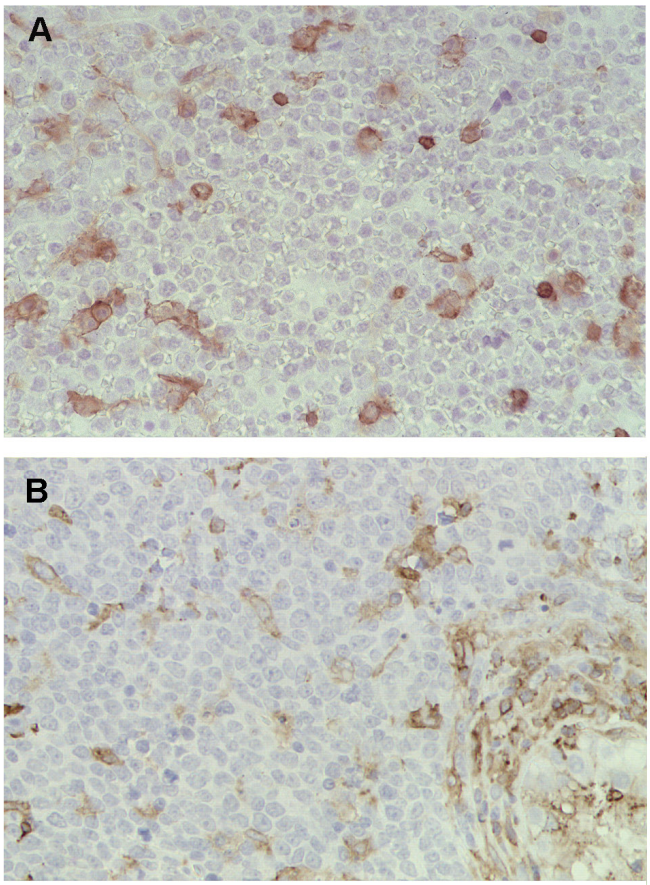

Figure 1. Immunohistochemical analysis of representative testicular DLBCL cases. Immunohistochemical staining was performed using (A) the MoAb HCA2 (anti-HLA-A) (T20) and (B) the MoAb clone B8.11.2 (HLA-DR) (T18) (original magnification × 40). Note the positive (brown) staining in the internal control cells scattered through the mass of negative lymphoma cells.

heterogeneity was likely present in cases T11, T13, T16, T18 and T25 showing variable percentages of cells with loss of one or both HLA allele specific signals (Table 2). For instance in case T11 a small fraction (9-15.5%) of all cells contained a homozygous deletion for probes 93N13 and 172K2, whereas a larger fraction (35-58%) contained a hemizygous deletion covered by the probes 93N13, 172K2 and M31A (Table 2).

For the hemizygous deletions (loss of one HLA specific signal while both centromere signals were retained), the individual cut off levels on the normal tonsils varied between 8.5% and 20.4%. Using a general cut off level of >21%, 7 of 18 extranodal DLBCL and one of 7 nodal cases (N3 and N4) contained a hemizygous deletion of covered by PAC 93N13. Similarly, 9 of 18 extranodal and one of 7 nodal DLBCL contained a hemizygous deletion covered by PAC 172K2. In all but one case (T3), these deletions were part of much larger hemizygous deletions also involving the region containing the HLA-DP genes and/or the HLA class III and even the HLA class I region (see below). The percentage of cells with hemizygous deletions varied between 24% and 79.5% (Table 2).

Combining all data for the HLA class II region covered by PAC 93N13 and/or 172K2, 4 of 18 extranodal cases contained exclusively homozygous deletions, 2 cases showed only hemizygous deletions and 7 cases showed combined homo- and hemizygous deletions. In contrast, only 2 of 7 nodal DLBCL contained a small hemizygous deletion in this region.

HLA class I and class III genes were mainly affected by large hemizygous deletions (Table 2). Only case T25 contained 12% of cells with loss of both signals for C109K2118 covering HLA-A suggesting a small tumour subclone with a homozygous deletion in the class I region. With respect to the class III region covered by M31A, 9 of 18 extranodal versus none of 7 nodal DLBCL contained hemizygous deletions. For the class I region covered by C109K2118, these numbers were 8 of 18 extranodal versus none of 7 nodal DLBCL.

Aneuploidy of chromosome 6 was observed in 6 cases. Five centromeric signals were seen in T3 and C11 (data not shown). T20 showed monosomy for chromosome 6. T23 showed trisomy 6 and loss of one allele upon LOH analysis, indicating that one of the two chromosomes was lost and the other present in triplicate. In addition, T18 and T25 contained subclones with aneuploidy for chromosome 6 (Figure 3).

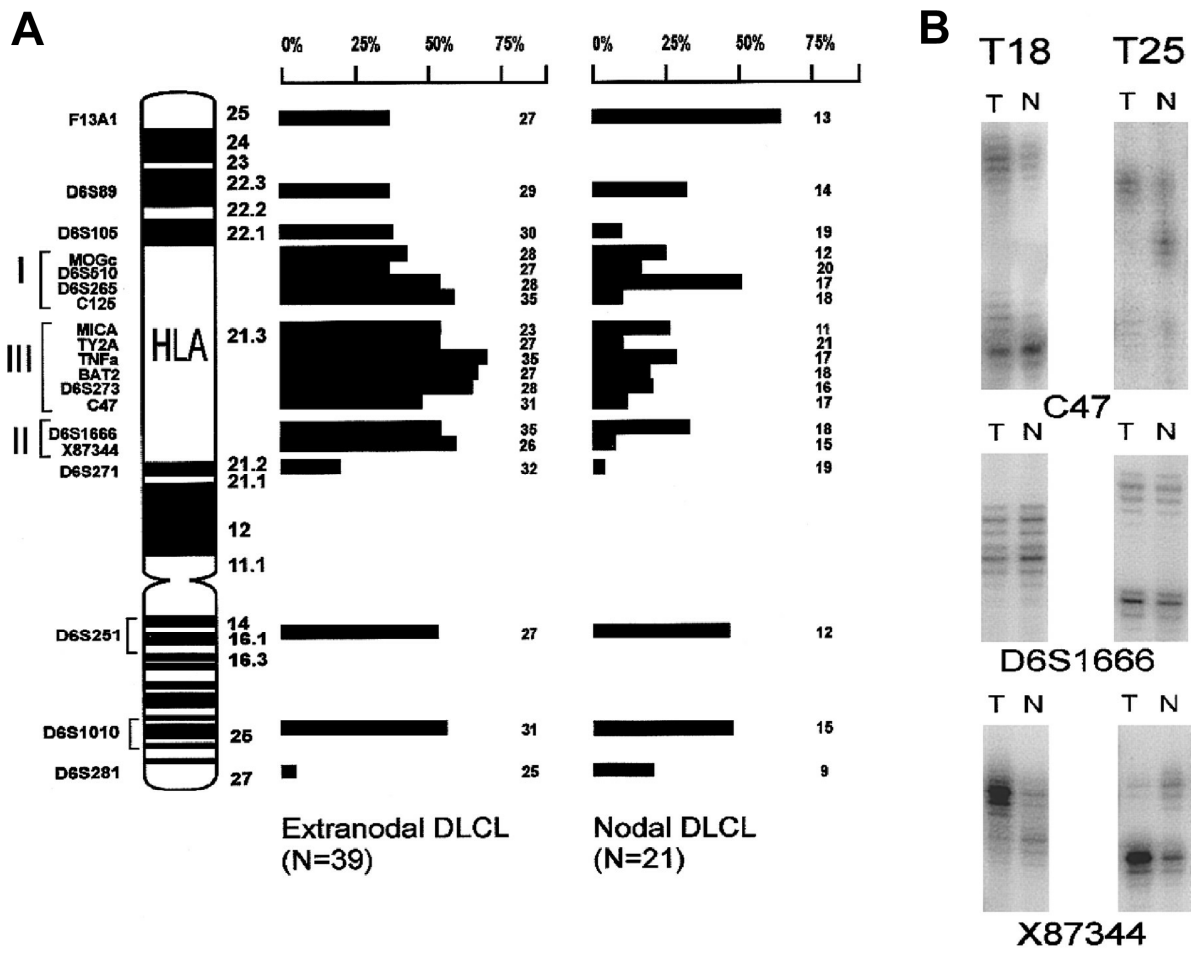

Figure 2. LOH analysis. (A) Ideogram depicting the chromosome 6p and q arms. All microsatellite markers used for LOH analysis and their location are indicated. On the right hand side the LOH data of the extranodal and nodal series of DLBCL are summarized. Each bar represents the percentage of informative cases with LOH (their number depicted at the right). (B) Example of 2 testicular lymphomas (T18 and T25) showing retention of heterozygosity at marker D6S1666 near HLA-DQB1 while allelic imbalance at both flanking markers C47 and X87344. This suggests a homozygous deletion. Allelic imbalance was determined by comparing the ratio between alleles of tumour DNA (T) and normal DNA (N).

	HOMOZYGOUS DELETION						HEMIZYGOUS DELETION						EXPRESSION		
	619pWE1 DPB	223H1 TAP	93N13 DQA1	172K2 DRA	M31A TNF-?	C109K2118 HLA-A	619pWE1 DPB	223H1 TAP	93N13 DQA1	172K2 DRA	M31A TNF-?	C109K2118 HLA-A	HLA-DQ	HLA-DR	HLA-A
T2	3.0	1.5	48.5	49.0	1.0	3.0	27.0	12.5	16.0	14.0	19.0	14.0	h	h	-
T3*	0.0	1.0	5.0	1.5	3.0	1.0	5.0	15.0	30.0	10.0	10.0	2.0	+	+	+
T11	1.5	0.0	9.0	15.5	3.5	1.0	13.0	5.5	58.0	49.5	35.0	20.0	-	+	-
T13	1.5	2.5	0.5	16.0	1.0	0.0	24.0	25.5	26.5	40.0	42.0	35.5	-	-	-
T14	1.5	2.5	69.0	58.5	2.5	0.0	15.0	48.0	13.5#	5.0#	57.5	58.5	-	-	-
T16	0.0	0.0	22.5	8.0	2.5	3.0	23.5	18.5	15.0	25.5	27.0	27.5	-	-	-
T18	0.0	7.0	64.5	71.5	1.0	2.5	18.5	17.0	2.0	2.0	7.5	23.5	-	-	h
T19	2.5	3.0	16.5	3.0	0.5	1.5	19.0	15.0	11.0	11.0	13.5	14.0	-	-	-
T20	0.0	0.0	59.5	3.0	2.0	6.5	70.0	70.0	25.5	79.5	75.5	53.0	-	-	-
T22	1.0	0.5	1.5	6.0	1.0	0.0	8.5	1.0	11.5	10.0	6.0	7.0	-	w	+
T23*	0.0	0.0	0.0	0.0	1.0	0.0	2.5	4.0	14.5	14.5	21.0	4.0	-	w	+
T25	0.0	6.0	60.5	11.5	2.5	12.0	58.0	33.5	9.0	45.0	52.0	26.0	-	-	-
T26	0.0	0.0	2.0	5.5	1.0	0.0	11.0	10.0	2.5	6.5	9.5	12.5	nd	h	h
T30	2.0	1.5	0.5	0.5	0.5	0.0	16.0	12.0	10.0	15.5	9.0	30.5	-	+	-
C1	0.0	0.5	27.5	26.0	1.0	2.0	3.0	16.0	32.5	15.0	22.0	17.0	-	-	-
C2	0.0	0.0	0.0	0.0	3.0	1.0	8.5	9.5	72.0	72.5	68.5	68.5	-	-	-
C9	0.0	30.0	44.0	52.0	1.0	0.0	10.0	13.5	27.0	22.0	56.0	8.5	nd	-	-
C11*	0.0	0.0	0.0	0.0	0.0	0.0	6.0	14.0	15.0	3.5	5.0	6.0	+	+	-
N3	2.0	0.0	0.0	0.5	2.0	0.0	5.0	12,5	12.0	24.0	16.5	9.0	-	-	-
N4	0.0	2.0	0.0	0.0	0.0	1.5	9.5	5.0	32.0	16.5	9.5	9.0	-	+	-
N8	0.0	1.0	0.0	0.0	0.5	1.0	15.0	9.5	7.0	7.0	17.0	16.0	+	+	+
N9	0.0	0.0	0.0	1.0	0.0	4.5	12.0	8.5	8.5	21.0	20.0	13.0	+	+	+
N13	0.0	0.0	0.0	0.0	0.0	0.0	10.0	9.0	10.5	9.0	13.0	8.0	+	+	h
N15	0.0	0.0	0.0	0.0	0.0	2.0	11.0	12.0	6.0	5.0	4.5	7.0	+	+	+
N18	0.0	0.0	0.5	0.0	2.0	0.0	9.0	7.0	5.0	4.0	16.5	9.0	?	h	+
co1	0.0	0.5	0.0	0.0	1.0	0.0	2.0	1.5	8.0	5.0	9.0	2.0	nd	nd	nd
co2	0.0	5.0	0.0	0.0	0.0	0.0	4.0	8.0	1.5	3.5	8.5	7.5	nd	nd	nd
co3	1.0	0.0	0.0	0.0	0.0	0.0	4.0	4.0	9.0	8.0	9.0	4.0	nd	nd	nd
co4	0.0	0.0	0.5	0.0	0.0	0.0	4.0	5.0	9.0	5.0	7.0	5.0	nd	nd	nd
co5	0.0	0.0	1.0	7.0	0.0	0.0	5.5	5.0	2.0	1.0	4.5	5.5	nd	nd	nd
co6	0.0	0.0	2.0	0.0	0.0	0.0	2.5	3.5	6.0	7.0	4.0	4.0	nd	nd	nd
co7	0.0	3.0	1.0	1.0	1.0	0.0	6.0	7.0	5.0	4.0	3.5	6.0	nd	nd	nd
co8	2.0	1.0	0.0	0.0	0.0	0.0	4.0	5.0	5.5	1.0	6.5	10.5	nd	nd	nd
co9	0.0	1.0	0.0	0.0	0.0	0.0	6.0	3.0	5.0	8.0	8.0	9.5	nd	nd	nd
co10	0.0	1.0	0.0	0.0	0.5	0.0	1.5	3.0	3.0	10.5	19.0	9.5	nd	nd	nd

Table 2. Interphase FISH results in DLBCL and comparison with HLA protein expression. The table shows the percentages of nuclei with loss of one or two HLA region specific signals in combination with retention of the centromeric signals for chromosome 6. In case of aneuploidy for chromosome 6 (cases T3, T23, C11, indicated by *), this was corrected per cell for the number of centromere 6 specific signals. In each experiment 200 nuclei were counted. T: testicular DLBCL; C: CNS DLBCL; N: nodal DLBCL; co: normal control (tonsil) tissue. Shaded boxes indicate loci with an abnormal percentage of cells with homozygous or hemizygous deletions. These percentages were significantly higher than those obtained in the ten normal controls. For homozygous deletions the cut off level was >6% cells, for hemizygous deletions >21% (see text). #: These and many other percentages are relatively low since the tumour was also affected by a homozygous deletion. HLA expression is shown in the three columns on the far right hand side. Immunohistochemistry was performed on frozen tissue sections for HLA-DR and -DQ and on paraffin embedded tissue sections for HLA-A. -: completely negative; +: positive: w: weakly positive; h: tumour heterogeneity with distinct tumour parts being completely negative; ?: undeterminable; nd: not done.

DNA fiber FISH mapping of the homozygous deletions in the HLA-DR and -DQ region.

To determine the size of the deletions of each allele, 10 cases were also analyzed by high resolution DNA fiber FISH using a probe set covering approximately 900 kb of the class II region. Within the normal HLA class II region the genomic organization of the different DR haplotypes differs in size between HLA-DRB1 and HLA-DRA. The size of DR 4, 7 and 9 haplotypes is approximately 110 kb and of DR 1, 2 and 10 haplotypes 30 kb larger compared to the size of DR 3, 5 and 6 haplotypes.[40] To determine these polymorphisms, HLA-DR and DQ typing of the patients was performed using PCR and oligo-hybridization according to standard protocols (courtesy Prof. F.H.J. Claas, Dept. of Immunohematology, LUMC).[42,43]

In cases T11 and T13 no abnormal fibers were observed, likely because of the too low percentage of aberrant cells (15.5% and 16% as determined by interphase FISH for 172K2; Table 2). In all other 8 cases (T2, T14, T16, T18, T19, T20, T25, C9) the deletions were confirmed (Figure 4). In all cases except T16 one allele was affected by a deletion involving the entire probe set. In 2 cases (C9 and T18) the deletion of the other allele involved all functional HLA-DQ and HLA-DR genes, the other HLA-DQ genes outside the deletion representing pseudogenes. Furthermore, in 2 cases (T2 and T14) the deletion involved HLA-DQA1 and all HLA-DR genes, in 3 cases (T19, T20 and T25) it involved both HLA-DQ genes and a part of the HLA-DR genes. In one case (T16) the allele with the smallest deletion involved only the DQB1, DQA1 and DRB1 genes covered by cosmid DV19 and PAC 93N13, while the other allele had a deletion involving DQA1 and all DR genes. In cases T16 and T25, interphase FISH showed homozygous deletion of the area covered by PAC 93N13 and in a small fraction (8% and 11,5%) also of the area covered by PAC 172K2. Probably, due to the relative insensitivity, this deletion of PAC 172K2 was not detected using fiber FISH.

As shown in Figure 4, the deletions were relatively variable at the telomeric side but never extended to the class III genes covered by PAC71117. In T20 (HLA-DR typed DR6) with monosomy 6 (as detected by interphase FISH for the centromere of chromosome 6), a deletion of PAC 93N13 and cosmid U16 was detected by fiber FISH. However, PAC 172K2 was not on the same fiber as PAC 223H1 suggesting a chromosomal breakpoint in addition to the deletion. This was in agreement with the absence of co-localization of these PACs as determined by interphase FISH (not shown).

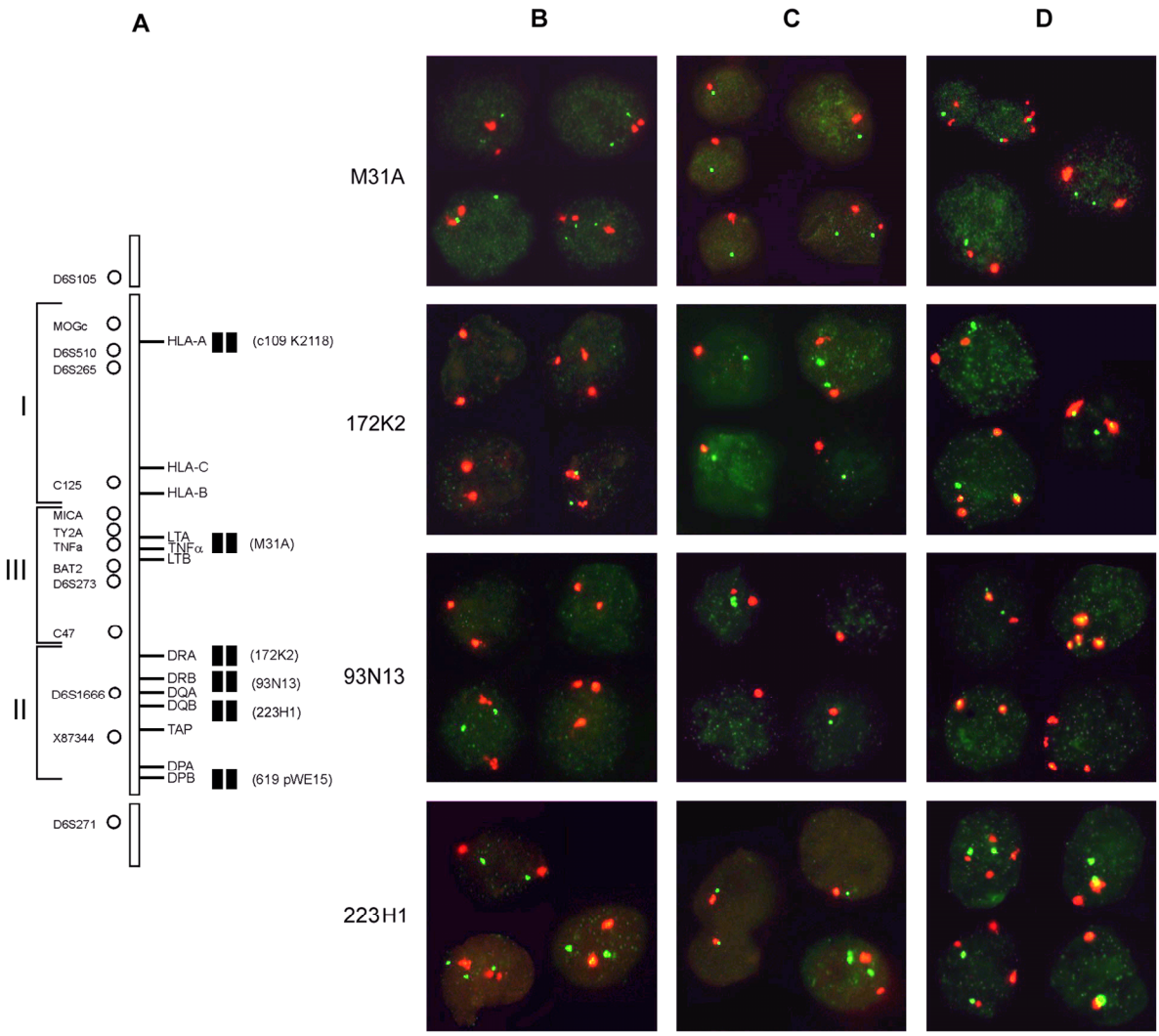

Figure 3. Homozygous and hemizygous deletions within the HLA-region determined by interphase FISH. (A) Schematic representation of the HLA-region on chromosome 6p21.3. On the left hand side the localization of the microsatellite markers used for LOH and on the right hand side the 6 PAC/cosmid probes used for interphase FISH (B through D) Examples of interphase FISH. Each panel represents a composite of 3-4 individually captured nuclei, including at least one control nucleus without a deletion. In all cases the red signal is derived from centromere 6 and the green signal is derived from one of the 6p21.3 specific probes as indicated on the left. (B) Testicular lymphoma T18 showing homozygous deletions of PACs 93N13 and 172K2 indicated by the presence of 2 centromere 6 signals and no detectable PAC signal. The homozygous deletion was also present in the 20% of nuclei with trisomy 6. (C) Testicular lymphoma T20 showing monosomy 6 and a deletion of PAC 93N13 in 75% of the nuclei with monosomy 6. (D) Testicular lymphoma T25 showing a homozygous deletion of PAC 93N13 including all cells with tetrasomy (25% of cells), and a large hemizygous deletion including PACs 223H1, 172K2 and cosmid M31A.

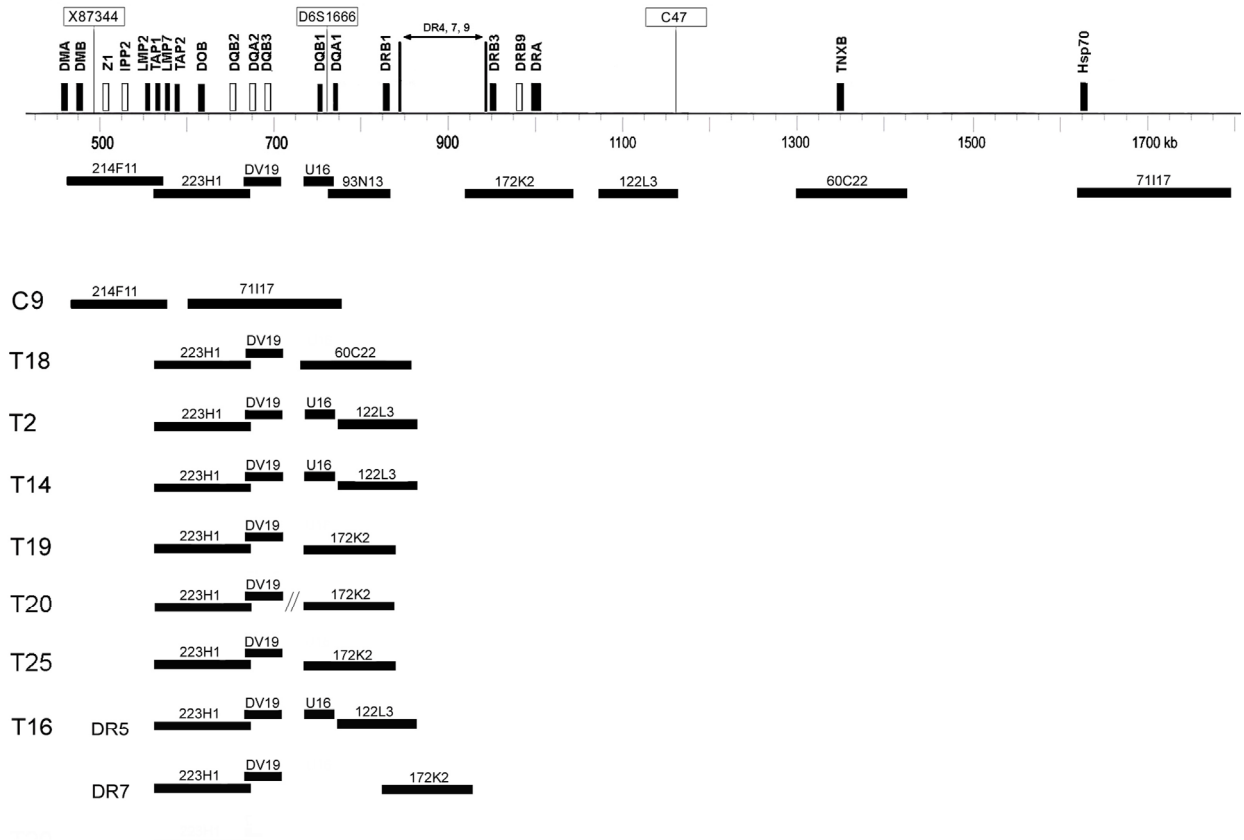

Figure 4. DNA fiber FISH detection of HLA-DQ and DR deletions.
Schematic representation of part of the HLA class II and class III region of a DR 4,7 or 9 haplotype showing the approximate localization of clones used for fiber FISH. Black bars represent expressed genes and white bars represent pseudogenes in the class II region. Micro-satellite markers X87344, D6S1666, C47 are boxed. DNA-fiber FISH of 8 extranodal lymphoma cases with various deletions in the HLA class II region is depicted. In all cases except case T16, one allele contained a deletion involving all used probes. In these cases the allele with a small deletion is shown. In case T16 both alleles DR5 and DR7 contained a deletion as illustrated.

Correlation between homozygous deletions and loss of HLA class II expression.

Table 3 shows the association between the homo- and hemizygous deletions in the class II region and the loss of HLA-DR and HLA-DQ expression as assessed on frozen tissue sections. Most importantly, in 8 of 15 cases without HLA-DQ expression homozygous deletions of PAC 93N13 were present and in 3 additional cases a hemizygous deletion was found. In 4 cases we did not find any gross genetic alterations. Similarly, 10 of 14 DLBCL cases with loss of HLA-DR expression showed homozygous deletions of PAC 172K2, while in 2 cases extensive hemizygous deletions were present. In only 2 DLBCL cases we did not find any explanation for loss of expression.

	HLA-DQ expression (n= 22)		HLA-DR expression (n=25)	
	+	-	+	-
No deletion	6	7	8	6
Homozygous deletion	0	9	1	10

Table 3. Correlation between homozygous deletions and loss of HLA class II expression.
For correlation analysis of loss of HLA-DQ expression, homozygous deletions of PAC 93N13 covering the only functional HLA-DQA gene DQA1 were taken into account ($p<0,01$, as determined by the chi-square test); similarly, for HLA-DR expression PAC 172K2, covering the only HLA-DRA gene was considered ($p<0,01$).

Discussion

We show a high frequency of homozygous deletions within the HLA class II region of primary testicular and CNS lymphomas which accounts for an important mechanism of loss of HLA-DR and HLA-DQ expression in these tumours. In addition, in most cases large hemizygous deletions and/or mitotic recombination involved the entire HLA region.

In solid tumours and corresponding cell lines, investigators have mainly focussed on loss of HLA class I expression as a route for tumour cells to escape killing by class I-restricted CD8+ cytotoxic T-cells.[10,11] According to these studies using pan-class I, locus- or allele-specific antibodies, between 39% and 88% of the epithelial tumours are completely or partially deficient in HLA class I expression. Mainly based on studies of cell lines, distinct phenotypes can be related to separate molecular mechanisms: (i) absence or strongly reduced class I expression is often attributed to respectively absence of β_2M molecules or defects in TAP expression;[22,23] (ii) loss of an haplotype can result from loss of one copy of chromosome 6 or from large deletions or mitotic recombination;[43-45] (iii) decreased or completely abolished expression of HLA-A or B may result from transcriptional downregulation;[46-48] (iv) finally, absence of expression of a single allele can result from point mutations, partial deletion or somatic recombination within the HLA locus.[24,45,49]

In our series of primary DLBCL of the testis and CNS, aberrant class I expression was very frequent with more than half of these lymphomas showing complete loss of HLA-A expression. Our data are in line with previous studies showing complete loss of class I expression in 6-30% of aggressive B-cell lymphomas. In those series, loss of expression was associated with extranodal presentation, and also with a relatively poor prognosis.[51,52] In the present series, this loss could be explained by absence of β_2M or TAP1 expression in

more than half of the cases assessed for class I expression (W6/32). In 5 class I negative extranodal lymphomas without loss of β_2M or TAP1, large hemizygous deletions involving the entire class I region were present. Loss of expression of the remaining haplotype was probably due to mutations, small homozygous deletions, or alterations in the methylation status of the genes, but it could also be explained by defects in TAP2 or LMP-2 and LMP-7 expression, especially since also these genes are often involved in hemizygous deletions or mitotic recombination.

So far, little attention has been paid to loss of HLA class II expression in cancer, probably due to the fact that class II molecules are not constitutively expressed on normal epithelial cells. We showed loss of HLA class II expression in approximately half of the testicular and CNS lymphomas but in only 5% of the nodal cases. Previously, loss of HLA class II expression has been reported in DLBCL,[18-20] especially those presenting at extranodal sites.[17] In primary testicular and CNS lymphoma homozygous deletions are a very important mechanism for loss of class II expression since more than half of these DLBCL contained such homozygous deletions (Table 2). In addition to these homozygous deletions, several class II negative DLBCL showed large hemizygous deletions. Similar to the situation in the class I region, other structural abnormalities such as point mutations or small homozygous deletions undetectable by FISH, or methylation of the promoter region might have caused loss of expression of the other allele. Of note, several cases showed tumour heterogeneity with different subpopulations containing homo- and hemizygous deletions. In few cases this was also represented by heterogeneous staining pattern for HLA-DR and -DQ.

The homozygous deletions within the HLA class II region covered a minimal region of approximately 100 kb and always included the HLA-DQA1 and HLA-DRB1 genes (see Figure 4). Since HLA-DQB2, DQA2 and DQB3 are pseudogenes, loss of HLA-DQA1 and in consequence the inability to form a heterodimer with HLA-DQB1, is sufficient to explain the total loss of expression of HLA-DQ. The situation is almost similar but more complex for HLA-DR, since the smallest homozygously deleted areas involved PAC 93N13 containing HLA-DRB1 and a variable area downstream of this PAC containing DRB3-5, the other functional alleles.[40] The presence of a length polymorphism in this area and the lack of appropriate probes for this polymorphic region hampered the exact deletion mapping within this region. However, it should be noted that at least 4 cases showed homozygous deletion of all HLA-DR genes.

Interestingly, in EBV-transformed irradiated human B-lymphoblastoid cell lines selected for absence of HLA class II expression, similar homozygous deletions within the HLA class II region have been observed.[52] In one cell line, the homozygously deleted region only contained the HLA-DRB1 and HLA-DQA1 genes, while in 2 other cell lines the homozygous

deletion also included the HLA-DQB1 gene. No other genes have been identified in the recently published complete sequence of this region.[53] This suggests that the HLA-DR and HLA-DQ genes are the real target of the deletion and that loss of these genes results in a growth advantage through immune-selection. Furthermore, in none of the cases the class III region was affected suggesting that genes essential for B-cell lymphomagenesis reside in this region.

What could be the implication of loss of HLA class II expression in lymphoma cells? HLA class I expression on tumour cells is essential for killing by CD8+ cytotoxic T-cells. However, recent studies also suggest an important role for cytotoxic CD4+ effector T-cells and thus for HLA class II expression on tumour cells.[12-14] Additionally, cytotoxic CD8+ cells are only effective after activation by CD4+ helper T-cells, i.e. when both CD8+ and CD4+ cells recognize tumour specific antigenic determinants on the same APC's.[16,56,57] Like normal B-cells, neoplastic B-cells may function as APC's as they can express both class I and II molecules, CD40 and costimulatory molecules.[58] This is supported by the observation that murine lymphoma cells can present their own idiotype to CD4+ T-cells.[59] In consequence, loss of both HLA class I and II expression may result in a impaired activation of cytotoxic CD8+ T-cells, and facilitate the growth of the tumour cells.

Tumour cells growing in immune privileged sites such as the CNS, testis and the eye experience a relative protection from immune attack. Various mechanisms may account for this effect, including the presence of an organ-blood barrier and the specific microenvironment with local production of immunosuppressive neuropeptides and cytokines[2,3] as well as expression of Fas ligand on pre-existing cells.[2] However, immune protection at immune privileged sites is not absolute as inoculated tumours are eventually killed by adoptively transferred cytotoxic T-cells.[21] This suggests that not only host factors but also tumour cell characteristics such as loss of HLA class I and II expression contribute to the phenomenon of immune privilege. Interestingly, primary DLBCL of the CNS preferentially express the immunoglobulin heavy chain gene V4-34.[60-63] The V4-34 gene product, which is implied in several autoimmune disorders might therefore function as a specific tumour antigen in these lymphomas and one could speculate that the lack of presentation in HLA molecules might give the tumour cells a selective immune privilege.

Strategies for the treatment of B-cell lymphomas are currently focussing on T-cell mediated anti-tumour responses based on recognition of tumour-specific antigens.[9] Our study shows that assessment of the HLA expression status and irreversible HLA gene defects is important to choose the optimal therapeutic regimen as the extranodal lymphomas we describe here likely will not be susceptible to T-cell mediated immunotherapy.

References

1) Drillenburg P, Pals ST. Cell adhesion receptors in lymphoma dissemination. Blood. 2000;95:1900-1910.
2) Ferguson TA, Griffith TS. A vision of cell death: insights into immune privilege. Immunol.Rev. 1997;156:167-184.
3) Owens T, Renno T, Taupin V, Krakowski M. Inflammatory cytokines in the brain: does the CNS shape immune responses? Immunol.Today. 1994;15:566-571.
4) Touroutoglou N, Dimopoulos MA, Younes A, et al. Testicular lymphoma: late relapses and poor outcome despite doxorubicin-based therapy. J.Clin.Oncol. 1995;13:1361-1367.
5) Hochberg FH, Miller DC. Primary central nervous system lymphoma. J.Neurosurg. 1988;68:835-853.
6) Herrlinger U. Primary CNS lymphoma: findings outside the brain. J.Neurooncol. 1999;43:227-230.
7) Kwak LW, Campbell MJ, Czerwinski DK, Hart S, Miller RA, Levy R. Induction of immune responses in patients with B-cell lymphoma against the surface-immunoglobulin idiotype expressed by their tumours. N.Engl.J.Med. 1992;327.1209-1215.
8) Nelson EL, Li X, Hsu FJ, et al. Tumour-specific, cytotoxic T-lymphocyte response after idiotype vaccination for B-cell, non-Hodgkin's lymphoma. Blood. 1996;88:580-589.
9) Schultze JL, Nadler LM. T-cell mediated immunotherapy for B-cell lymphoma. J.Mol.Med. 1999;77:322-331.
10) Garrido F, Ruiz Cabello F, Cabrera T, et al. Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. Immunol.Today. 1997;18:89-95.
11) Ruiz Cabello F, Garrido F. HLA and cancer: from research to clinical impact. Immunol.Today. 1998;19:539-542.
12) Ohminami H, Yasukawa M, Kaneko S, et al. Fas-independent and nonapoptotic cytotoxicity mediated by a human CD4+) T-cell clone directed against an acute myelogenous leukemia- associated DEK-CAN fusion peptide. Blood. 1999;93:925-935.
13) Yasukawa M, Ohminami H, Arai J, Kasahara Y, Ishida Y, Fujita S. Granule exocytosis, and not the Fas/Fas ligand system, is the main pathway of cytotoxicity mediated by alloantigen-specific CD4+) as well as CD8+) cytotoxic T lymphocytes in humans. Blood. 2000;95:2352-2355.
14) Falkenburg JH, Wafelman AR, Joosten, et al. Complete remission of accelerated phase chronic myeloid leukemia by treatment with leukemia-reactive cytotoxic T lymphocytes. Blood. 1999;94:1201-1208.
15) Toes RE, Ossendorp F, Offringa R, Melief CJ. CD4 T-cells and their role in antitumor immune responses. J.Exp.Med. 1999;189:753-756.
16) Keene JA, Forman J. Helper activity is required for the in vivo generation of cytotoxic T lymphocytes. J.Exp.Med. 1982;155:768-782.
17) Momburg F, Herrmann B, Moldenhauer G, Moller P. B-cell lymphomas of high-grade malignancy frequently lack HLA-DR, -DP and -DQ antigens and associated invariant chain. Int.J.Cancer. 1987;40:598-603.
18) Slymen DJ, Miller TP, Lippman SM, et al. Immunobiologic factors predictive of clinical outcome in diffuse large-cell lymphoma. J.Clin.Oncol. 1990;8:986-993.
19) Guy K, Krajewski AS, Dewar AE. Expression of MHC class II antigens in human B-cell leukemia and non-Hodgkin's lymphoma. Br.J.Cancer. 1986;53:161-173.
20) O'Keane JC, Mack C, Lynch E, Harrington D, Neiman RS. Prognostic correlation of HLA-DR expression in large cell lymphoma as determined by LN3 antibody staining. An Eastern Cooperative Oncology Group ECOG) study. Cancer. 1990;66:1147-1153.
21) Schurmans LR, den Boer AT, van der Voort EI, et al. Successful immunotherapy of an intraocular tumour in mice. Cancer Res. 2000;59:5250-5254.
22) D'Urso CM, Wang ZG, Cao Y, Tatake R, Zeff RA, Ferrone S. Lack of HLA class I antigen expression by cultured melanoma cells FO-1 due to a defect in B2m gene expression. J.Clin.Invest. 1991;87:284-292.
23) Spies T, DeMars R. Restored expression of major histocompatibility class I molecules by gene transfer of a putative peptide transporter. Nature. 1991;351:323-324.
24) Koopman LA, van der Slik A, Giphart MJ, Fleuren GJ. Human leukocyte antigen class I gene mutations in cervical cancer. J.Natl.Cancer Inst. 2000;91:1669-1677.
25) Isaacson PG: The revised European-American lymphoma REAL) classification [editorial]. Clin.Oncol.R.Coll.Radiol. 1995;7:347-348.
26) Kramer MH, Hermans J, Parker J, et al. Clinical significance of BCL2 and P53 protein expression in diffuse large B-cell lymphoma: A population based study. J.Clin.Oncol. 1996;14:2131-2138.
27) de Boer CJ, Schuuring E, Dreef E, et al. Cyclin D1 protein analysis in the diagnosis of mantle cell lymphoma. Blood. 1995;86:2715-2723.
28) Cromme FV, Airey J, Heemels MT, et al. Loss of transporter protein, encoded by the TAP-1 gene, is highly correlated with loss of HLA expression in cervical carcinomas. J.Exp.Med. 1994;179:335-340.
29) Watson AJ, DeMars R, Trowbridge IS, Bach FH. Detection of a novel human class II HLA antigen. Nature. 1983;304:358-361.
30) Rebai N, Malissen B, Pierres M, Accolla RS, Corte G, Mawas C. Distinct HLA-DR epitopes and distinct families of HLA-Dr molecules defined by 15 monoclonal antibodies mAb) either anti- DR or allo-anti-Iak cross-reacting with human DR molecule. I. Cross-inhibition studies of mAB-cell surface fixation and

differential binding of mAb to detergent-solubilized HLA molecules immobilized to a solid phase by a first mAb. Eur.J.Immunol. 1983;13:106-111.
31) Isola J, DeVries S, Chu L, Ghazvini S, Waldman F. Analysis of changes in DNA sequence copy number by comparative genomic hybridization in archival paraffin-embedded tumour samples. Am.J.Pathol. 1994;145:1301-1308.
32) Foissac A, Cambon Thomsen A. Microsatellites in the HLA region: 1998 update. Tissue Antigens. 1998;52:318-352.
33) Kersemaekers AM, Kenter GG, Hermans J, Fleuren GJ, van de Vijver MJ. Allelic loss and prognosis in carcinoma of the uterine cervix. Int.J.Cancer. 1998;79:411-417.
34) Vaandrager JW, Schuuring E, Raap AK, Philippo K, Kleiverda KK, Kluin PM. Interphase FISH detection of BCL2 rearrangement in follicular lymphoma using breakpoint-flanking probes. Genes Chromosomes.Cancer. 2000;27:85-94.
35) Halder T, Pawelec G, Kirkin AF, et al. Isolation of novel HLA-DR restricted potential tumour-associated antigens from the melanoma cell line FM3. Cancer Res. 1997;57:3238-3244.
36) Vaandrager JW, Schuuring E, Zwikstra E, et al. Direct visualization of dispersed 11q13 chromosomal translocations in mantle cell lymphoma by multicolor DNA fiber fluorescence in situ hybridization. Blood. 1996;88:1177-1182.
37) Johansson B, Mertens F, Mitelman F. Cytogenetic evolution patterns in non-Hodgkin's lymphoma. Blood. 1995;86:3905-3914.
38) Newton MA, Gould MN, Reznikoff CA, Haag JD. On the statistical analysis of allelic-loss data. Stat.Med. 1998;17:1425-1445.
39) Cairns P, Polascik TJ, Eby Y, et al. Frequency of homozygous deletion at p16/CDKN2 in primary human tumours. Nat.Genet. 1995;11:210-212.
40) Dunham I, Sargent CA, Dawkins RL, Campbell RD. An analysis of variation in the long-range genomic organization of the human major histocompatibility complex class II region by pulsed-field gel electrophoresis. Genomics. 1989;5:787-796.
41) Olerup O, Zetterquist H. HLA-DR typing by PCR amplification with sequence-specific primers PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. Tissue Antigens. 1992;39:225-235.
42) Kennedy LJ, Poulton KV, Dyer PA, Ollier WE, Thomson W. Definition of HLA-C alleles using sequence-specific oligonucleotide probes PCR-SSOP). Tissue Antigens. 1995;46:187-195.
43) Koopman LA, Mulder A, Corver WE, et al. HLA class I phenotype and genotype alterations in cervical carcinomas and derivative cell lines. Tissue Antigens. 1998;51:623-636.
44) Torres MJ, Ruiz Cabello F, Skoudy A, et al. Loss of an HLA haplotype in pancreas cancer tissue and its corresponding tumour derived cell line. Tissue Antigens. 1996;47:372-381.
45) Browning M, Petronzelli F, Bicknell D, et al. Mechanisms of loss of HLA class I expression on colorectal tumour cells. Tissue Antigens. 1996;47:364-371.
46) Versteeg R, Kruse Wolters KM, Plomp AC, et al. Suppression of class I human histocompatibility leukocyte antigen by c-myc is locus specific. J.Exp.Med. 1989;170:621-635.
47) Peltenburg LT, Schrier PI. Transcriptional suppression of HLA-B expression by c-myc is mediated through the core promoter elements. Immunogenetics. 1994;40:54-61.
48) Soong TW, Hui KM. Locus-specific transcriptional control of HLA genes. J.Immunol. 1992;149:2008-2020.
49) Browning MJ, Krausa P, Rowan A, et al. Loss of human leukocyte antigen expression on colorectal tumour cell lines: implications for anti-tumour immunity and immunotherapy. J.Immunother. 1993;14:163-168.
50) Moller P, Lammler B, Herrmann B, Otto HF, Moldenhauer G, Momburg F. The primary mediastinal clear cell lymphoma of B-cell type has variable defects in MHC antigen expression. Immunology. 1986;59:411-417.
51) Amiot L, Onno M, Lamy T, et al. Loss of HLA molecules in B lymphomas is associated with an aggressive clinical course. Br.J.Haematol. 1998;100:655-663.
52) DeMars R, Chang CC, Shaw S, Reitnauer PJ, Sondel PM. Homozygous deletions that simultaneously eliminate expressions of class I and class II antigens of EBV-transformed B- lymphoblastoid cells. I. Reduced proliferative responses of autologous and allogeneic T-cells to mutant cells that have decreased expression of class II antigens. Hum.Immunol. 1984;11:77-97.
53) Complete sequence and gene map of a human major histocompatibility complex. The MHC sequencing consortium. Nature. 2000;401:921-923.
54) Tischfield JA. Loss of heterozygosity or: how I learned to stop worrying and love mitotic recombination. Am.J.Hum.Genet. 1997;61:995-999.
55) de Nooij van Dalen AG, van Buuren van Seggelen VH, et al. Isolation and molecular characterization of spontaneous mutants of lymphoblastoid cells with extended loss of heterozygosity. Mutat.Res. 1997;374:51-62.
56) Ossendorp F, Mengede E, Camps M, Filius R, Melief CJ. Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumours. J.Exp.Med. 1998;187:693-702.
57) Bennett SR, Carbone FR, Karamalis F, Miller JF, Heath WR. Induction of a CD8+ cytotoxic T lymphocyte response by cross- priming requires cognate CD4+ T-cell help. J.Exp.Med. 1997;186:65-70.

58) Dorfman DM, Schultze JL, Shahsafaei A, et al. In vivo expression of B7-1 and B7-2 by follicular lymphoma cells can prevent induction of T-cell anergy but is insufficient to induce significant T-cell proliferation. Blood. 1997;90:4297-4306.
59) Weiss S, Bogen B. B-lymphoma cells process and present their endogenous immunoglobulin to major histocompatibility complex-restricted T-cells. Proc.Natl.Acad.Sci.U.S.A. 1989;86:282-286.
60) Montesinos-Rongen M, Kuppers R, Schluter D, et al. Primary central nervous system lymphomas are derived from germinal- center B-cells and show a preferential usage of the V4-34 gene segment. Am.J.Pathol. 1999;155:2077-2086.
61) Thompsett AR, Ellison DW, Stevenson FK, Zhu D. VH) gene sequences from primary central nervous system lymphomas indicate derivation from highly mutated germinal center B-cells with ongoing mutational activity. Blood. 1999;94:1738-1746.
62) Silberstein LE, Jefferies LC, Goldman J, et al. Variable region gene analysis of pathologic human autoantibodies to the related i and I red blood cell antigens. Blood. 1991;78:2372-2386.
63) Baranzini SE, Jeong MC, Butunoi C, Murray RS, Bernard CC, Oksenberg JR. B-cell repertoire diversity and clonal expansion in multiple sclerosis brain lesions. J.Immunol. 1999;163:5133-5144.