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Loss of heterozygosity, a frequent but a non-exclusive mechanism responsible for HLA dysregulation in non-Hodgkin's lymphomas

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Summary

The frequent alteration of human leucocyte antigen (HLA) class I molecule expression observed in non-Hodgkin's lymphomas (NHL), similarly to solid tumours, has been reported to favour tumoral escape from the immune system. In order to identify the underlying mechanisms, we analysed 15 HLA defective NHL including partial ($n = 10$) and total class I ($n = 5$) loss, as well as HLA class II defects ($n = 5$). The HLA defect concerned HLA-A and -B antigens in 14 of 15 cases. In the cases with partial defect, the use of specific allelic monoclonal antibodies detected a defect of both alleles of A or B loci in six of seven tested cases. Allelic reverse transcription polymerase chain reaction (RT-PCR) demonstrated defects in six of nine cases, including four alterations of both A and B mRNA alleles. Real-time quantitative RT-PCR (RQ-PCR) did not detect the HLA-DR transcript in the two negative HLA-DR lymphomas, contrasting with the presence of CMH II transactivator (CIITA) transcript. Loss of heterozygosity (LOH) was detected in nine of 14 cases through variable pattern of nine microsatellites markers of the HLA locus. Taken together, these findings demonstrate the complexity and the variability of the mechanisms underlying HLA protein deficiencies with a high frequency of LOH. The diversity of these mechanisms indicates the importance of positive selection of HLA altered clones in the development of these NHL cases.

Keywords: lymphoma, human leucocyte antigen, loss of heterozygosity, flow cytometry, immune escape.

Human leucocyte antigen (HLA) molecules are frequently altered in different solid tumours, such as melanoma, breast cancer, cervical carcinoma and head and neck carcinomas (Garrido *et al*, 1993; Ferrone & Marincola, 1995). The loss of HLA molecules may induce a tumoral escape from the immune system, by the lack of peptide interaction between tumoral cell and specific lymphocytes (Garrido *et al*, 1997). We have previously described the alteration of HLA expression in non-Hodgkin's lymphoma (NHL). The loss of HLA class I molecules could be total or partial with a respective frequency of 2–10% (Amiot *et al*, 1998; Drenou *et al*, 2002). In some cases, HLA class II losses are associated with the decrease of HLA class I molecules. These findings were associated with poor outcome and appeared frequently as an acquired event during lymphomagenesis.

Down-regulation or loss of HLA protein expression may be conferred by multiple mechanisms characterized by different genes alterations. They could act at gene transcription level or in other cases at protein level (translation or processing). First, the HLA locus located at 6p21.3 could be altered. These alterations of HLA genes may be caused by mutations or deletions within the two alleles. Deletion of one allele has been studied in different tumours using the detection of loss of heterozygosity (LOH) (Feenstra *et al*, 2000; Koopman *et al*, 2000). Using this methodology, it has been shown that chromosome loss is the most frequent mechanism contributing to HLA haplotype loss in human tumours (Jimenez *et al*, 1999). Furthermore, numerous genes interacting with HLA expression are located in the same locus, such as TAP1, TAP2, LMP2, LMP7 or tapasin (Ortmann *et al*, 1997; Stephens *et al*, 1999). Furthermore, non-HLA genes

are also involved in the regulation of HLA genes. For example, the β2-microglobulin molecule, the light chain of HLA class I molecule encoded by a gene located on chromosome 15, could be deleted or truncated, generating total or partial decrease of class I expression as demonstrated in the Daudi cell line (Rosa *et al*, 1983). Similarly, defects in transcription or translation of β2-microglobulin or *TAP* genes could also have been involved in class I alteration. HLA class II molecules and, to a lesser degree, HLA class I molecules, are co-ordinately regulated by a set of conserved *cis*-acting promoter DNA elements, known as the W, X1, X2 and Y boxes (Gobin *et al*, 1998; Ting & Trowsdale, 2002). The regulatory factors of the RFX family (RFX-ANK, RFX-AP, RFX5) bind to the X1 box in association with the CMH II transactivator (CIITA), generating an active complex.

We have previously detected abnormalities of *TAP* or β2microglobulin protein in HLA defective NHL (Amiot *et al*, 1998). However, the mechanisms responsible for HLA molecule alteration have not yet been extensively studied in NHL. In order to identify the mechanisms involved in these HLA defective cases, we performed analysis to detect abnormal HLA expression in 15 selected NHL cases. Using allelic-specific monoclonal antibodies (MoAbs), specific allelic reverse transcription polymerase chain reaction (RT-PCR), real time RT-PCR (RQ-PCR) and LOH, we observed that the mechanisms involved in HLA defects are complex and variable according to each case, suggesting an important role for immune selection in lymphomagenesis.

Material and methods

Patients

Fifteen cases of malignant lymphoid proliferations diagnosed in the Department of Cytometry of the University Hospital of Rennes and characterized by a decrease of HLA class I molecules were selected on the basis of available frozen cells. Thirteen of the 15 cases were selected from a previous study (Drenou *et al*, 2002). These cases have been classified according to the Revised European–American Classification of Lymphoid Neoplasms (Harris *et al*, 1994). Their main characteristics are summarized in Table I. They consisted of follicular lymphoma (FL; $n = 4$), *de novo* diffuse large B-cell lymphoma (DLBCL; $n = 4$), secondary DLBCL [$n = 5$ derived from three FL, one mucosa-associated lymphoid tissue lymphoma, one Waldenström macroglobulinaemia], B chronic lymphoid leukaemia ($n = 1$) and a case of $CD4^+$ $CD56^+$ plasmacytoid dendritic cell proliferation of lymphoid lineage, as previously reported (Feuillard *et al*, 2002).

Analysis of HLA protein expression using flow cytometry

The cell purification and staining procedures have been previously reported (Amiot *et al*, 1998). Briefly, triple immunofluorescence assay was performed using flow cytometry with the pan HLA class I MoAb W6/32 (Parham *et al*, 1979), CD2 and CD19. W6/32 linearized mean fluorescence intensity (MFI) of tumoral cells was compared with that obtained from reactive cells, in order to distinguish complete (severe) and partial (minor) HLA class I defects as previously reported (Table I) (Drenou *et al*, 2002). Thus, W6/32 MFI was relevant because the lymphomatous cell population was homogeneous in the studied cases. Similar experiments were also performed using a β2 microglobulin MoAb, HLA-DR, -DP and -DQ in place of W6/32 (Dako, Trappes, France). In order to quantify HLA protein expression, indirect immunofluorescence was performed using an extensive panel of locus and allele-specific anti-HLA MoAb at saturating concentration (Tongio *et al*, 1997; Table II).

Table I. Characteristics of the patients.

	B-CLL	FL	B-DLCL	Secondary B-DLCL	PDC NHL
Partial defect ($n = 10$)	1	3	3	2	1
Total defect ($n = 5$)	0	1	1	3	0

B-CLL, B-chronic lymphocytic leukaemia; FL, follicular lymphoma; B-DLCL, B diffuse large cell lymphoma; PDC, plasmacytoid dendritic cell.

Secondary B-DLCL cases were derived from FL ($n = 3$), Waldenström macroglobulinaemia ($n = 1$) and mucosa-associated lymphoid tissue non-Hodgkin's lymphoma (NHL) ($n = 1$).

Separation of cell populations

The purification of tumoral and normal cells was performed using CD2 or CD19 coated immunomagnetic beads and Minimacs separation columns, according to the manufacturer's instructions (Miltenyii, Bergisch Gladbach, Germany). Both tumoral and reactive fractions were used to obtain RNA or DNA for transcriptional or LOH analysis.

DNA and RNA extraction

The DNA from purified normal cells and tumoral cells was isolated using the Trizol reagent (Gibco BRL, Cergy Pontoise, France) according to the manufacturer's instructions. Total RNA from the purified tumoral ($CD19^+$) and reactive fractions were isolated using an RNA isolation reagent (Promega, Charbonnières, France) that avoids genomic DNA contamination. cDNAs were prepared by hexamer random priming from 3 μg of RNA using the Superscript II Rnase reverse transcriptase (Gibco BRL).

Genotyping

The HLA class I genotyping was performed by the PCR single strand polymorphism method using specific primers provided in the course of the 12th International Histocompatibility Workshop (Kennedy *et al.*, 1997).

Table II. Anti-HLA monoclonal antibodies used in the study (Tongio *et al*, 1997).

MoAb	Specificity	Origin
W6/32 (PE labelled)	HLA class I	Dako (Glostrup, Denmark)
W6/32	HLA class I	12th Workshop Bodmer (UK)
GRH1	β2 microglobulin	12th Workshop Garrido (Spain)
IMMU357	HLA-DR	Immunotech (Marseille, France)
HI43	HLA-DP	Pharmingen/ Becton-Dickinson (San-Jose, CA, USA)
HK19	HLA-DQ	Sigma (L'Isle d'Abeau, France)
A131	HLA-A	12th Workshop Kornbluth (USA)
Tu155	HLA-A	12th Workshop Zigler (Germany)
H289-1	HLA-B	12th Workshop Ferrone (USA)
Q6/64	HLA-B	12th Workshop Ferrone (USA)
CR-11-351	HLA-A2/A28	12th Workshop Ferrone (USA)
BB7.2	HLA-A2/A69	12th Workshop Bodmer (UK)
160-30	HLA-A3	12th Workshop Gelsthorpe (UK)
361-1	HLA-A3	12th Workshop Gelsthorpe (UK)
HA41	HLA-A23-A24	12th Workshop One Lambda
H213	HLA-A25-A26	12th Workshop One Lambda
KS4	HLA-B7	12th Workshop Ferrone (USA)
BB7.1	HLA-B7	12th Workshop Bodmer (UK)
404HA-1	HLA-B8	12th Workshop One Lambda
MRE 4	HLA-B8	12th Workshop Fauchet (France)
H0129	HLA-B13-B15	One Lambda (Canoga Park, CA, USA)

Loss of heterozygosity analysis

All cases except one [unique patient number (UPN) 2] were studied. The microsatellite markers used included TAP1, DQ-CAR, D6S273, C141, C125, C143, D6S265, D6S510 and D6S276, spanning the region 6p21.3 of chromosome 6. The characteristics of the selected primers and the locations of the microsatellite markers on chromosome 6 are given in Table III. The 5′ site of one of the primers of each set was tagged with either FAM or TET (Eurogentec, Seraing, Belgium). PCR was performed in 50 μl reactions containing 1 × PCR II buffer (Perkin Elmer, Foster City, CA, USA), 100 ng DNA, 12 pmol of each primer combination, 0·8 μmol/l dNTPs, 0·4 μl Amplitaq DNA polymerase (5 U/ml) (Perkin Elmer) and 1·5–3 mmol/l $MgCl_2$. Amplification was performed in 30 cycles as follows: 94°C (30 s), 55–64°C (30 s), 72°C (45 s). $MgCl_2$ concentration and annealing temperature was optimized for each primer set (Table III). Amplification was preceded by a denaturation step (94°C, 4 min) and completed with an extension step (72°C, 5 min).

Microsatellite allele size and height were analysed on ABI 310 (Applied Biosystems, Foster City, CA, USA), a capillary electrophoretic method using genotype software. Loss was calculated using the ratio of the height of normal and tumoral alleles. LOH was assigned when the normal and tumoral ratio differed more than 25% (Feenstra *et al*, 2000).

Transcriptional studies

Each cDNA was amplified using appropriate HLA class I specific primers in order to test both HLA-A and HLA-B allelic expression. PCR products were run in a 2% agarose ethidium bromide stained gel. In order to compare the transcription levels in tumoral and normal cell fractions, the amount of cDNA target was normalized after quantification of 18S cDNA levels by TAQMAN methodology (Applied Biosystems).

Table III. Microsatellite characteristics.

Microsatellite	Location	Specificity	Size	Sequence	Ta (°C)
TAP1	INTRON 3 TAP1	TAP	188–206	5′GCTTTGATCTCCCCCCTC3′ 5′GGGGATGACGAATTATTCACTAACT3′	57
DQ-CAR	Cent./HLA-DQA1	DQ	178–200	5′TTGAGAGGTGTGCATGTTAC3′ 5′GCATTTCTCTTCCTTATCACTTC3′	57
D6S273	6p21.3	CLASS III	120–140	5′GCAACTTTTCTGTCAATCCA3′ 5′ACCAAACTTCAAATTTTCGG3′	55
C141	Cent. (6 kb)/HLA-B	HLA-B region	213–229	5′CGAGAGAACAACTGGCAGGACTG3′ 5′GACAGTCCTCATTAGCGCTGAGG3′	64
C125	Tel. (62 kb)/HLA-B Cent. (19 kb)/HLA-C	HLA-C/B region	178–220	5′CAGTAGTAAGCCAGAAGCTATTAC3′ 5′AAGTCAAGCATATCTGCCATTTGG3′	57
C143	Tel. (26 kb)/HLA-C	HLA-E/C region	413–473	5′TAGAAAACGCAATCTCGGCC3′ 5′CTGGATTAACCTGGAGACTC3′	57
D6S265	Cent. (70–100 kb)/HLA-A	HLA-E region	118–140	5′ACGTTCGTACCCATTAACCT3′ 5′ATCGAGGTAAACAGCAGAAA3′	55
D6S510	Cent.(25 kb)/HLA-A	HLA-A	178–196	5′AATGGGCTACTACTTCACACC3′ 5′CAACACACTGATTTCCATAGC3′	57
D6S276	Tel. (6000 kb)/HLA-A	6p21.3	198–230	5′TCAATCAAATCATCCCCAGAAG3′ 5′GGGTGCAACTTGTTCCTCCT3′	64

Real-time quantitative reverse-transcription PCR

Each cDNA (1 μl) was analysed in duplicate using the ABI PRISM 7000 sequence detector (Applied Biosystems, Courtaboeuf, France). Quantitative assessment of DNA amplification was detected through the SYBR green fluorescence dye on binding to the amplicon's double strand DNA. The RQ-PCR reactions were carried out in a total volume of 25 μl using the SYBR green Universal Master Mix (Applied Biosystems) and 300 nmol/l of each primer. Oligonucleotides used to detect HLA-DRA and total CIITA mRNA have been previously reported (Landmann *et al*, 2001). As an endogeneous control, a set of primers and probe specific for 18S ribosomal DNA (Applied Biosystems) was used. Samples were quantified using relative standard curves established with the cDNA of the HLA-DR positive RAJI cell line. The results were normalized with respect to the internal control (RNA 18S) and were expressed according to the RAJI cell line transcript levels using the cycle threshold (CT) of each sample ($2^{-\Delta\Delta CT}$). Thus the normal value obtained using RAJI cell line was 1·00 for both HLA-DRA and CIITA. The HLA-DR negative plasma-cell line SK-MM-2 [Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ: number ACC 430, Braunschweig, Germany), ACC 430, Germany], characterized by both HLA-DR and CIITA down-regulation was also used as negative control. The normalized HLA-DRA or CIITA transcript quantifications were, respectively, assigned as 0·00 and 0·01 in this cell line (Table IV).

Results

Characteristics of altered HLA class I cell surface expression in selected cases

The selected cases were representative of the different type of the HLA abnormalities described in a larger series of NHL. The decrease of surface β2 microglobulin appeared similar to that of W6/32 epitope expression and a total loss was observed in some lymphomas, particularly in cases with total loss of Class I.

In the 10 cases of HLA class I partial defect (UPN 1–10; Table V) the intensity of the HLA-A or B decrease appears to be comparable with that of total HLA class I detected by W6/32 in four cases (UPN 3, 5, 8, 10; Table V; Fig 1). In contrast, the remaining cases exhibited different intensity of staining (UPN 1, 2, 4, 6, 7, 9). The use of specific allele MoAbs to study five HLA-A alleles and two HLA-B alleles, demonstrated the type of the defective molecule in six cases (Table V). In the first group, defined by comparable HLA total and locus MFI decrease, allelic specific MFI was similarly altered ($n = 3$, UPN 3, 5, 10) in contrast to the three remaining cases ($n = 3$). Thus, in UPN 1, the majority of the strong HLA-A defect could be related to a nearly complete HLA-A2 loss, whereas allelic anti-HLA-B MoAbs were not available (Fig 1, UPN 1). In UPN 2 only the HLA-A3 allele was completely lost, suggesting a normal expression of HLA-A1 allele and the role of a specific HLA-A3 alteration, whereas the HLA-B proteins were normally expressed (Fig 1, UPN 2). The example of UPN 7 showed that the defect of HLA-B15 was stronger than that of B7 allele, contrasting with the similar results obtained using pan-A and pan-B markers. Finally, the 'coordinated pattern', as defined by a similar decrease of locus- or allele-derived proteins could be evoked in four of the 10 partial defects (example in Fig 1, UPN 3).

The value of the pan HLA-A and -B specific MFI in the five totally defective cases (UPN 11–15) was similar to that of W6/32, and confirmed the total HLA class I defect in all cases (Table V; Fig 1, UPN 11).

Table IV. RT-PCR detection of HLA-DR and CIITA mRNAs.

Cell line or UPN	HLA-DRA mRNA	Total CIITA mRNA
HLA-DR positive cells		
RAJI	1·00	1·00
UPN 3	2·22	1·66
UPN 14	1·55	5·22
HLA-DR negative cells		
SK-MM-2	0·00	0·01
UPN 10	0·09	4·52
UPN 15	0·08	1·91

Deficiencies of HLA class I gene expression at mRNA level

Specific class I mRNA levels were successfully examined in nine patients (UPN 2, 3, 8, 9, 10, 12, 13, 14, 15; Table V). Six patients showed a decreased HLA class I transcriptional level in tumoral cells, when compared with normal cells. In four of these patients, this deficiency involved one HLA-A allele and one B allele (UPN 3, 8, 10, 13) as illustrated for cases 10 and 13 (Fig 2C). Surprisingly, for two patients with total loss of HLA class I expression (cases 12 and 14) only one HLA-A allele mRNA was decreased. The three other patients (cases 2, 9, 15) showed no significant changes in the level of HLA class I specific mRNA allele.

HLA-DR protein defect is associated with absence of HLA-DR mRNA

Alterations of HLA class II molecules were found in five of the 15 cases including two cases with total HLA class II molecules defect (UPN 10 and UPN 15) and three cases characterized by only HLA-DQ or DP loss (UPN 5, UPN 9, UPN 14) as shown in Table V. In order to determine the mechanisms involved in HLA-DR defects, specific RQ-PCR was performed on tumoral samples to quantify HLA-DRA transcript. Results were normalized according to RAJI HLA-DRA mRNA level (Table IV). Two HLA-DR positive cases (UPN 3 and UPN 14) and two HLA-DR negative cases UPN 10 and UPN 15) were studied. Similar to the RAJI cell line, both positive cases expressed a high level of HLA-DRA

Table V. Protein and mRNA expression of HLA molecules on tumoral cells.

UPN	HLA Class I W6/32	β2 Micro.	DR	DP	DQ	HLA genotype	HLA-A	HLA-B	Specific defects	mRNA
1	0·75	0·91	+	+	w	A02; A24 B15; B39	0·38	0·67	A2: 0·05 A24: 0·55	NA
2	0·65	0·82	+	+	w	A01; A03 B35; B51	0·40	1·0	A3: 0·00	AABB *
3	**0·44**	**0·40**	+	+		A01; A26 B08; B45	**0·40**	**0·45**	**A26: 0·44**	**AMB**
4	0·42	0·38	+	+	+	A02; A09 B15; B18	0·28	0·28		NA *
5	**0·41**	**0·41**	+	+	−	A02; A68 B15; B44	**0·51**	**0·49**	**A2: 0·36**	**NA** *
6	0·33	0·52	+	+	w	A01; A02 B08; B18	0·50	0·56		NA *
7	0·25		+	+	+	A01; A29 B07; B15	0·13	0·19	B7: 0·29 B15: 0·05	NA *
8	**0·22**	**0·24**	+	+	+	A02 B15; B44	**0·21**	**0·19**		AB *
9	0·15	0·05	w	w	−	A01; A02 B37; B44	0·26	0·05		AABB
10	**0·06**	**<0·05**	−	−	−	A01; A31 B07; B51	**<0·05**	**0·05**	**A31: 0·02** **B7: 0·02;** **B18-51: 0·03**	**AB** *
11	**<0·05**	**<0·05**	+	+	w	A02; A24 B15; B49	**<0·05**	**<0·05**		NA *
12	**<0·01**	**0·05**	+	+	+	A02; A31 B51	**<0·05**	**<0·05**		ABB *
13	**<0·05**	**<0·05**	+	+	w	A02; A29 B07; B08/42	**<0·05**	**<0·05**		AB
14	**<0·01**	**0·07**	+	−	−	A02; A66 B41; B44	**<0·05**	**<0·05**		ABB *
15	**<0·01**		−	−	−	A03 A25 B07; B18	**<0·05**	**<0·05**		AABB *

*LOH as described in Fig 3.

UPN, unique patient number; NA, not available; AABB: normal mRNA expression for each HLA-A and HLA-B gene; ABB: decrease of one HLA-A transcript; AB: decrease of one HLA-A and one HLA-B transcript.

Intensity of HLA expression in tumoral cells is expressed using the ratio of the mean fluorescence intensity divided by the same parameter on reactive T cells for HLA class I molecules. For HLA class II molecules intensity is characterized by +, positive; −, absence; w, weak. Intensity of HLA class I antigens correspond to the mean of the values measured using A131 and Tu 155 MoAb for HLA-A, H284-1 and Q6/64 for HLA-B.

'Coordinated deficiencies' are given in bold.

mRNA. Both negative cases presented a low level of DRA mRNA (below 10% of the control), corresponding to residual reactive cells. These results were similar to the HLA-DR negative SK-MM-2 plasma cell line.

Furthermore, as the major function of CIITA is to regulate HLA-DR expression, we measured CIITA mRNA level in these four cases. In all cases, CIITA mRNA was strongly detected similarly to the RAJI cell line, in contrast to the plasma cell line SK-MM-2 (UPN 10: Fig 2, 1-B).

Characterization of HLA genes deficiencies using LOH

Using LOH methodology, no chromosome alteration was found in five of the 14 studied cases: UPN 5, 8, 10, 12, 14. In contrast, patterns of LOH were observed in eight of the 14 cases (UPN 1, 3, 4, 6, 7, 9, 11, 13, 15) but these were variable according the location or the number of altered or informative markers (Fig 3). Extensive LOH was defined by three different consecutive markers; four cases exhibited such a pattern: UPN 1, 3, 4, 13. One of these cases (UPN 4) also displayed normal markers. Furthermore, three cases were characterized by an alternate normal and LOH pattern: UPN 7, 11, 15. Those cases were difficult to interpret, because the normal pattern could represent normal residual alleles in cases where both alleles were deleted. The frequency of LOH detection was similar concerning HLA class II and class III loci (six of 14 and five of 14 respectively). However, LOH at HLA-B or C loci was frequent (seven of 14) compared with LOH at HLA-A locus,

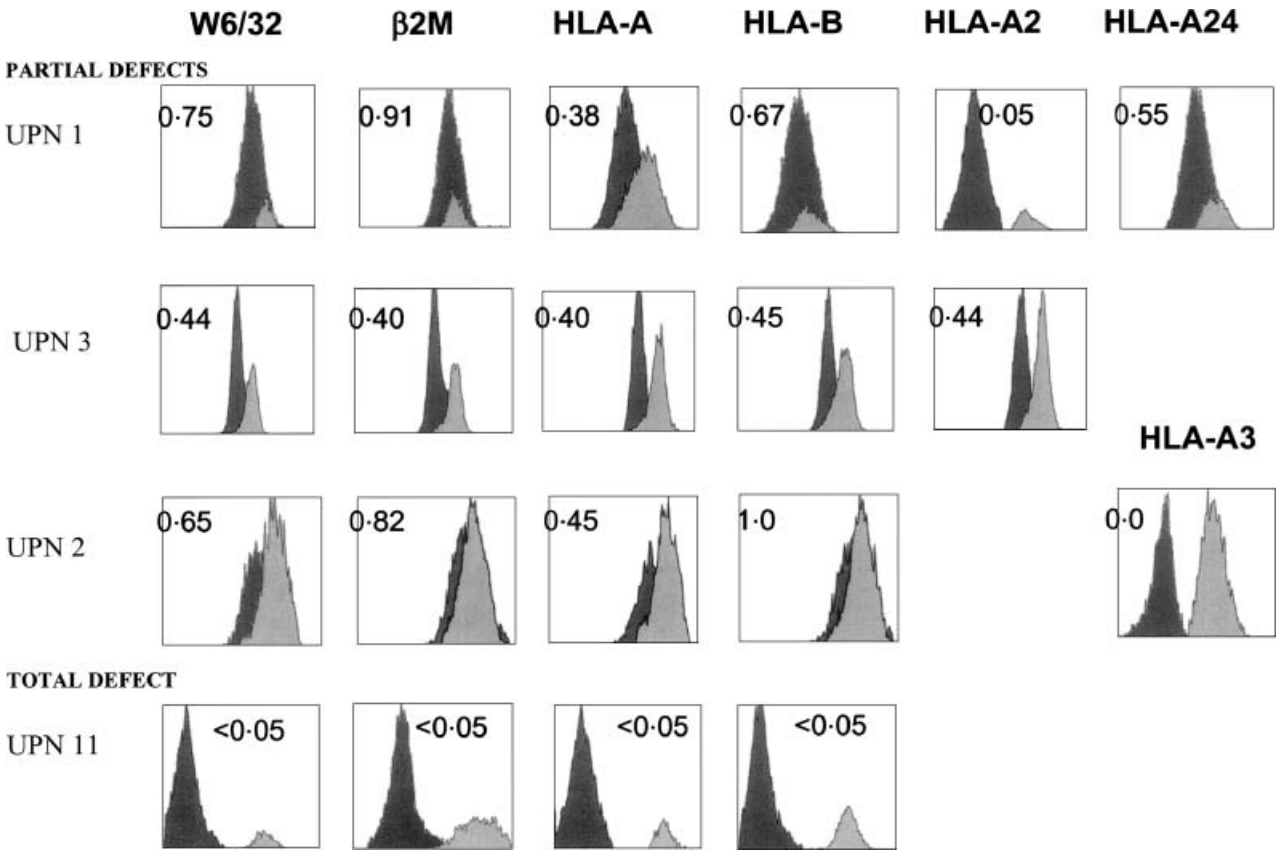

Fig 1. Flow-cytometric representative cases: data from four representative cases are represented by histogram overlay using a logarithmic scale. Dark grey shaded areas represent tumoral cells and light grey shaded areas indicate normal reactive cells. The number given for each histogram corresponds to the ratio of linearized MFI. UPN 1, 2 and 3 demonstrated a partial defect whereas UPN 11 showed complete HLA loss. UPN 3 exhibited a 'coordinated pattern' whereas UPN 1 displayed complex defects and UPN 2 an isolated HLA-A3 deficiency.

which was not observed. A representative case of LOH is shown in Fig 2A.

Discussion

As we have previously demonstrated the frequency of HLA class I alterations in NHL, the present report investigated the underlying mechanisms involved in these deficiencies at the genomic, transcriptional and protein level. LOH was frequently involved in NHL with HLA class I alterations (two of three of the different cases). The HLA gene transcription was usually altered in accordance with LOH findings (haplotype deficiencies). Coordinated defects concerning all the different HLA molecules at mRNA or protein level were observed in some cases, suggesting alterations of regulation mechanisms involved in the expression of HLA class I and, sometimes, class II molecules. However, protein abnormalities are not completely explained by these mechanisms, particularly the intensity of the protein defect, demonstrating the involvement of multiple mechanisms.

Loss of chromosome 6p21 regions, where HLA genes are located, has been demonstrated using LOH with nine microsatellite markers. The frequency of these gene alterations (nine of 14) could not be compared with that reported in variable types of solid tumours, because our study concerned only NHL cases that were selected on the basis of HLA class I alteration, which represents only 20% of total NHL in our experience. In the majority of these cases, LOH is not sufficient to explain the total HLA class I or HLA-DR defect detected by flow cytometry. The fact that approximately half of the cases did not have any evidence of HLA-DR alteration, but presented LOH in the class II locus could appear contradictory. However, in this case chromosomal amplification could have occurred because LOH cannot distinguish between loss or amplification (Koene, *et al* in press). Furthermore, genes frequently involved in HLA class I alteration in different tumours, such as *TAP1*, *TAP2*, *LMP2* and *LMP7* are also located in this locus. In some cases, LOH is observed in the HLA class III region, which is not directly relevant for HLA genes. These data should be compared with the different prognostic value according to tumour necrosis factor-α polymorphism in NHL (Warzocha *et al*, 1998; Juszczynski *et al*, 2002; Libura *et al*, 2002). Fluorescence *in situ* hybridization should be useful to demonstrate the absence of HLA

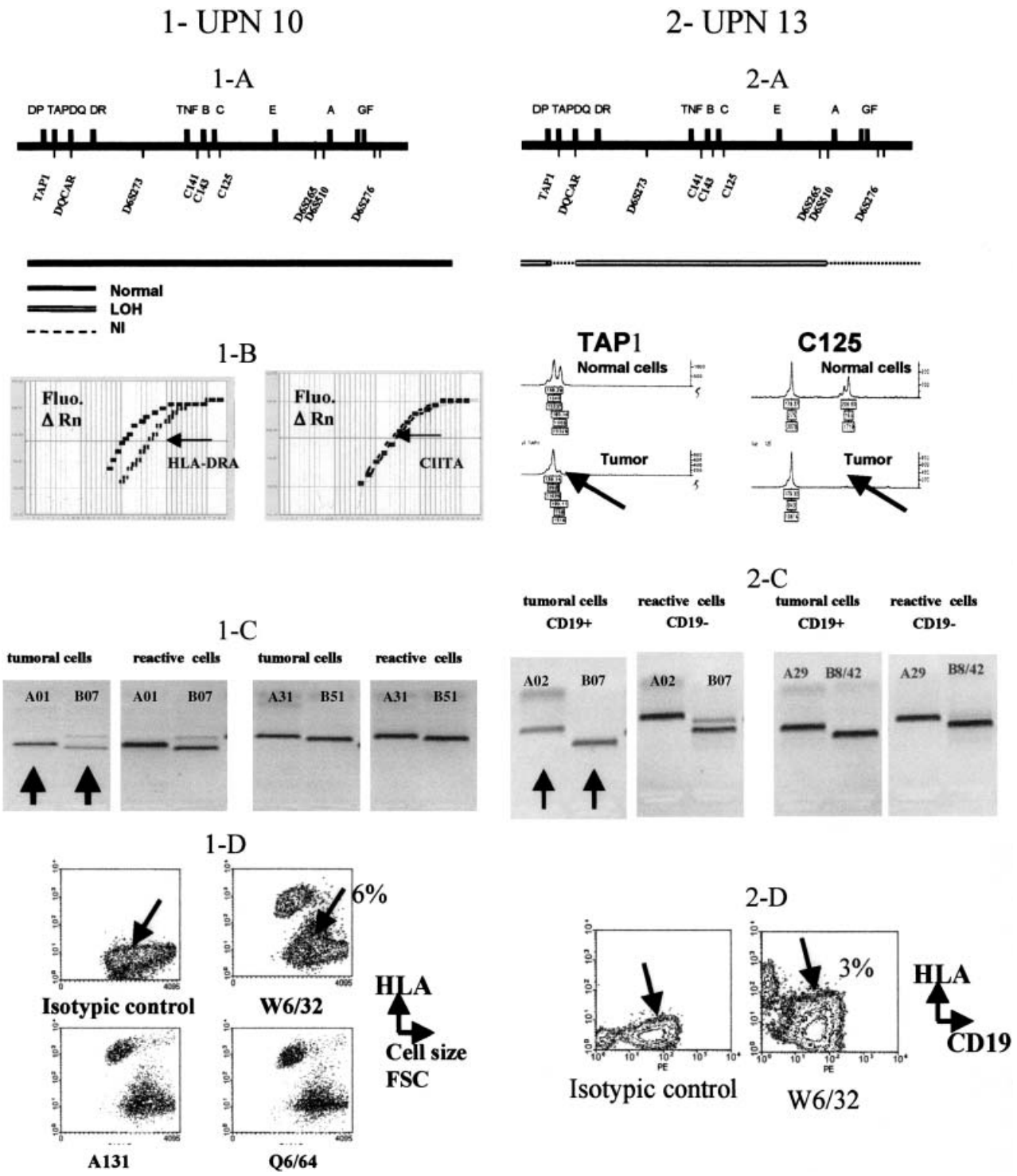

Fig 2. Two representative cases: 1-UPN 10, HLA class I and class II defective; 2-UPN 13, HLA class I decrease associated with normal HLA-DR expression. (A) Genomic analysis according to LOH studies: UPN 10 is normal (1-A) whereas extensive LOH is observed in UPN 13 (2-A); for C125 and TAP1 microsatellites, normal cells were characterized by two peaks, which represents the two alleles, whereas the lymphomatous cells have only one (2-A). (B) (only UPN 10) HLA-DRA expression is down-regulated at mRNA level, with a high cycle threshold (CT) compared with the RAJI cell line (solid bold curve). Similar levels are observed concerning CIITA expression. (C) Specific mRNA HLA-A or B alleles demonstrated lower intensity of the bands characterizing tumoral cells for all alleles (1-C) whereas only one haplotype expression was decreased in tumoral $CD19^+$ cells in the other case (2-C). (D) HLA class I cell surface expression is dramatically decreased in both cases (1-D; 2-D). Arrows pointed abnormalities in lymphoid tumoral cells.

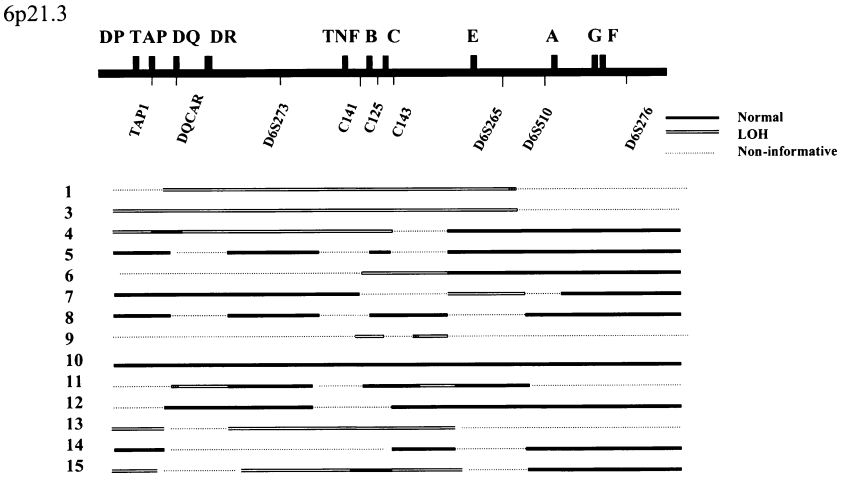

Fig 3. Summary of LOH data: LOH data for each case were represented by a line placed under the location of the different microsatellites markers on chromosome 6p. A solid black line indicates normal DNA expression or no LOH, whereas an open line indicates LOH. Dotted lines indicate non-informative data (homozygosity of a microsatellite in normal tissue or damaged DNA).

bi-allelic locus alteration in cases without LOH. However, the intensity of the different HLA transcripts detected either by standard or quantitative RT-PCR rule out the hypothesis of a double allelic deletion.

Few data have been reported concerning the loss of HLA loci in NHL. Using classical comparative genomic hybridization (CGH) or array-CGH to detect large loss or gain of genomic DNA on different chromosomes, deletion in 6p21 has not been frequently observed in multiple types of lymphomas (Hernandez *et al*, 2001; Stokke *et al*, 2001; Bea *et al*, 2002; Berglund *et al*, 2002; Martinez-Climent *et al*, 2002; Schop *et al*, 2002). In contrast and similar to our data, studies that employed microsatellite methodology also detected alteration of the chromosome 6p arm, suggesting that deletions involving the HLA locus are not particularly large and subsequently are not detected by cytogenetic methods (Rigaud *et al*, 2001). Two studies that used a large number of microsatellites markers located in the HLA locus also found frequent deletions in FLs or extra-nodal DLBCL (Randerson *et al*, 1996; Riemersma *et al*, 2000). However, to our knowledge, no data comparing DNA abnormalities to mRNA or protein expression are available. Finally, a recent study reported DLBCL that exhibited complete loss of class II molecules was explained by LOH for one allele and mutations in the HLA class II remaining allele (Jordanova *et al*, 2003).

The HLA class I mRNA transcript has been studied using specific allelic RT-PCR. As no accurate quantification could be determined, we were not able to observe an apparent parallel decrease between allele tumour-derived mRNA loss and the corresponding allele observed at the protein level using flow cytometry, or at the genomic DNA level using LOH. Half of the LOH positive cases studied (two of four) demonstrated a transcriptional loss of a haplotype corresponding to LOH. In contrast, we observed a down-regulation of a specific mRNA allele without corresponding DNA alteration in four cases: two cases demonstrated an mRNA defect involving one HLA-A and one HLA-B allele and two cases involved only HLA-A allele mRNA. These results demonstrate the complex genomic dysregulation that occurs in lymphomas. Furthermore, the presence of transcript without or with low level of protein was observed, suggesting a post-transcriptional mechanism of regulation. HLA class II mRNA was quantified using real time PCR with consensus primers. No HLA-DRA transcript was found in HLA-DR negative cases ($n = 2$) when compared with HLA-DR positive NHL used as controls ($n = 2$). It is interesting to observe that in UPN 15, this absence of transcript was not associated with a bi-allelic loss of DRA gene loci because LOH was detected at the HLA class II locus. These data suggest that an alteration of the HLA-DR regulation may be involved. Subsequently, the CIITA level, which is physiologically the major factor of HLA-DR positive regulation was investigated. No CIITA defect was detected, suggesting a potential alteration of another member of the RFX complex or co-stimulatory molecules such as cAMP responsive element binding proteins or chemokine-binding protein during lymphomagenesis.

As specific allelic anti-HLA class I MoAbs are available for flow-cytometric use, we approached the quantification of the different HLA class I alleles using this method. However, despite a valid methodology, flow-cytometric analysis did not predict mRNA or genomic findings because insufficient relevant MoAbs were available to identify clear allelic- or locus-specific defects. To our knowledge, no quantitative protein expression has been previously reported in neoplasias. Taken together, the different data demonstrated the complexity of the mechanisms necessary to induce HLA protein alterations. Similar data have been previously reported in cervical cancer using a cervical cancer cell line (Brady *et al*, 2000). Furthermore, these mechanisms appeared to differ between cases. Chromosomal aberrations with different mechanisms of down-regulation class I and class II gene expression were obviously found. These data suggest an important role for immune selection of the HLA defective lymphomatous cells by the immune system, because the selection of an HLA-altered clone occurs, whatever the involved underlying molecular mechanisms. Finally, these data and our previous findings, demonstrating association of different mechanisms involved during NHL evolution, provide a rational for the use of immunotherapeutic clinical trials at the early stages of the disease in non-HLA defective NHL.

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