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MUC1 (EMA) is preferentially expressed by ALK positive anaplastic large cell lymphoma, in the normally glycosylated or only partly hypoglycosylated form

R L ten Berge★, F G M Snijdewint★, S von Mensdorff-Pouilly, R J J Poort-Keesom, J J Oudejans, J W R Meijer, R Willemze, J Hilgers, C J L M Meijer

Department of Pathology, University Hospital Vrije Universiteit, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands
R L ten Berge
J J Oudejans
C J L M Meijer

Department of Obstetrics and Gynaecology, University Hospital Vrije Universiteit
F G M Snijdewint
S von Mensdorff-Pouilly
R J J Poort-Keesom

Department of Dermatology, University Hospital Vrije Universiteit
R Willemze

Department of Pathology, Rijnstate Hospital, 6815 AD Arnhem, The Netherlands
J W R Meijer

Biotech and Research Division, Sanbe Fama, Bandung, Indonesia
J Hilgers

★These authors contributed equally to this study.

Correspondence to:
Dr ten Berge
rl.tenberge@azvu.nl

Abstract
Aims—To investigate whether MUC1 mucin, a high molecular weight transmembrane glycoprotein, also known as epithelial membrane antigen (EMA), differs in its expression and degree of glycosylation between anaplastic large cell lymphoma (ALCL) and classic Hodgkin's disease (HD), and whether MUC1 immunostaining can be used to differentiate between CD30 positive large cell lymphomas.
Methods/Results—Using five different monoclonal antibodies (E29/anti-EMA, DF3, 139H2, VU-4H5, and SM3) that distinguish between various MUC1 glycoforms, high MUC1 expression (50–95% of tumour cells positive) was found in 13 of 17 anaplastic lymphoma kinase (ALK) positive systemic nodal ALCLs, and in one of 20 cases of classic HD. Scattered or focal staining (< 25% of tumour cells) was seen in two additional ALK positive systemic ALCLs, two additional classic HD cases, and in three of 20 cases of ALK negative systemic nodal ALCL. Primary cutaneous ALCL showed no staining with the anti-MUC1 antibodies. Antibodies detecting hypoglycosylated MUC1 were found to be absent in all lymphomas (SM3) or present in only six of 15 ALK positive ALCLs (VU-4H5).
Conclusions—MUC1 is preferentially expressed by a subtype of systemic nodal ALCL, characterised by ALK expression, but is found in only a few cases of classic HD and ALK negative ALCL. Therefore, although MUC1 could be used in a panel of markers for CD30 positive lymphomas, it is probably not a valuable tool to differentiate between ALK negative CD30 positive large cell lymphomas. Finally, the degree of MUC1 glycosylation in lymphomas is relatively high, compared with the aberrant hypoglycosylation found in adenocarcinomas.

(*J Clin Pathol* 2001;**54**:933–939)

Systemic (nodal) anaplastic large cell lymphoma (ALCL) is a CD30 positive non-Hodgkin's lymphoma of T cell or null cell phenotype, mainly arising in lymph nodes.[1] In 30–50% of cases, a chromosomal aberration such as the t(2;5)(p23;q35) translocation gives rise to expression of the anaplastic lymphoma kinase (ALK) protein,[2] identifying a subgroup of patients with systemic ALCL with excellent prognosis.[3–6] ALK expression seems specific for systemic nodal ALCL; it is not found in classic Hodgkin's disease (HD)[7–11] or primary cutaneous ALCL.[7 10 12 13] Morphologically, these lymphomas may closely resemble systemic ALCL, being also characterised by CD30 positive tumour cells with abundant cytoplasm, large irregular nuclei, and a prominent single nucleolus or multiple nucleoli.[1] Clinically, however, classic HD and primary cutaneous ALCL have a more favourable prognosis than ALK negative systemic ALCL.[12 14–16] Because of the differences in clinical behaviour and subsequent therapeutic strategies, differentiating ALK negative systemic ALCL from classic HD or primary cutaneous ALCL is very important.

Several markers can be used in the differential diagnosis of ALCL and other CD30 positive lymphomas. One such marker is MUC1 mucin, also known as epithelial membrane antigen (EMA).[17 18] MUC1 is a high molecular weight transmembrane glycoprotein, usually expressed on the luminal surface of glandular epithelia.[18–20] It consists of protein core with a constant cytoplasmic domain of 69 amino acids, and an extracellular domain with a variable number of 20 amino acid tandem repeats, containing serine and threonine residues, to which multiple oligosaccharide side chains are *O*-linked[21–24] (fig 1).

Since the first rabbit polyclonal antisera raised against human milk fat globule,[17] and the first monoclonal antibodies designated HMFG1 and HMFG2[25] were described, many anti-MUC1 monoclonal antibodies have been generated and characterised.[26] Most react with a dominant epitope within the variable number of tandem repeats of the protein core; namely, the hydrophilic sequence of PDTRPAP.[26–28] The specificity of antibody binding to the protein core depends largely on the extent of MUC1 glycosylation; during the ISOBM TD-4 workshop, clusters of monoclonal antibodies were identified that distinguish between the various glycoforms of MUC1.[29 30]

Although MUC1 is defined as an epithelial antigen, in 1984 Delsol *et al* reported that lymphoid cells and malignancies express EMA.[31] Since then, EMA (or more appropriately, MUC1) has been found to be consistently
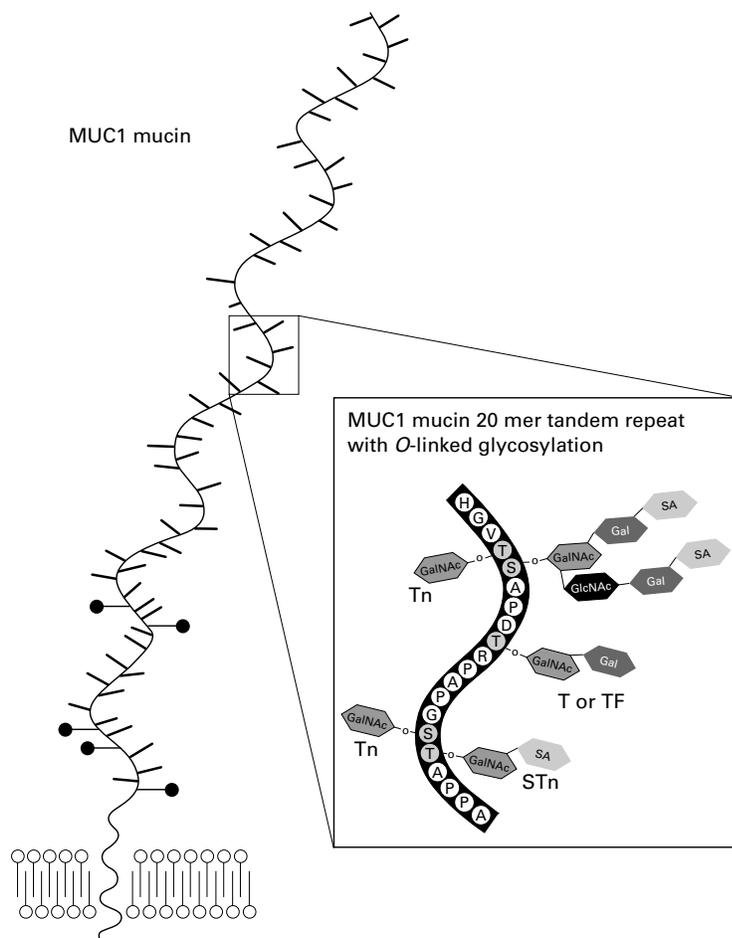
Figure 1   Extended MUC1 mucin molecule on a cell surface membrane with O-linked and N-linked glycosylated side chains. The insert shows a MUC1 20 mer single repeat peptide with O-linked hypoglycosylated side chains.

form,[22 38 39] and is related to tumour progression and increased metastatic potential.[40–42]

In our present study, we used the E29 antibody and four other MUC1 monoclonal antibodies that distinguish between the various glycoforms of MUC1. We investigated whether the expression and extent of glycosylation of MUC1 differs between ALCL of systemic and primary cutaneous origin and classic HD, and whether MUC1 is a valid tool in the differential diagnosis of these lymphomas. In addition, peripheral blood T and B cells, as well as a small number of T cell and B cell lymphomas, were investigated for MUC1 expression.

Methods
TISSUE SAMPLES
Formalin fixed, paraffin wax embedded tumour biopsies of ALK positive (n = 17) and ALK negative (n = 20) systemic nodal ALCL, primary cutaneous ALCL (n = 10), nodular sclerosing (NS) (n = 14), and mixed cellularity (MC) (n = 6) subtypes of classic HD were selected from the files of the Comprehensive Cancer Center Amsterdam, and from the department of pathology, Rijnstate Hospital, Arnhem, The Netherlands. We also selected samples of peripheral T cell lymphoma not otherwise specified (PTCL-NOS) (n = 5), T lymphoblastic lymphoma (T-LB) (n = 2), and nodal diffuse large B cell lymphoma (DLBCL) (n = 5). Cases were classified according to the proposed WHO classification[43] and systemic ALCL was subtyped as described previously.[44 45] ALK positive systemic ALCL could thus be divided into common type (n = 13), small cell variant (n = 3), and lymphohistiocytic type (n = 1); whereas ALK negative ALCL comprised common type (n = 11), small cell variant (n = 1), lymphohistiocytic type (n = 6), and giant cell rich variant (n = 2).

Standard immunophenotyping included CD30, ALK, CD15, B cell markers CD20/L26 and CD79A, and T cell markers CD3 and CD45RO/UCHL-1. By definition, systemic nodal ALCL, primary cutaneous ALCL, and classic HD were all CD30 positive. Two cases of DLBCL showed CD30 expression in a subset of the tumour cells. ALK expression was absent in all cases of classic HD, primary cutaneous ALCL, PTCL-NOS, T-LB, and DLBCL.

PBMC STIMULATION AND CELL SUBSET ISOLATION
PBMCs were isolated from the peripheral blood of healthy volunteer donors by density centrifugation on Ficoll-Hypaque (1.077 g/ml; Pharmacia, Uppsala, Sweden). Cells were washed three times with HBSS (Gibco, Paisley, UK) supplemented with 2% fetal calf serum (FCS; HyClone Laboratories, Logan, Utah, USA) and cryopreserved in IMDM (Gibco) with 50% FCS and 10% DMSO until use. PBMCs were thawed, washed three times in HBSS with 2% FCS, and cultured in IMDM plus 10% FCS and gentamycin (80 µg/ml) in the presence of PHA (Murex Diagnostic Limited, Dartford, UK) for four days. CD4+, CD8+, or CD19+ cells were isolated from the stimulated PBMCs using the corresponding

expressed on plasma cells[20 31–32] and immature erythroid cells in normal haematolymphoid tissues,[33 34] but not on CD34+ progenitor cells or resting T cells.[32 34] Among peripheral blood mononuclear cells (PBMCs), B cells show variable MUC1 expression,[32 34] but T cells express MUC1 only after activation, for instance, by stimulation with phytohaemagglutinin (PHA).[31 35] Cumulative experience on MUC1 expression in lymphoid malignancies, recently reviewed by Chittal *et al*,[36] shows that plasma cell neoplasms and T/null cell ALCLs have the highest expression of MUC1 (approximately 85% and 60% of cases, respectively). In contrast, MUC1 expression was found to be low in classic HD and in cutaneous ALCL. Thus, based on the literature, MUC1 would seem a valuable tool for differentiating systemic nodal ALCL from classic HD or primary cutaneous ALCL.

However, data on MUC1 expression in lymphomas are skewed in favour of the commercially available E29 (anti-EMA) monoclonal antibody[37] because this is the anti-MUC1 antibody used most frequently in haematopathology. Furthermore, E29 binds to MUC1 independently of its degree of glycosylation,[29] so little is known about the glycosylation pattern of MUC1 in lymphomas. In adenocarcinomas, MUC1 is overexpressed in its hypoglycosylated

Table 1 Characteristics of the anti-MUC1 monoclonal antibodies and staining dilutions used

Antibody	Mouse isotype	Immunogen	Specificity	Material and staining dilution	Refs
E29 (anti-EMA)	IgG2a	Human milk fat globule membrane	APDTRP	Purified protein, 1/1000	26, 37
139H2	IgG1	Human milk fat globule membrane	APDTRPAP	Ascites material, 1/1000	46
DF3	IgG1	Membrane from breast metastasis	APDTRPAP	Purified protein, 1/1000	26, 47
VU-4H5	IgG1	60 mer MUC1 peptide–BSA	APDTRPAP	Ascites material, 1/1000	48
SM3	IgG1	Deglycosylated milk mucin	APDTRP	Culture supernatant, undiluted	26, 38

E29 and 139H2 detect MUC1 irrespective of the degree of glycosylation.
DF3 also detects MUC1 irrespective of the degree of glycosylation, with the exception of strongly hyperglycosylated MUC1, as present in small intestine.
VU-4H5 detects MUC1 when the threonine residue is non-glycosylated.
SM3 detects MUC1 when it is hypoglycosylated; that is, contains short sugar chains.
EMA, epithelial membrane antigen.

mouse antihuman monoclonal antibodies coupled to magnetic beads (Dynal, Oslo, Norway). Cytospins with 2×10^5 cells/slide were made, dried, and fixed in 3.7% formalin/phosphate buffered saline (PBS) for 15 minutes, washed three times in PBS, fixed in −20°C ethanol for five minutes, washed three times in PBS, and stored at −20°C in PBS containing sucrose/glycerol until immunohistochemistry.

MUC1 MONOCLONAL ANTIBODIES
The five anti-MUC1 monoclonal antibodies selected are all directed at the immunodominant tandem repeat region of the protein core, but have different affinities, depending on the mode and extent of MUC1 glycosylation. Table 1 describes the immunogens used for the generation of and other characteristics of the monoclonal antibodies. E29 and 139H2 detect all forms of MUC1, irrespective of its degree of glycosylation.[29 46] DF3 also binds to MUC1 relatively independently of its degree of glycosylation; however, it does not detect hyperglycosylated MUC1, as present in normal small intestine, and has a preference for membrane MUC1 staining above Golgi MUC1 staining.[47] VU-4H5[48] can only detect MUC1 if the threonine residue in PDTRPAP of the tandem repeat region is non-glycosylated. SM3[38] only detects MUC1 when it is hypoglycosylated; that is, contains relatively short sugar side chains. All monoclonal antibodies are commercially available, with the exception of DF3 (kindly provided by Dr DW Kufe, Dana-Farber Cancer Institute, Boston, USA) and 139H2, which was developed in the laboratory of one of the authors (JH).

IMMUNOHISTOCHEMISTRY
Paraffin wax embedded sections (4 µm) were mounted on poly-L-lysine coated slides. After dewaxing, sections were immersed in methanol containing 0.3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity. Slides were pretreated with an antigen retrieval method by heating the slides in a microwave oven in citrate buffer (10 mM, pH 6.0) for 10 minutes (360 W). After rinsing in PBS, slides were preincubated with normal rabbit serum, diluted 1/50 in PBS with 1% bovine serum albumin (BSA), for 10 minutes. The anti-MUC1 monoclonal antibodies were used at the dilutions given in table 1, and incubated for one hour at room temperature. Subsequently, slides were incubated with biotinylated rabbit antimouse antibody (Dako,

Glostrup, Denmark) diluted 1/500 in PBS/BSA for 30 minutes, followed by incubation with 1/200 streptavidin biotinylated horseradish peroxidase complex (Dako) in PBS/BSA for one hour; 3'3-diaminobenzidine was used as chromogen and haematoxylin as the counterstain.

INTERPRETATION OF RESULTS
After immunostaining, cases were examined under the light microscope. MUC1 expression on tumour cells of the lymphomas was scored as follows: negative (−), scattered or focal staining of less than 25% of tumour cells (+/−), staining of 25–50% of tumour cells (+), staining of 50–75% of tumour cells (++), or staining of more than 75% of tumour cells (+++). Qualitative variables were analysed by Pearson χ^2 test or by the Fisher exact test, when appropriate. All p values are based on two tailed statistical analysis, and p values below 0.05 were considered to be significant. All analyses were performed using the SPSS statistical software (SPSS Inc, Chicago, Illinois, USA).

Results
SYSTEMIC NODAL ALCL
In general, E29 (anti-EMA), DF3, and 139H2, which bind to normally glycosylated and adenocarcinoma associated hypoglycosylated MUC1, gave similar staining patterns in the tissue sections, although in some cases staining with 139H2 was slightly lower than with DF3

Table 2 Staining for anti-MUC1 monoclonal antibodies in tumour cells of ALK positive systemic nodal anaplastic large cell lymphoma

Case	SM3	VU–4H5	DF3	139H2	E29
1	−	−	−	−	−
2	−	−	+++	+++	+++
3	−	++	+++	++	+++
4	−	+/−	+++	++	+++
5	−	−	+++	+	+++
6	−	−	+++	+++	+++
7	−	+++	+++	+++	+++
8	−	−	+++	++	+++
9	−	−	+/−	+/−	+/−
10	−	++	+++	+++	+++
11	−	+/−	++	+	++
12	−	−	+++	+++	+++
13	−	−	+/−	+/−	+/−
14	−	−	+++	+++	+++
15	−	−	−	−	−
16	−	−	+++	++	+++
17	−	+	+++	++	+++

+++, 75–95% positive tumour cells; ++, 50–75% positive tumour cells; +, 25–50% positive tumour cells; +/−, focal or scattered staining of <25% of tumour cells; −, no staining of tumour cells.
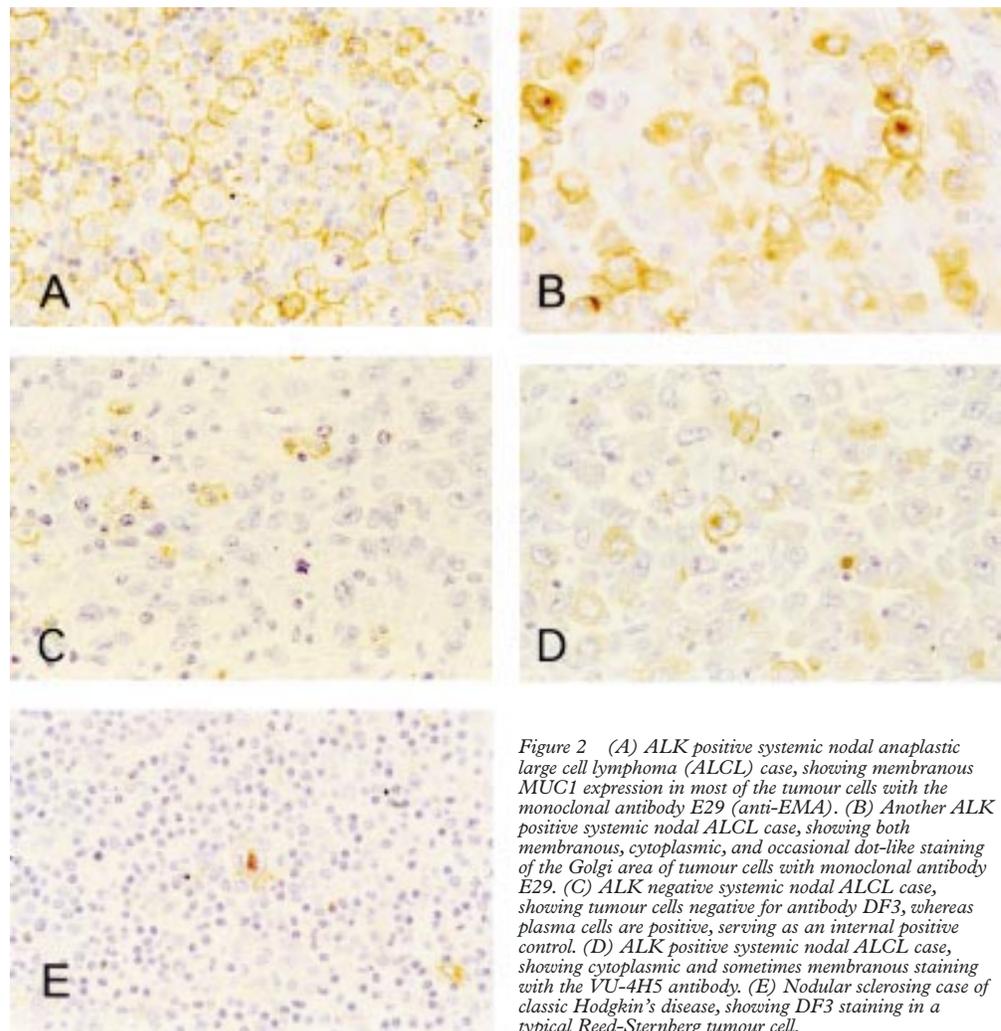
Figure 2 (A) ALK positive systemic nodal anaplastic large cell lymphoma (ALCL) case, showing membranous MUC1 expression in most of the tumour cells with the monoclonal antibody E29 (anti-EMA). (B) Another ALK positive systemic nodal ALCL case, showing both membranous, cytoplasmic, and occasional dot-like staining of the Golgi area of tumour cells with monoclonal antibody E29. (C) ALK negative systemic nodal ALCL case, showing tumour cells negative for antibody DF3, whereas plasma cells are positive, serving as an internal positive control. (D) ALK positive systemic nodal ALCL case, showing cytoplasmic and sometimes membranous staining with the VU-4H5 antibody. (E) Nodular sclerosing case of classic Hodgkin's disease, showing DF3 staining in a typical Reed-Sternberg tumour cell.

or E29 (table 2). Staining was usually localised to the tumour cell membrane (fig 2A), although sometimes it was both membranous and cytoplasmic, with occasional dot-like staining of the Golgi area (fig 2B).

Staining with these three monoclonal antibodies was seen in 15 of 17 ALK positive systemic nodal ALCL cases (table 2), with 13 cases showing staining of 50% to > 90% of tumour cells, and two cases showing scattered staining of < 25% of tumour cells. In contrast, only three of 20 ALK negative systemic nodal ALCL cases showed MUC1 expression: staining in these cases was focal or scattered, in < 25% of tumour cells (table 3). When MUC1 expression was defined as "high" (> 50% of tumour cells staining positively) or "low" (0–50% of tumour cells), ALK expression was significantly related to high MUC1 expression: 13 of 17 ALK positive cases showed high MUC1 expression, whereas all ALK negative cases showed low MUC1 expression ($p < 0.001$) (fig 2C).

Cases staining positively with E29, DF3, and 139H2 were all negative for SM3, but in six ALK positive systemic nodal ALCLs staining with VU-4H5 was found, with the percentage of positive tumour cells ranging from a few to > 50% (table 2; fig 2D).

PRIMARY CUTANEOUS ALCL

None of 10 primary cutaneous ALCL stained positively with the anti-MUC1 monoclonal antibodies.

CLASSIC HD

In classic HD, MUC1 expression as detected by E29, DF3, and 139H2 was found in three of 20 cases (table 3). MUC1 staining was high in one, with > 50% of tumour cells staining positively (fig 2E), but low in the other two cases, showing only scattered staining of < 25% of tumour cells. None of the classic HD cases showed staining for either SM3 or VU-4H5.

The staining pattern was the same as seen in systemic nodal ALCL—in most cases membranous, but sometimes membranous and cytoplasmic, with occasional dot-like staining of the Golgi area.

T AND B CELL LYMPHOMAS

One of five PTCL-NOS cases showed focal, membranous staining with E29, DF3, and 139H2. SM3 and VU-4H5 were negative in all PTCL-NOS cases. The two T-LB cases showed no staining with the five anti-MUC1 monoclonal antibodies (table 3). Of five nodal DLBCL cases, one showed membranous MUC1 expression, staining positively for E29,

Table 3 Number of cases staining positively for anti-MUC1 monoclonal antibodies in tumour cells of ALK negative lymphomas

Lymphoma type	SM3	VU-4H5	DF3	139H2	E29
ALK negative systemic ALCL	–	–	3+/–	3+/–	3+/–
Primary cutaneous ALCL	–	–	–	–	–
Classic HD-NS	–	–	1+++, 1+/–	1++, 1+/–	1+++, 1+/–
Classic HD-MC	–	–	1+/–	1+/–	1+/–
PTCL-NOS	–	–	1+/–	1+/–	1+/–
T-LB	–	–	–	–	–
DLBCL	–	1+/–	1+++	1+++	1+++

+++, 75–95% positive tumour cells; ++, 50–75% positive tumour cells; +/–, focal or scattered staining of <25% of tumour cells; –, no staining of tumour cells.
ALCL, anaplastic large cell lymphoma; DLBCL, diffuse large B cell lymphoma; HD-MC, Hodgkin's disease, mixed cellularity subtype; HD-NS, Hodgkin's disease, nodular sclerosing subtype; PTCL-NOS, peripheral T cell lymphoma, not otherwise specified; T-LB, T lymphoblastic lymphoma.

Table 4 MUC1 expression on peripheral blood mononuclear cells (PBMCs)

PBMCs	Culture conditions	SM3	VU-4H5	DF3	139H2
Total population	Non-stimulated	–	–	–	–
Total population	PHA stimulated	–	–	+++	+
CD4+ subset	PHA stimulated	–	–	+++	+++
CD8+ subset	PHA stimulated	–	–	+++	+++
CD19+ subset	PHA stimulated	–	–	–/+	–/+

+++, most cells positive; +, subpopulation of cells positive; –/+, scattered blast-like cells positive; –, no staining of cells.
PHA, phytohaemagglutinin.

DF3, and 139H2 in most of the tumour cells, and focally for VU-4H5 (table 3). Interestingly, this case also expressed CD30 on tumour cells. Another CD30 positive DLBCL was MUC1 negative.

PERIPHERAL BLOOD MONONUCLEAR CELLS
As shown in table 4, unstimulated PBMCs derived from normal healthy donors showed no MUC1 expression. However, when cells had been cultured in the presence of PHA, both the CD4+ and CD8+ subsets of PBMCs showed strong staining with DF3 and 139H2, but no staining with SM3 or VU-4H5. In the stimulated CD19+ subset of PBMCs, sporadic CD19+ blast-like cells stained positively with DF3 and 139H2.

Discussion
Previously, several groups have investigated the expression of MUC1 in malignant lymphomas. However, data on MUC1 expression in lymphomas are skewed in favour of the E29 (anti-EMA) monoclonal antibody,[37] the one used most frequently in haematopathology. Furthermore, E29 does not distinguish between hyperglycosylated and hypoglycosylated MUC1[29]; therefore, little is known about the MUC1 glycoforms present on lymphoma tumour cells. In our present study, we have used a panel of five monoclonal antibodies to study the expression and extent of glycosylation of MUC1 in ALCL and classic HD. As already described, these five antibodies all react to the immunodominant PDTRP(AP) sequence within the MUC1 protein core, but have different binding affinities, depending on the mode of glycosylation of MUC1.[26 29 30 46]

Our results show that MUC1, as detected by E29, DF3, and 139H2, is preferentially expressed in a subtype of systemic nodal ALCL, characterised by the presence of the ALK protein. E29 and DF3 showed similar strong staining of most of the tumour cells, whereas 139H2 in some cases stained a lower number of tumour cells. No staining was found with SM3, showing that the MUC1 present in ALK positive systemic nodal ALCL contains long sugar side chains. Approximately 40% of cases staining positively for E29, DF3, and 139H2 also showed VU-4H5 staining, indicating that in these cases the PDTRPAP region of the MUC1 protein core is non-glycosylated. Thus, MUC1 is aberrantly glycosylated in only a proportion of ALK positive systemic nodal ALCL cases, in contrast to adenocarcinomas, which usually show overexpression of hypoglycosylated MUC1.

Primary cutaneous ALCL totally lacked MUC1 expression, and only a few ALK negative systemic nodal ALCL and classic HD cases showed MUC1 expression, usually present as focal, scattered staining with E29, DF3, and 139H2 of < 25% of tumour cells. These cases stained for neither VU-4H5 nor SM3, again suggesting that MUC1 in lymphomas is usually normally (hyper)glycosylated.

Systemic nodal ALCL, primary cutaneous ALCL and classic HD are considered separate disease entities,[1 43] with the last two entities showing good prognosis compared with ALK negative systemic nodal ALCL.[12 14–16] However, because they share many similarities with regard to morphology and immunophenotype (CD30 expression), in some instances the initial diagnosis may pose a problem. EMA, a MUC1 epitope, is one of the markers currently used for differential diagnosis. Previous studies reported that it was highly expressed in systemic ALCL, but low in classic HD, and absent in primary cutaneous ALCL.[16 31 33 36 49–52] However, our study shows that the high MUC1 expression in systemic nodal ALCL is largely attributable to the ALK positive cases. Other papers also show a strong association between MUC1 (EMA) and ALK expression, although this was never explicitly stated.[45 53–55] Thus, MUC1 may be included in a panel of markers for CD30 positive large cell lymphomas (for which purpose the commercially available E29 antibody seems well suited), but is probably not a useful tool in differentiating between ALK negative CD30 positive lymphomas.

MUC1 expression is related to late differentiation stages in normal B cells[32 34] and is probably an activation related phenomenon because it is usually coexpressed with other activation markers, such as CD30.[20 56] In line with the literature,[31 32 34 35] we found that normal peripheral blood T cells expressed MUC1 only after activation. Like MUC1 found in most ALK positive ALCL, MUC1 in these T cells was normally hyperglycosylated, as demonstrated by the absence of SM3 or VU-4H5 staining. The strong MUC1 expression by ALK positive ALCL might be explained by this lymphoma subtype being a neoplastic equivalent of activated T cells. Previously, we and others showed that ALK expression is highly associated with the expression of cytotoxic proteins, such as granzyme B and/or TIA-1.[53 57] Thus, ALK positive systemic ALCLs are probably

derived from activated cytotoxic T lymphocytes (CTLs) that express MUC1 along with cytotoxic granules.

The clinical relevance of MUC1 expression in lymphomas is not yet known. In human adenocarcinomas, MUC1 is abundantly overexpressed and hypoglycosylated,[22 38 39] and the overexpression is related to advanced stage, increased metastatic potential, and invasive growth,[40–42] as a result of interference with adhesion processes.[58 59] Furthermore, MUC1 has both immunostimulatory and immunosuppressive effects. On the one hand, MUC1 is capable of eliciting a humoral[60] and a cytotoxic[61–65] immune response; on the other hand, soluble MUC1 inhibits CTL proliferation and causes T cell anergy.[35 62 66–67] In systemic nodal ALCL, we found that MUC1 expression was related to a good prognosis; however, when analysed together with ALK expression in multivariate analysis, MUC1 expression lost its predictive value (data not shown). Thus, the prognostic value of MUC1 in systemic ALCL might be attributed to its strong association with ALK expression. Further studies are required to elucidate the possible role of MUC1 expression in lymphomagenesis.

We conclude that MUC1 expression is preferentially found in ALK positive systemic nodal ALCL, but is low to absent in ALK negative systemic nodal ALCL, primary cutaneous ALCL, and classic HD. As such, MUC1 immunostaining is not a valuable tool to distinguish between these three lymphoma subtypes. Finally, in contrast to adenocarcinomas, malignant lymphomas express MUC1 that is normally (hyper)glycosylated or shows hypoglycosylation in only a proportion of cases.

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