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B and CTL responses to the ALK protein in patients with ALK-positive ALCL

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Anaplastic lymphoma kinase (ALK)-positive anaplastic large cell lymphoma (ALCL) has a good prognosis compared to ALK-negative ALCL, possibly as a result of the immune recognition of the ALK proteins. The aim of our study was to investigate the presence of both a B and cytotoxic T cell (CTL) response to ALK in ALK-positive ALCL. We confirmed the presence of an antibody response to ALK in all 9 ALK-positive ALCL patients investigated. An ELISpot assay was used to detect a γ-interferon (IFN) T cell response after short term culture of mononuclear blood cells with 2 ALK-derived HLA-A*0201 restricted peptides: ALKa and ALKb. A significant γ-IFN response was identified in all 7 HLA-A*0201-positive ALK-positive ALCL patients but not in ALK-negative ALCL patients ($n = 2$) or normal subjects ($n = 6$). CTL lines (>95% CD8-positive) raised from 2 ALK-positive ALCL patients lysed ALK-positive ALCL derived cell lines in a MHC-Class I restricted manner. This is the first report of both a B cell and CTL response to ALK in patients with ALK-positive ALCL. This response persisted during long-term remission. The use of modified vaccinia virus Ankara (MVA) to express ALK is also described. Our findings are of potential prognostic value and open up therapeutic options for those ALK-positive patients who do not respond to conventional treatment.

Key words: ELISpot; tumour-associated antigens; CTL responses; ALK; ALCL

Anaplastic large cell lymphoma (ALCL) comprises 30–40% of pediatric large cell lymphomas. Approximately 53–80% of ALCL are associated with the (2;5)(p23;q35) translocation resulting in the expression, by the neoplastic cells, of the tumour-associated oncogenic nucleophosmin-anaplastic lymphoma kinase (NPM-ALK) protein.[1–3] These ALK-positive ALCL have an improved prognostic outlook compared to ALK-negative ALCL.[4] Given the extremely restricted normal tissue distribution of the ALK protein (present only in scattered cells in the adult brain), ALK may function as a tumour-associated antigen (TAA). Indeed, evidence confirming the immunogenicity of ALK has been obtained from preliminary studies reporting an antibody response to ALK in ALK-positive ALCL.[5] Borisch *et al.*[6] also reported the presence of circulating antibodies to ALK in ALK-positive ALCL patients. The improved prognosis of ALK-positive ALCL compared to ALK-negative tumours may, therefore, reflect the existence in patients of an immune response to ALK.[5]

It is well established that peptide epitopes derived from TAA can be recognised by cytotoxic T cells (CTL) in the context of MHC molecules.[7] CD8-positive CTL have been shown to play a major role in the cell-mediated recognition of TAA such as NY-ESO-1,[8] tyrosinase,[9] gp 100,[10] Melan-A/Mart-1[11] and tyrosine-related proteins 1 and 2 (TRP 1-2)[12,13] in melanoma, PR1[14] and BCR-ABL[15] in chronic myeloid leukaemia and WT1[16] in acute myeloid leukaemia. A recent study also identified 2 HLA-A*0201 immunogenic ALK peptides that were able to stimulate *in vitro* specific CTL responses in peripheral blood mononuclear cells (PBMC) from 3 healthy donors.[17] The existence of such an effector arm of the immune response has raised the possibility of immunotherapy in a range of human tumours.[18,19]

The aim of our study was to investigate the presence of both CTL and B cell responses to ALK in patients with ALK-positive ALCL. B cell responses were identified using an immunocytochemical labeling technique with antibodies from patients' plasma on cells transfected with the ALK protein, whereas 2 ALK peptides were studied for their efficacy in eliciting CTL responses in patients. Evidence for both a CTL and B cell response was obtained. These results may provide valuable information on prognostic outcome and also open up the possibility of improved therapeutic methods for those 20–30% patients with ALK-positive ALCL or other ALK-positive tumours who fail to respond to current methods of treatment.

Material and methods

Subjects

ALK-positive ALCL. Nine patients were studied and all cases were diagnosed according to the WHO classification.[4] Blood was obtained from patients attending the Haematology Departments of the John Radcliffe Hospital, Oxford, UK (Patients 1–5), Liverpool General Hospital, Liverpool, UK (Patients 6 and 9), the Royal Marsden Hospital, London, UK (Patient 7), the Merkur Hospital, Zagreb and Croatia (Patient 8). Patients 6, 7 and 9 were obtained from the ongoing Biological Studies project BS001 run by the United Kingdom Children's Cancer Study Group (UKCCSG). The ALK-positive ALCL patients presented with differing stages of the disease and blood samples were obtained at varying times after diagnosis (Table I). Patients were treated with combination chemotherapy according to standard protocols (Table I).

Control samples. Blood was obtained from 2 patients (10 and 11) with ALK-negative ALCL attending the John Radcliffe Hospital, Oxford whereas 6 blood samples were provided by healthy volunteer subjects. Tissue typing was carried out at the Oxford Transplant Centre, Oxford. Ethical approval and written consent was obtained for all samples.

Peptides

Peptides were synthesized by standard chemistry on a multiple peptide synthesizer (Invitrogen, UK). All peptides were >90%

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TABLE I – CLINICAL DETAILS OF PATIENTS WITH ALK-POSITIVE ALCL[1]

Patient no.	Age	Gender	Stage	Treatment	Time of blood sample after diagnosis	Follow-up
1	46	M	II	CHOP	47 months	CR (58 months)
2	34	F	II	CHOP	52 months	CR (56 months)
3	17	M	I	ALCL 98 protocol[2]	47 months	CR (61 months)
4	50	F	IV	MACOP-B	163 months	CR (174 months)
5	23	F	II	CHOP, MTX, Ara C	63 months	CR (78 months)
6	16	F	I	ALCL 99 protocol[2]	1 months	Regression during treatment
7	5	M	II	ALCL 99 protocol	4 months	Regression during treatment
8	57	M	I	ABVD	At diagnosis	CR (32 months)
9	9	NA	II	ALCL 99 protocol	Diagnosis	Regression during treatment

[1]CR, complete remission; CHOP, cyclophosphamide, adriamycin, vincristine, prednisolone; MACOP-B, methotrexate, adriamycin, cyclophosphamide, vincristine, prednisolone and bleomycin; MTX, methotrexate; Ara-C, Cytarabidine; E, etoposide; ABVD, adriamycin, BCNU, vinblastine, decarbazine.–[2]ALCL 98 and 99 use a modification of the NHL-BFM-B 90[20] treatment.

pure as indicated by analytical HPLC. Lyophilized peptides were diluted in dimethyl sulfoxide and stored at −20°C. The three ALK peptides tested in our experiments were those predicted to bind to HLA-A2*0201 using the BIMAS (http://bimas.dcrt.nih.gov/molbio/hla_bind/index.html) and SYPETHI (ww.syfpethi.de) web-based programmes to identify those peptides predicted to bind to HLA-A2*0201 with the highest affinity. The chosen peptides were present within the kinase domain, $ALKa_{aa280–289}$: SLAMLDLLHV, $ALKb_{aa282–290}$: AMLDLLHVA and $ALKc_{aa242–p250}$: CIGVSLQSL. It is of note that ALKa and ALKb peptides were also predicted to be potentially immunogenic by Passoni *et al.*[17] Control peptides predicted to bind to HLA-A2*0201 consisting of the Flu Matrix Peptide $(FMP)_{58–66}$ (GILGFVFTL)[21] and a peptide derived from the HIV-1 reverse transcriptase (ILKEPVHGV)[22] were also used. Experimental binding was confirmed by using a T2 binding assay as described previously.[23]

Antibodies

Monoclonal antibodies. Anti-CD3 (DAKO-3D4), anti-CD4 (DAKO-CD4), anti-CD20 (DAKO-CD20) and anti-CD56 (DAKO-CD56) were obtained from DAKOCytomation (Ely, Cambridgeshire, UK). Antibodies to ALK (ALK1), CD8 (X-107) and CD68 (KP1), were obtained from the authors' laboratory. Anti-HLA-A2*0201 (BB7.2) was purchased from BD Biosciences (Oxford, UK).

Polyclonal antibodies. Rabbit anti-CD3 (diluted 1:100), horseradish peroxidase (HRP)-conjugated goat anti-mouse Ig (diluted 1:50), HRP-conjugated goat anti-rabbit Ig (diluted 1:100) and HRP-conjugated rabbit anti-human IgG reagents (diluted 1:100) were all purchased from DAKOCytomation (Glostrup, Denmark).

Tumour-derived cell lines

The t(2;5)-positive ALCL-derived cell lines SUDHL-1 and KARPAS-299 were obtained from by Drs M.L. Cleary (Stanford, CA) and A. Karpas (Cambridge, UK), respectively. The COS-1 monkey epithelial cell lines were provided by the Sir William Dunn School and the TAP2-deficient T2 and lymphoblastoid JY cells were obtained from the German Collection of Microorganisms and Cell Cultures, (Braunschweig, Germany). All cell lines were maintained in culture in RPMI-1640 (Sigma-Aldrich Co. Ltd, Dorset, UK) containing 10% FCS (Invitrogen Ltd, Paisley, UK) as described previously.[5]

Detection of the antibody response to ALK

COS cells were transfected transiently with cDNA (1-2 μg) encoding for either ALK, NPM-ALK or the pcDNA vector alone[5] using the FuGENE 6 transfection reagent following manufacturer's instructions (Roche Diagnostics, Indianapolis, IN). Cytocentrifuge preparations of cells were made after 24 hr culture. These were then stained using patients' plasma in an indirect immunoperoxidase labeling procedure.[5] Briefly, the cytocentrifuge preparations were incubated with patients' plasma (diluted between 1:50 to 1:547,500) for 30 min. After washing in PBS, the slides were then incubated with HRP-rabbit anti-human IgG and the antigen:antibody complexes visualised using diaminobenzidine-tetrahydrochloride. Positive controls consisted of the inclusion of a plasma sample from an ALK-positive patient with a known level of antibodies to ALK to test the reproducibility of the dilution steps whereas transfectants were also labeled using monoclonal anti-ALK followed by HRP-conjugated goat anti-mouse Ig to confirm ALK expression. The cut-off for a positive result was taken as the highest dilution before the labeling of the NPM-ALK or ALK transfectants disappeared. Each experiment was repeated twice.

Preparation of PBMC

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Histopaque 1077, (Sigma-Aldrich Co. Ltd., Dorset, UK). Cytocentrifuge preparations were made and PBMC were adjusted to 2×10^6 cells/ml in complete RPMI (RPMI 1640, supplemented with 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, [Invitrogen Ltd, Paisley, UK] with 10% FCS). A total of 2×10^5 PBMC (in 200 μl) were added to each well of a 96-well flat-bottomed plate (Fisher Scientific Ltd, Leicester, UK), and incubated for 3–10 days with 1–10 μM of one of the following peptides: ALKa, ALKb, $FMP_{58–66}$ or HIV-1. Cultures containing 10 μg/ml of phytohemagglutinin (PHA) (Sigma-Aldrich Co. Ltd.) or no peptides were also set up. Twenty IU/ml of interleukin-2 (rIL-2) (Roche Diagnostics) and 25 ng/ml of recombinant human IL-7 (R&D Systems, Minneapolis, MN) were added on Days 2 and 5.

ELISpot assay

Briefly, cells were incubated for 18 hr in complete RPMI at 37°C in 5% CO_2 with one of the following: ALKa, ALKb, $FMP_{58–66}$, HIV-1, PHA or medium only. γ-IFN-releasing cells were then quantified by the standard γ-IFN release ELISpot assay according to the manufacturer instructions (Mabtech, Stockholm, Sweden). Spots were counted using an automated AID ELISPOT reader (Autoimmun-Diagnostika, Strassberg, Germany). Results were considered positive if the number of spots in the test wells were twice those present in the control wells and assays were excluded if there were more than 25 spots per well in the absence of peptide. In some experiments, CD8+ T cells were enriched from the PBMC using magnetic beads coated with anti-human CD8 antibody according to manufacturer's instructions (Dynabeads, Dynal, Oslo, Norway), before assay. In other experiments, the anti-HLA-A2*0201 antibody (BB7.2) was added at a concentration of 10 μg/ml for 1 hr to block the γ-IFN CTL release.

Generation of CTL lines

PBMC were cultured in RPMI 1640-10% FCS at a density of 2×10^6 cells/ml and stimulated with 10 μM of ALKa or ALKb peptide. After 72 hr, an equal volume of RPMI 1640-10% FCS containing 50 IU of rIL-2/ml was added to each culture well and every 3 days thereafter half of the medium was removed and replaced with fresh medium. Lymphocytes were then restimulated

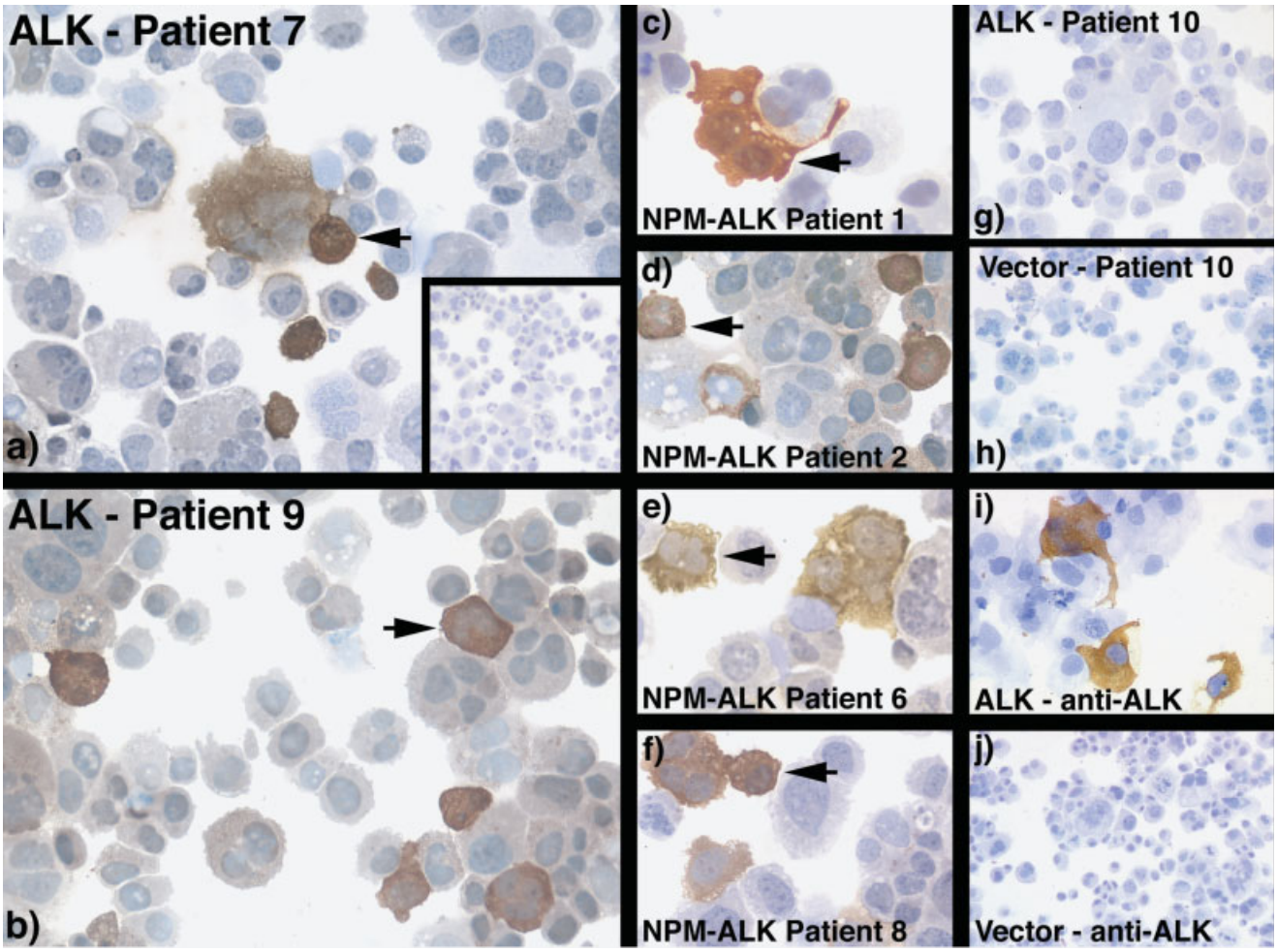

FIGURE 1 – Examples of immunoperoxidase labeling of ALK or NPM-ALK COS transfectants using patients' sera. Plasma from Patients 7 (*a*) and 9 (*b*) (diluted 1:2,250) strongly label transfected COS cells expressing ALK protein (arrow). In contrast, plasma from neither patient labeled COS cells transfected with the ''empty'' *pcDNA3* vector only (example of Patient 7 shown in *inset*). (*c–f*) Immunolabeling of NPM-ALK transfectants using plasma from Patients 1, 2, 6 and 8 (arrow). Plasma from these patients also labeled ALK transfectants thus confirming the presence of circulating antibodies to ALK in these patients. No comparable labeling was seen of COS cells transfected with vector only. (*g,h*) Examples of the lack of staining observed of ALK or vector only transfected COS cells using plasma from patients with ALK-negative ALCL (Patient 10). The patterns of labeling of ALK and vector-only transfectants using the positive control anti-ALK monoclonal antibody ALK1 are shown in (*i,j*), respectively.

weekly in the same way. After 28 days, some cells were harvested and used either in an ELISpot assay or to prepare cytocentrifuge preparations for immunophenotyping. These slides were then stained for CD3, CD4, CD8, CD20, CD22, CD56, CD68 and γ-IFN using an indirect immunoperoxidase technique as previously described. The remaining cells were maintained in culture for another 4 weeks, before being tested in the cytolytic assays.

Preparation of MVA-ALK

The entire intracytoplasmic region of ALK present in the NPM-ALK fusion protein was sub-cloned into the vaccinia vector pSCII30R.2 (kindly provided by Prof A. Townsend, Oxford, UK). Plasmid 5′ l520 *NPM/ALK* (kindly provided by Dr. S. Morris, Memphis, TN) was digested with *Xho*I and then end-filled with the Klenow fragment of DNA polymerase. The cDNA was then digested with *Nco*I and the 1.5 kb partial *ALK* cDNA fragment was sub-cloned into the *Nco*I and *Stu*I sites of the vaccinia virus insertion vector pSCII30R.2 to create the plasmid pKP1. The additional 5′ end of the intracellular *ALK* sequence was amplified by PCR using the primers 5′-ccccccatggtgtaccgccggaagcaccag and 5′-ggcgccatggcccagaccccgaatgagggt and plasmid 5′ λ 520 *NPM/ALK* as the template. The 177 bp PCR product was sub-cloned into pGEM-T Easy (Promega) and validated by DNA sequencing. The PCR product was then excised using *Nco*I and inserted into the *Nco*I site of pKP1 to create plasmid pKP2. Plasmid pKP2 was used to recombine the intracytoplasmic *ALK* cDNA into the thymidine kinase gene of MVA using previously described techniques[24] to produce MVA-ALK. The recombinant MVA was grown in bulk and sucrose-cushion purified. MVA containing β-galactosidase through insertion of the empty vector pSCII30R.2 into the thymidine kinase gene (MVA-βGal) was used as a control.

JY cells were incubated at 37°C with 10^6 plaque forming units (p.f.u.) of MVA-ALK or MVA-βGal (control) at a ratio of 2 p.f.u./cell. After 90 min, the cells were washed and re-suspended at 5×10^3 cells/well for the cytolytic assay. Some cells were used to make cytocentrifuge preparations whereas the majority of the infected cells were used as targets in a cytolytic assay. The expression of ALK protein in JY cells infected by MVA-ALK but not in cells infected with MVA-βGAL was confirmed in an indirect immunolabeling technique on cytocentrifuge preparations using the ALK1 monoclonal antibody.

TABLE II – SUMMARY OF THE CTL AND B CELL IMMUNE RESPONSE TO ALK IN ALCL PATIENTS AND NORMAL DONORS[1]

Patient no.	MHC subtype	Titre of circulating anti-ALK[2]	γ-IFN response to peptides					
			ALKa	ALKb	HIV	None	FMP	PHA
ALK+ve								
1	A*0201	1:6,750	158 ± 8	212 ± 35	14 ± 5	12 ± 4	120 ± 12	280 ± 42
2	A*0201	1:6,750	34 ± 9	51 ± 18	11 ± 2	7 ± 3	72 ± 24	128 ± 28
3	A*0201	1:250	10 ± 3	12 ± 3	3 ± 2	2 ± 1	42 ± 8	42 ± 10
4	A*0201	1:2,250	22 ± 8	24 ± 4	8 ± 2	10 ± 4	8 ± 4	108 ± 16
5	A*0201	1:250	16 ± 5	26 ± 7	6 ± 1	8 ± 3	22 ± 5	124 ± 12
6	A*0201	1:20,250	24 ± 2	28 ± 7	8 ± 2	5 ± 3	34 ± 10	134 ± 18
7	A*0201	1:6,750	13 ± 1	15 ± 3	4 ± 1	3 ± 1	10 ± 2	116 ± 12
8	A*0203	1:547,500	7 ± 2	10 ± 4	10 ± 2	12 ± 5	3 ± 2	32 ± 4
9	A*0203	1:6,750	10 ± 4	12 ± 2	9 ± 2	10 ± 3	12 ± 3	146 ± 12
ALK−ve								
10	ND	—	5 ± 1	4 ± 1	5 ± 2	3 ± 2	8 ± 2	16 ± 4
11	A*0201	—	8 ± 1	8 ± 2	7 ± 3	7 ± 1	24 ± 8	56 ± 12
Normal 1	A*0201	—	3 ± 1	4 ± 2	1 ± 1	2 ± 1	21 ± 2	34 ± 4
Normal 2	A*0201	—	5 ± 3	4 ± 1	2 ± 1	4 ± 2	40 ± 3	248 ± 32
Normal 3	A*0201	—	2 ± 1	3 ± 1	3 ± 2	2 ± 1	12 ± 6	8 ± 2
Normal 4	A*0201	—	10 ± 4	12 ± 3	14 ± 1	13 ± 5	10 ± 5	38 ± 6
Normal 5	ND	—	7 ± 6	5 ± 4	3 ± 2	5 ± 3	5 ± 1	56 ± 12
Normal 6	ND	—	13 ± 7	12 ± 3	8 ± 3	11 ± 5	9 ± 3	12 ± 2

[1]A significant γ-IFN response to ALKa and ALKb peptides with respect to the HIV peptide and medium only controls were found only in the 7 patients with ALK-positive ALCL who were HLA-A2*0201-positive (Patients 1-7). No significant response was detected in the HLA-A2*0201-negative patients with ALK-positive ALCL (Patients 8,9) even though circulating antibodies to ALK were present in these subjects. PBMC from the ALK-negative ALCL patients or healthy controls did not recognise the ALK peptides. All cultures were carried out in triplicate and peptides were used at 10 μM. The results ± SD are from triplicate ELISpot cultures. The SD was calculated using standard techniques. –[2]Highest dilution before labeling of NPM-ALK or ALK transfectants disappeared.

Cytolytic assay

The ability of CTL generated from PBMC of patients to lyse target cells was monitored in a conventional ^{51}Cr-labeling release assay. Target cells consisted of (*i*) T2 cells pulsed with either 10 μM of ALKa, ALKb or the control HIV-1 peptide for 90 min during radio-labeling with 100 μCi ^{51}Cr, (*ii*) ^{51}Cr-labeled SUDHL-1 and KARPAS-299 ALCL-derived cell lines or (*iii*) the ^{51}Cr-labeled JY lymphoblastoid cells infected with either MVA-ALK or MVA-βGal. After washing, the target cells were added to the CTL cell lines (at an effector target ratio of 3:1 to 5:1) in 96-well microplates. The plates were incubated for 4 hr at 37°C in a humidified atmosphere with 5% CO_2. Maximum chromium release was determined by the addition of 10% Triton-X to the radiolabeled cells and spontaneous release was assessed by adding complete medium (RPMI 1640 + 10% FCS) to the target cells. The supernatant was harvested and counted in a gamma counter (Beckmann, Heidelberg, Germany). The percentage of specific lysis was calculated as the following: (experimental cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm) × 100.

Statistical analysis

The Student's *t*-test was used to analyse the results obtained in the ELISpot and cytolytic assays.

Results

T2 binding assay

ALKa and ALKb peptides were able to bind efficiently to the HLA-A*0201 molecule on the surface of T2 cells using flow cytometric methods (not shown). In contrast, ALKc peptide demonstrated no binding to T2 cells and was, therefore, not included in further experiments.

Presence of the antibody response to ALK

Circulating IgG antibodies that recognised the ALK protein expressed by ALK and NPM-ALK transfectants were recorded in the sera of all ALK-positive ALCL patients regardless of their MHC Class 1 subtype (Patients 1–9). Examples of the results obtained are shown in Figure 1. The titres varied between 1:250 and 1:547,500. No such antibodies could be observed in the 2 ALK-negative ALCL patients or in the 6 healthy donors. The results are summarized in Table II.

γ-IFN release assay

Results demonstrated that the optimal γ-IFN responses were obtained from ALK-positive patients (Patients 1–4) after 3 days of culture with peptides whereas the best response for Patients 5–7 was observed after 7 days. No significant changes in the γ-IFN release by PBMC from the two ALK-negative ALCL and 6 normal controls were detected during 3–7 days of culture and the results described here for these patients were those obtained after 3 days culture. Significant γ-IFN release to the ALKa and ALKb peptides compared to the response obtained from the HIV and medium only controls ($p < 0.05$) was detected in all ALK-positive, HLA-A2*0201-positive patients (Table II). The responses of PBMC from Patients 1 and 2 to the ALK peptides were particularly vigorous. In contrast, no significant γ-IFN responses to the ALK peptides were detected in PBMC from the 2 ALK-positive ALCL patients that were HLA-A*0201–negative, the 2 ALK-negative ALCL patients or the 6 normal controls. No significant response was identified to the HIV irrelevant peptide in any of the subjects (Table II).

Generation of T-cell lines specific for ALKa and ALKb peptides

To further characterize the functional capacity of the ALK-reactive cells, cell lines recognizing the ALK peptides were generated from PBMC of Patients 1 and 2 (this was made possible by the availability of larger volumes of blood samples collected from these patients). The cell lines were re-stimulated weekly with ALKa and ALKb peptides and rIL-2. After 6 rounds of stimulation, these cultures were tested for their γ-IFN secreting activity in response to both ALK and control peptides in an ELISpot assay. The cell lines showed an increased γ-IFN response to the ALK peptides compared to that obtained after short-term culture of PBMC (Fig. 2*a*). The CTL demonstrated a minimum of 30% increase in γ -IFN release from the CTL lines compared to the PBMC. Immunophenotyping of the responding cell population demonstrated greater than 95% of the cells to express CD3 and CD8 and less than 1% of the cells were CD56-positive.

Depletion of the CD8-positive cell population using CD8-coated magnetic beads (Dynal) (Fig. 2*b*) or the addition of BB7.2

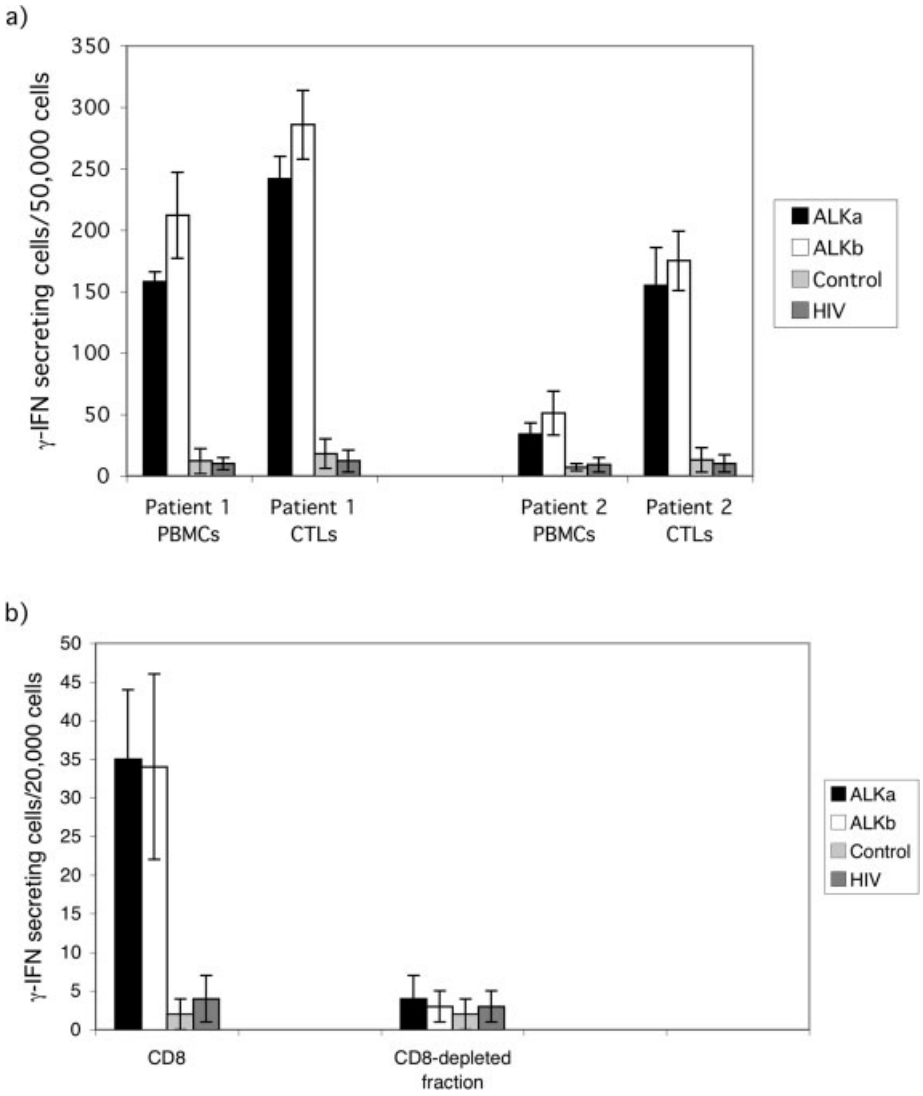

Figure 2 – PBMC and CTL line responses of ALK-positive ALCL patients 1 and 2 to the ALK peptides. (*a*) PBMC were maintained in culture and stimulated with ALKa and ALKb peptides for 72 hr. CTL lines were raised from 2 ALK-positive patients (Patients 1 and 2) after 6 weeks of repeated stimulation with ALK peptides. Cells were then tested in an overnight ELISpot assay for a γ-IFN response to the ALK peptides. The CTL lines raised demonstrated a significantly increased γ-IFN response to both ALKa and ALKb peptides when compared to PBMC cultures from the same individuals ($p < 0.05$). No significant response to the control HIV peptide was observed in either patient. (*b*) CTL lines from Patient 1 were enriched for CD8-positive T cells using a magnetic depletion step. After an overnight incubation with the ALK peptides in an ELISpot assay, the CD8-positive cell fraction responded to both ALKa and ALKb peptides. No significant response γ-IFN to the peptides was obtained using cells from the CD8-depleted cell fraction. The results are the mean ± SD and were obtained from triplicate ELISpot cultures.

antibody directed against HLA-A*0201 (not shown) abrogated the γ-IFN release completely.

Functional activity of CTL cell lines

As shown in Figure 3*a*, CTL lines specific for ALKa and ALKb obtained from Patients 1 and 2 demonstrated significant peptide-specific killing of the ALK peptide-pulsed T2 cells ($p < 0.001$). No significant lysis of T2 cells loaded with the irrelevant control peptide (HIV-1) was observed. The ALKb-generated CTL demonstrated a greater cytolytic effect than the ALKa- stimulated cells, with maximal lysis ranging between 71 ± 8% to 77 ± 12% (for Patient 1) and 51 ± 16% to 56 ± 18% (for Patient 2). ALKa-specific CTL killing ranged between 58 ± 10% to 65 ± 14 % (for Patient 1) and 45 ± 9% to 52 ± 11% (for Patient 2).

It is possible that the results obtained above could arise from the presence of CTL with high affinity for the ALKa and ALKb peptides but that they may not recognise cells expressing naturally occurring ALK peptides that were processed endogenously. Therefore, we examined further the ability of the ALKa and ALKb peptide-specific CTL to lyse ALK-positive ALCL tumour cells. ALKa-peptide stimulated CTL efficiently lysed the HLA-A*0201-positive SUDHL-1 target cells with maximal lysis ranging between 71 ± 12% (Patient 1) and 63 ± 10% (Patient 2). ALKb-specific CTL also lysed SUDHL-1 cells with maximal killing between 48 ± 10% (Patient 1) and 57 ± 18% (Patient 2) (Fig. 3*b*). No significant cytolytic activity against the HLA-A*0201-negative ALK-positive KARPAS-299 cell line could be detected with the CTL obtained from either patient thus demonstrating the MHC Class I dependence of the lytic response (Fig. 3*b*).

In addition to ALK-positive ALCL, the ALK protein can also be aberrantly expressed in a number of tumours such as neuroblastomas and inflammatory myofibroblastic tumours. Recognition of ALK-derived peptides by specific CTL could, therefore, mediate killing of other cancer types. To investigate this possibility, the ALK-negative JY cell line, known to process and efficiently present immunogenic antigens in the context of MHC Class I molecule, was infected with either MVA-ALK or MVA-βGAL and was used as a target in a ^{51}Cr-labeled release assay. The results from an experiment are shown in Figure 3*c*. Even at an effector: target ratio of 3:1, CTL from Patients 1 and 2 were able to lyse the MVA-ALK infected target cells effectively. No significant lysis was observed of the cells infected with MVA-βGal only.

Discussion

Although we have reported previously an antibody response to ALK in patients with ALK-positive ALCL,[5] this study is the first description of the presence of a CTL response in patients with ALK-positive ALCL. This response was limited to patients with

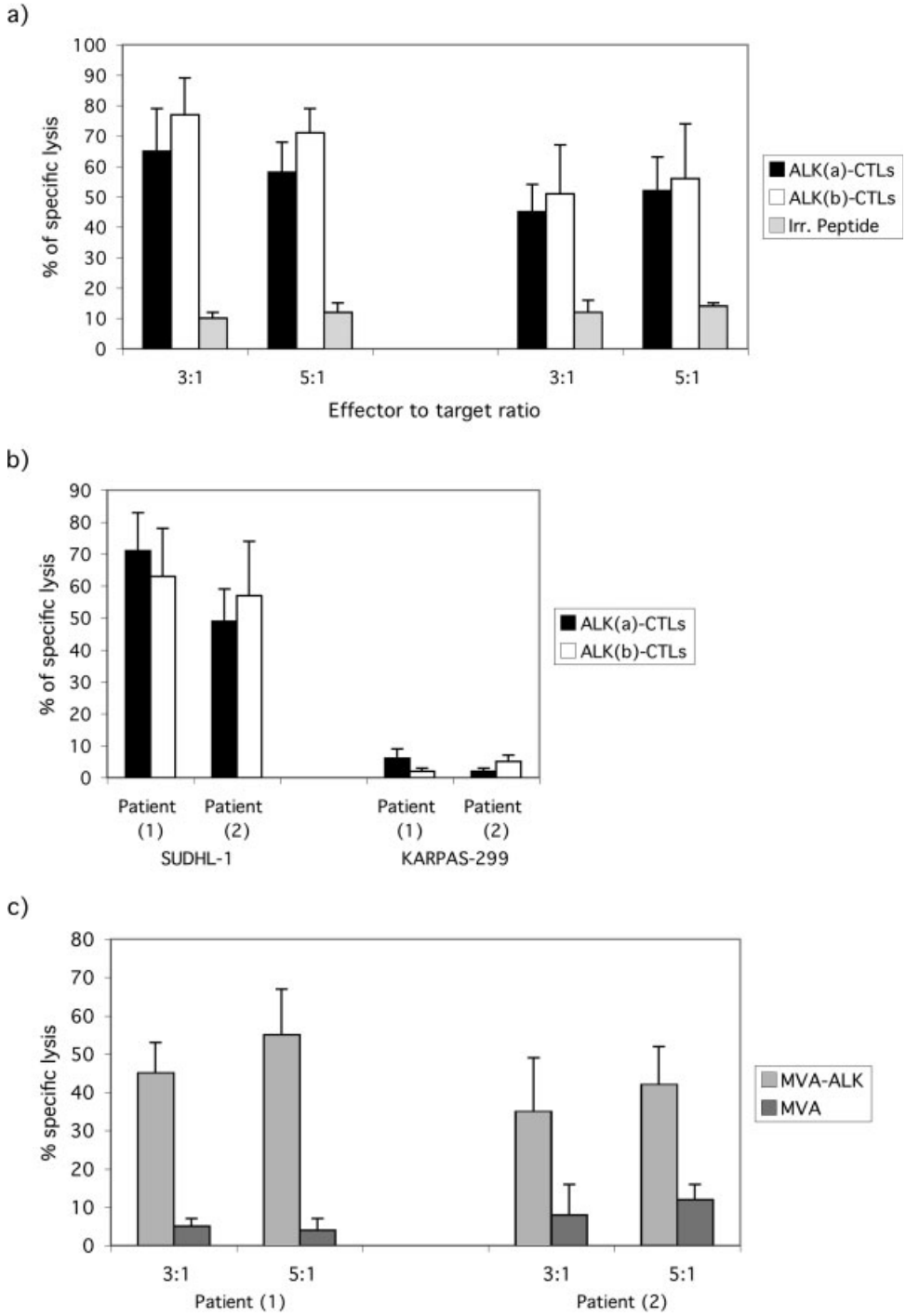

FIGURE 3 – Cytolytic activity of ALK-specific CTL derived from ALK-positive ALCL patients. The functional activity of the CTL raised from the 2 ALK-positive ALCL Patients 1 and 2 against ALKa and ALKb peptides were tested in a standard 4-hr Cr-release assay on (*a*) 10 μM peptide pulsed T2 cells, (*b*) the HLA-A*0201-positive SUDHL-1 and HLA-A3*0301-positive Karpas 299 ALCL-derived cell lines (both NPM-ALK-positive) and (*c*) MVA-ALK and MVA-control infected JY cells. In (*a*) significant lysis was observed in cultures containing T2 cells pulsed with either ALKa or ALKb peptides. This was in contrast to the lack of significant response seen with the HIV peptide pulsed T2 cells. (*b*) Lysis of the NPM-ALK- positive cell lines by CTL (E:T ratio of 3:1) raised against the ALKa and ALKb peptides from both patients was observed only for the HLA-A*0201-positive SUDHL-1 cells. (*c*) The CTL from both patients lysed the MVA-ALK infected JY cells but not the MVA-βGal JY cells that did not express ALK. The results are the mean ± SD for triplicate wells.

ALK-positive ALCL suggesting that it was a tumour-driven immune response. Although the presence of an antibody response to TAA has been correlated with the existence of a CTL-mediated immune response in tumours such as melanomas[8] and B cell lymphomas,[25] there are reports of CTL responses in the absence of an antibody response.[8,26] We were, however, able to confirm the presence of both a B cell mediated and MHC Class 1 restricted response to ALK in ALK-positive patients. This is important because there is increasing evidence that effective tumour immunity requires interplay between the humoral and cellular arms of the immune response.[27]

Our demonstration of a γ-IFN response to ALK peptides by PBMC from ALK-positive, HLA-A2*0201-positive patients after only short-term culture suggests that ALK peptide-specific CTL precursors were present in these individuals. A similar situation was described for HER-2/neu.[28] Although the ability of ALK peptides to be recognised by CTL in normal human subjects and after immunisation in murine studies has been reported previously, these authors had to maintain PBMC from normal subjects in long-term culture in the presence of dendritic cells before a CTL response could be detected.[17] Of interest was our finding that, whereas patients with ALK-positive ALCL were able to recognise both the ALKa and ALKb peptides, Passoni et al. found no evidence of CTL response to ALKb in normal subjects.[17] It is noteworthy that those patients (Patients 1 and 2), who displayed the strongest γ-IFN responses to the ALK peptides, were in remission

during the course of this study. The persistence of the T cell responses in a number of patients in remission suggests the presence of a pool of memory T cells to the ALK protein that could be involved in protective immunity.

Although results from the ELISpot technique have shown close correlation with those obtained from cytolytic studies,[29] the possibility remains that the γ-IFN response and lysis reported here might have been limited only to peptides synthesized exogenously. Peptides processed endogenously by the tumour cells may not be recognised due to the low concentrations of epitopes present on the surface of tumour cells or, more likely, to inefficient processing of antigen by the tumour cells.[30,31] We were, however, also able to demonstrate that the CTL lines raised here against the ALK peptides were capable of recognising ALK epitopes presented as naturally processed peptides on ALK-positive ALCL-derived tumour cells in an MHC Class I restricted manner. Indeed, these CTL demonstrated cytolytic properties comparable to those obtained in other studies where much higher effector:target ratios were necessary. Furthermore, results using MVA-ALK demonstrated that the cytolytic activity of the CTL generated was not restricted to ALK-positive ALCL. Our results and those of Passoni *et al*.,[17] therefore provide further evidence that CTL activity may be of relevance in other ALK-positive tumours such as neuroblastoma,[32] inflammatory myofibroblastic tumours,[33] rhabdomyosarcoma,[34] ALK-positive B cell lymphomas[35] and various other tumours.[36]

Reliable assays are needed to identify those high-risk patients at time of diagnosis and to monitor the effectiveness of treatment. The ELISpot technique has been used previously to study the presence and induction of T cell responses to tumour antigens.[8,37] Our ELISpot experiments using short-term culture may, therefore, constitute a simple and effective means to monitor the immune response at time of diagnosis as well as during remission to provide information on prognostic outcome in ALK-positive ALCL. Such methodology uses only a small number of cells, an important consideration when only limited samples of blood can be obtained from subjects such as pediatric patients. Furthermore, the production of tetramers incorporating the ALK peptides is being investigated and could be used to monitor the presence of circulating anti-ALK CTL.[38]

Studies in mice and *in vitro* studies in humans have demonstrated the powerful potential of immunotherapy as an alternative form of treatment.[39–41] Clinical trials in humans have, however, demonstrated more limited success. There are a variety of reasons that may account for this. For example, patients entered into the new therapy studies are in an advanced stage of disease and may therefore be immunocompromised. Second, the tumour cells may evade the immune system through a variety of mechanisms.[42–46] ALK expression is, however, considered to be a primary oncogenic event and it would be predicted that the lack of this protein would not be compatible with the continued growth of ALK-positive ALCL. The restricted expression in normal tissues also suggests the absence of peripheral tolerance. Our studies and those of Borisch *et al*.[6] reporting ALK to be highly immunogenic in patients with ALK-positive ALCL suggest that ALK protein represents an excellent target for immunotherapy with limited toxicity for healthy tissue. Previously, MVA has been used for vaccination purposes due to its safety, even in immunocompromised hosts.[47–52] We have taken the first steps to study MVA in ALK-positive ALCL and our data from the CTL cytolytic study showing the recognition of MVA-ALK infected JY cells raises the possibility of using MVA containing ALK epitopes as a potential vaccination vector for ALK-expressing tumours.

The presence of IgG antibody responses to TAA detected in the sera of melanoma patients is indicative of the presence of cognate CD4 T-cell helper responses.[53] The presence of circulating IgG antibodies to ALK in our current study thus provides evidence for the existence of ALK-specific CD4 T-cell helper cells. Studies have demonstrated the potential of using epitopes predicted to bind to both MHC Class I and II to obtain more effective immune responses[54,55] and work is in progress to investigate this possibility.

In conclusion, we have demonstrated not only a B cell response to ALK but, for the first time, the presence of circulating CTL specific for 2 ALK peptides in a number of ALK-positive ALCL patients. The CTL response was shown to be MHC Class I restricted and was limited to patients with ALK-positive ALCL. These results open up the possibility of using ALK as a therapeutic target for patients with ALK-positive ALCL and also for patients with other ALK-expressing tumours. These results pave the way for further detailed *in vitro* and *in vivo* investigations on the CTL and CD4 T cell responses to the ALK protein.

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