Cancer Immunol Immunother (2004) 53: 1119–1126
DOI 10.1007/s00262-004-0558-0

ORIGINAL ARTICLE

**Christian Kersten · Jan Delabie · Gustav Gaudernack
Erlend B. Smeland · Alexander Fosså**

Analysis of the autoantibody repertoire in Burkitt's lymphoma patients: frequent response against the transcription factor ATF-2

Received: 7 April 2004 / Accepted: 22 April 2004 / Published online: 4 June 2004

Abstract In the last few years, serological identification of tumour-associated antigens (TAAs) by recombinant cDNA expression cloning (SEREX) has enabled the mapping of humoral immune responses against TAAs in various types of cancer. The present paper describes the application of SEREX to Burkitt's lymphoma (BL), a malignancy not previously characterized by SEREX. By using a cDNA library from a BL cell line that does not express IgG, technical difficulties related to background immunoglobulin clones were overcome. Screening with sera from three BL patients revealed immunoreactivity against seven different gene products, six of which represent known human genes. Five proteins had previously been characterized by SEREX in other malignancies or identified as targets of autoantibodies in autoimmune disease. Seroreactivity against ATF-2, a member of the AP-1 transcription factor family, was validated by enzyme-linked immunosorbent assay (ELISA) and Western blot analysis using recombinant ATF-2 protein. Autoantibody responses against ATF-2 were detected by ELISA in 6 of 8 BL patients, compared with 6 of 13 patients with T-cell non-Hodgkin's lymphoma (T-NHL), 5 of 23 patients with follicular lymphoma and 2 of 27 diffuse large B-cell lymphoma patients. In contrast, reactivity was found in only 1 of 50 healthy volunteers. Next, we showed by immunohistochemistry that the activated form of ATF2 (ATF-2_{pp}) was highly expressed in six different BL samples. We conclude that the SEREX approach with a B-cell cDNA source is applicable in NHL. Furthermore, we identified genes with possible involvement in the pathogenesis of BL using this technique.

C. Kersten (✉) · G. Gaudernack · E. B. Smeland · A. Fosså
Department of Immunology, The Norwegian Radium Hospital, Montebello, 0310 Oslo, Norway
E-mail: christik@klinmed.uio.no
Tel.: +47-22-934000
Fax: +47-22-500730

J. Delabie
Department of Pathology, The Norwegian Radium Hospital, Montebello, 0310 Oslo, Norway

Keywords ATF-2 · Burkitt's lymphoma · Non-Hodgkin's lymphoma · SEREX · Tumour immunology · Tumour-associated antigen

Introduction

Development of immunotherapeutic strategies for cancer is greatly aided by the identification of TAAs, capable of eliciting long-lasting immune response in patients. While most immunotherapies for cancer have focused on generating tumour-specific $CD8^+$ cytotoxic T lymphocytes, a mounting body of evidence suggests that stimulation of antitumour $CD4^+$ T cells may be required for highly effective therapy [37]. In that respect, there is a growing recognition that some tumour antigens elicit a strong integrated immune response involving both cellular and humoral immunity. Of particular interest are the cancer germline antigens (CGAs), a family of proteins expressed in a variable subset of patients with a given tumour entity and in testicular germ cells, but not, or at low levels, in other normal tissue [30, 45]. Humoral immune responses against TAAs have been mapped with the help of serological cDNA expression cloning (SEREX) in various types of cancer [11, 14, 31, 43, 44] including haematological neoplastic conditions such as leukaemia, Hodgkin's lymphoma and non-Hodgkin's lymphoma (NHL) [12, 21, 28, 40]. Some previous studies have reported technical difficulties applying SEREX to B-cell NHL [28]. Most B-NHL cells synthesize immunoglobulins, regularly of the IgG class, and together with antibodies from tumour-infiltrating normal B cells, this results in a high background of immunoglobulin clones during screening.

The present paper describes the application of SEREX to Burkitt's lymphoma (BL), a malignancy not previously characterized by SEREX. BL is a highly aggressive B-NHL entity that includes endemic, sporadic and human immunodeficiency virus (HIV)–associated subtypes [4]. All subtypes are characterized by chromosomal rearrangements involving the c-*myc* proto-oncogene,

leading to its deregulated overexpression. In endemic BL, tumour cells are latently infected with Epstein-Barr virus (EBV). In contrast, only a subset of sporadic and HIV-associated BLs are EBV-associated [22]. BL was chosen for SEREX analysis, since BL is quite common in immunosuppressed patients, and therefore immunosurveillance possibly plays a role in the pathogenesis of this disease. Furthermore, by using a cDNA library from a BL cell line that does not express IgG, but IgM, the described technical difficulties related to background immunoglobulin clones should be reduced.

By screening this library with pooled sera from three BL patients, seven different antigens were identified. Seroreactivity against the transcription factor ATF-2 was further validated, and its serological profile in patients with different NHL entities, as well as immunohistochemical studies, indicated a possibly important pathophysiological role in BL. EBV and v-*myc* are known to activate ATF-2 and the present study indicates a high expression of the activated ATF-2 in BL and demonstrates frequent autoantibody responses against this molecule in BL.

Material and methods

Sera, peripheral blood leukocytes, tumour and tonsil tissue and cell lines

Tumour biopsy samples, tonsil tissue and sera were obtained during routine clinical procedures with formal agreement by the patients and with approval by the regional ethics committee. Sera from three patients with histologically and cytogenetically verified BL were available for screening. Sera from 68 other patients with different NHL entities and 50 healthy volunteers were provided by the Central Laboratory, the Norwegian Radium Hospital (NRH), Oslo, Norway, and by the Blood Bank, Ullevål University Hospital. Formalin-fixed paraffin-embedded biopsy samples of six BL were obtained from the Department of Pathology, NRH. In all patients, the lymphoma diagnosis was established according to the WHO classification. The following cell lines from human lymphoid malignancies were used and maintained in RPMI 1640 supplemented with 10% foetal bovine serum, 100 U/ml penicillin G, and 100 U/ml of streptomycin sulphate: pre-B cell line Reh (ATCC CRL 8286), pro-B cell lines BV173 (DSZM ACC20) and Tom-1 [38], EBV-negative BL cell lines Ramos (ECACC 85030802) and Bjab (Dr G. Moldenhauer, University of Heidelberg, Heidelberg, Germany), EBV-positive BL cell lines Rael [27] and Namalva (ECACC 87060801) and human B-cell lymphoma U-698 (DSZM ACC 4).

cDNA library construction and immunoscreening

Total RNA was extracted from the cell line Ramos using the acid guanidine thiocyanate method and mRNA isolated with oligo-dT-Dynabeads (Dynal, Oslo, Norway) [15]. The cDNA library was constructed in the λZAP expression vector (Stratagene, LaJolla, CA, USA) with 1.5×10^6 primary recombinant clones amplified once before screening. Sera from three BL patients were used for immunoscreening as described previously [15]. In brief, sera were diluted 1:20 and extensively absorbed against *Escherichia coli* XL-1MRF'Blue cells transfected with wild-type λZAP phage and further diluted to a final titre of each serum of 1:200. Recombinant plaques (5×10^5) were screened with this dilution. After washing, filters were incubated with alkaline phosphatase–conjugated goat antihuman IgG, and immunoreactive plaques were visualised by incubation with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium. Positive plaques were subcloned until homogeneity, converted to plasmid clones in pBluescript using the Ex-Assist helper phage (Stratagene) and sequenced by means of BigDye Terminator Cycle Sequencing (Perkin Elmer Applied Biosystems, Warrington, UK) on an ABI Prism 310 Genetic Analyser (Perkin Elmer). DNA and deduced protein sequences were analysed using DNASIS for Windows 2.1 (Hitachi Software Engineering America, San Bruno, CA, USA) and compared with sequences in GeneBank and other public databases using BLAST, and with entries of the SEREX database (http://www.licr.org/SEREX.html). Searches within the SEREX database were done both for identical sequences and functionally related genes.

Expression analysis of ATF-2 in tissues and cell lines

Immunohistochemistry

Five-micrometre sections of formalin-fixed, paraffin-embedded tissue from six BL and five normal tonsils were analysed for ATF-2 expression. The deparaffinized sections were microwaved eight times in 0.01-M sodium citrate, pH 6.0, for 5 min to unmask epitopes. Immunostaining was performed using the EnVision + Kit (DAKO, Glostrup, Denmark), according to the manufacturer's protocol. Antibodies anti-ATF-2 (reacting with ATF-2 protein irrespective of the level of phosphorylation) and anti-phospho-ATF-2 (Thr69/71) (reacting only with ATF-2 phosphorylated at Thr69 and Thr71) (all, Cell Signalling Technology, Beverly, MA, USA) were diluted 1:25 and incubated overnight at 4°C. Dilutions of antibodies were made in phosphate-buffered saline (PBS), pH 7.4, 10 mg/ml BSA containing 1 mg/ml NaN_3. Sections were counterstained with hematoxylin, dehydrated and mounted in Eukitt (Kindler, Freiburg, Germany).

Immunoblotting

Cells ($3–5\times10^6$) from cell lines were lysed in sample buffer (glycerol 10%, β-mercaptoethanol 5%, 0.0625 M-Tris-HCl [pH 6.8], sodium dodecyl sulphate [SDS] 2.5% w/vol). Total protein (30 μg) from each sample was run

through SDS/polyacrylamide gel electrophoresis (SDS/PAGE) and blotted onto nitrocellulose filters. Blocking, washing and incubation of the filters with antibodies anti-ATF-2 (Cell Signalling Technology) and anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were done according to the manufacturers' protocols. After washing with PBS / 1 mg / ml Tween 20 (PBS-T), the filters were incubated with horseradish peroxidase (HRP) coupled to goat antirabbit IgG (DAKO) for 60 min at room temperature (RT). Enzyme activity was visualised by the enhanced chemiluminescence system ECL + Plus (Amersham, Buckinghamshire, UK).

Northern analysis

Total RNA was isolated from cell lines as above. For Northern analysis of ATF-2 mRNA, 4-μg total RNA was separated by formaldehyde/agarose gel electrophoresis and transferred to nitrocellulose membranes. Hybridisation was performed with a ^{32}P-labelled fragment covering the complete open reading frame (ORF) of the ATF-2 gene. The quality of RNA was confirmed by hybridisation to a ^{32}P-labelled fragment of β-actin cDNA [15]. Quantification was performed on a Storm 840 Phosphorimager using ImageQuant 5.0 (Molecular Dynamics, Krefeld, Germany).

Serological analysis for anti-ATF-2 antibodies

An enzyme-linked immunosorbent assay (ELISA) was established to quantify IgG reactivity in serum against recombinant ATF-2. A PCR fragment equipped with appropriate restriction sites at the 5′ and 3′ ends and covering the complete ATF-2 ORF was cloned into the pQE30 vector (Qiagen, Hildesheim, Germany). The protein was produced in *E.coli* M15 cells (Qiagen) and purified using 8-M urea as the denaturing agent according to the manufacturer's protocol, followed by dialysis in PBS. MaxiSorp plates (Nunc, Rochester, MN, USA) were coated overnight at 4°C with 50-μl ATF-2 protein in PBS (μg/ml). Plates were washed (Scan washer 400; Skatron Instruments, Norway) and blocked with PBS-T/50 mg/ml milk powder (MP). Sera were preincubated at 1:100 in PBS/10 mg/ml MP supplemented with 0.25 μg/μl *E.coli* M15 lysate for 2 h at RT, and 50 μl was subsequently added to each well for 2 h incubation at RT. Plates were washed again and incubated 1 h at RT with HRP-conjugated goat antihuman IgG (Sigma-Aldrich, Steinheim, Germany) diluted 1:5,000 in PBS-T/10 mg/ml MP. After washing, HRP reactivity was visualised using 1,2-phenylenediamine dihydrochloride (OPD) tablets (DAKO), and absorption at 492 nm read using a MultiscanEX photometer (Labsystems, Helsinki, Finland). All sera were tested in duplicate. Selected sera were retested in a competition assay, with preincubation of serum as above, but with addition of soluble recombinant ATF-2 or a similarly produced irrelevant protein (p352) [14].

Reduction of OD_{492} reading by 30% or more for ATF-2 competition compared with irrelevant protein was considered significant. Selected sera were tested by Western blot analysis, performed essentially as described above, but applying 0.2-μg recombinant ATF-2 and 0.2-μg p352 per lane in SDS/PAGE. Sera were diluted 1:100, and HRP-conjugated goat antihuman IgG (Sigma-Aldrich) diluted 1:5,000 in PBS-T/1% w/vol MP was used as secondary antibody.

Results

Immunoscreening of the Ramos cDNA library

Approximately 5×10^5 clones of the Ramos cDNA library were screened with pooled sera from three BL patients. Twelve independent clones were identified and sequenced. Sequence analysis and BLAST searches in publicly available databases revealed that these 12 clones were transcribed from seven different genomic loci, of which six are functionally characterized human genes (Table 1). Four different clones encoded the activation transcription factor ATF-2. All four clones covered at least 90% of the ORF of the ATF-2 gene, and the sequence of all clones was identical to that previously reported for ATF-2 (data not shown). ATF-2 is a member of the basic region leucine zipper (bZIP) transcription factor family, and is a component of the AP-1 complex [49].

Three independent clones encoded nonmetastatic protein 23-H2 (Nm23-H2), a multifunctional protein with aberrant, and partly augmented expression, in human tumours [18, 32]. Furthermore, Nm23-H2 has been shown to be involved in the deregulation of the translocated c-*myc* allele in BL cells [24]. Proteins encoded by one clone each included a component of the small nuclear ribonucleoprotein (snRNP) U2, ribosomal protein S2, triosephosphate isomerase (TPI) and *S*-adenosine-homocysteine hydrolase (AHCY). TPI is a ubiquitously expressed enzyme of the glycolytic pathway, shown to be overexpressed in bladder, colon and lung carcinomas [6, 10, 35]. AHCY is a key enzyme of transmethylation, a mechanism also involved in controlling the replication of several viruses such as EBV [7, 16, 46]. The clone identical to EST AA195197 encoded a putative protein with significant homology to retroviral reverse transcriptases. The human genome contains a high number of elements with identical or near identical nucleotide sequences to this clone. This suggests that the clone is derived from a transcribed endogenous retroviral sequence within the human genome.

Searches for identical clones in the public part of the SEREX database

A search of SEREX (http://www.licr.org/SEREX.html) revealed that five of the identified antigens have been previously identified. Of note, most of the SEREX-defined antigens do not serve exclusively as TAAs. Many

Table 1 Identity of clones isolated by serological screening

Gene identified/ accession no.	Positive clones (*n*)	Functional significance	Expression pattern	Identical and related entries in the SEREX database
Activation transcription factor 2/X15875	4	Oncogenic AP1- transcription factor, interacts with c-*myc* and EBV	Ubiquitously expressed	One entry for ATF-3/NM_001674 (breast cancer)
Nm23-H2/NM_002512	3	Nucleoside diphosphate kinase/transcription factor for c-*myc*	Overexpressed in tumours	Two entries for Nm23-H2 (glioma and colon cancer), two further entries for Nm23-H1(NM_000269)
S-adenosylhomocysteine hydrolase/M61831	1	Hydrolase involved in transmethylation	Ubiquitously expressed	One entry for AHCYNM_000687 (cDNA from testis, unclassifiable patient)
Triosephosphate isomerase 1/NM_000365	1	Enzyme of the glycolytic pathway	Overexpressed in tumours	Three entries for TPI1 (unclassifiable patient and breast cancer)
U2 snRNP-specific A′ protein/X13482	1	Role in hnRNA splicing/ known autoantigen	Ubiquitously expressed	Four entries for U2 small nuclear ribonucleo-protein auxiliary factor/ NM_005089 (renal cancer, breast cancer, stomach cancer, and Hodgkin's disease)
Ribosomal protein S2/ NM_002952	1	Ribosomal protein	Ubiquitously expressed	Two entries for ribosomal protein S2 (rps2) in prostate cancer
Unknown/AA195197	1	Endogenous retroviral sequence?		No entry with similarity

of these antigens frequently elicit antibody responses in autoimmune diseases or in apparently healthy humans. These antigens therefore also classify as autoantigens and their possible role in human malignancies remains to be clarified [37].

For Nm23-H2, the occurrence of autoantibodies has been described both in cancer patients and healthy volunteers [47]. Ribosomal protein S2 has been detected as an autoantigen in prostate cancer. TPI has been identified by SEREX in breast cancer and also as a mutated antigen recognized by $CD4^+$ T cells in a patient with malignant melanoma [41]. In addition to these identical entries, molecules related to the snRNP U2 have been isolated by SEREX, and snRNPS, including components of U2, are frequently recognised by autoantibodies in patients with autoimmune disease [8, 29, 50]. AHCY is a key enzyme of transmethylation, a mechanism also involved in controlling the replication of several viruses such as EBV [7, 46]. Although no entries identical to the ATF-2 gene were found in the SEREX database, the related family member ATF-3 has been identified as an antigen eliciting humoral immune response in breast cancer patients.

Detection of anti-ATF-2 antibodies in sera

We chose to focus on ATF-2 in our further studies, because it has not been previously described as a SEREX antigen. Furthermore, ATF-2 may be linked to the development of BL, since it has previously been implicated to be involved in oncogenesis and to interact with c-*myc* and EBV [24, 34]. We performed an ELISA to reveal the occurrence of IgG autoantibodies against ATF-2. Sera from 50 healthy volunteers, 8 patients with BL (including the 3 patients used for screening) and 63 patients with other NHL entities were tested. Using an arbitrary OD_{492} of 0.2 as cutoff, 22 of 71 (31%) of patient sera displayed anti-ATF-2 reactivity, in contrast to 1 of 50 (2%) healthy volunteers (Fig. 1A). All sera with reactivity in ELISA and several negative cases were subjected to competition ELISA, where sera were preincubated with purified ATF-2 or irrelevant recombinant protein. Sera from 19 NHL patients and 1 healthy volunteer were confirmed as positive (Fig. 1B), whereas three patient sera could not be confirmed. Ten positive sera were further tested by Western blot analysis using purified recombinant ATF-2 as antigen and four sera with highly positive ELISA results showed reactivity against ATF-2 in immunoblotting (Fig. D). Sera with negative ELISA results were also negative by Western blot analysis.

The frequency of anti-ATF-2 antibodies in patients with different NHL entities is shown in Table 2. Six of eight BL patients tested (75%) showed seroreactivity against ATF-2, as did 6 of 13 patients with T-NHL (46%). The frequency of ATF-2 autoantibodies appeared lower in DLBCL and FL, with 7 and 22% positive sera identified, respectively.

Analysis of ATF-2 expression

Earlier reports have revealed ubiquitous expression of ATF-2 mRNA-transcripts in normal human tissues, with highest levels in brain, testis and thymus [52], and to our knowledge, no evidence for ATF-2 mRNA overexpression in B-cell lymphomas as compared to normal tissues has been reported [2]. Our own analysis of different lymphoma cell lines could not detect significant differences in ATF-2 mRNA levels or total ATF-2 protein in different NHL cell lines. In particular, we were unable to detect different expression levels when

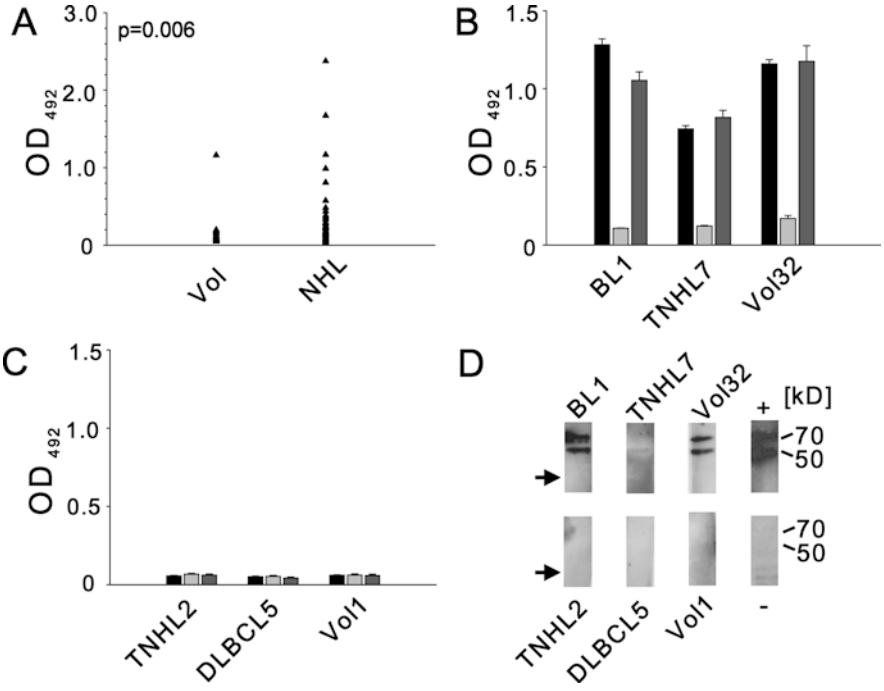

Fig. 1A–D Analysis of anti-ATF-2 antibodies in sera from healthy volunteers and patients with non-Hodgkin's lymphoma (*NHL*). **A** Results of enzyme-linked immunosorbent assay (*ELISA*) using recombinant ATF-2 as target antigen. *Arrow* depicts mean of duplicate experiments for each serum from healthy volunteers (*Vol*) and patients (*NHL*). OD_{492}, optical density at 492 nm. **B**, **C** Results of competition ELISA shown for individual positive (**B**) and negative (**C**) sera. *Bars* denote results in ELISA (*black*), after preincubation of serum with recombinant ATF-2 (*light grey*) or irrelevant protein p352 (*dark grey*) with *error bars* representing standard error of the mean of duplicate experiments. *BL* Burkitt's lymphoma, *T-NHL* T-cell lymphoma, *DLBCL* diffuse large B-cell lymphoma. **D** Western blot analysis of individual sera from **B** and **C**. Expected protein bands corresponding to recombinant ATF-2 are indicated at 70 and 50 kDa. No reactivity to p352 (*arrow*) is seen. Lane titled "+" represents experiment using anti-ATF-2 primary antibody and appropriate secondary antibody (see text). Lane titled "−" shows reactivity of BL1 serum toward a lysate of *E. coli* M15 cells prepared in parallel with lane BL1

comparing cell lines form BL with other cell lines (data not shown).

ATF-2 is regulated at the post-translational level by phosphorylation of the amino acid residues threonine-69 and threonine-71, mainly mediated by stress-activated protein kinases (SAPKs) including Jun-NH2-terminal kinase (JNK) [19]. Therefore, to determine the level of activation of ATF-2 in BL, paraffin-embedded tissue from six patients with BL was analysed by immunohistochemistry for expression of total ATF-2 protein and the doubly phosphorylated isoform ATF-2_{pp} (Thr69/71). All lymphomas expressed ATF-2_{pp} in nearly 100% of viable tumour cells and at high levels, comparable to a subset of centroblasts in the normal germinal centre of tonsils. Most B cells in normal tonsils except for the subset of centroblasts expressed low levels of ATF-2_{pp} (Fig. 2). In contrast, the total ATF-2 protein levels appeared similar in BL cells and most normal germinal centre B cells (data not shown).

Discussion

The present paper describes the use of the SEREX technology for identification of TAAs in BL. By screening a Ramos cDNA library with pooled sera of three BL patients, we detected humoral responses against seven antigens, showing that humoral immune responses to nonidiotypic antigens take place in BL.

The use of a IgM-expressing B cell line as a cDNA source reduced problems with false positive Ig clones, a problem frequently encountered when using SEREX in B-cell malignancies [28].To identify CGAs and to overcome the mentioned technical obstacles, others have used testis tissue as a cDNA source for SEREX, but no clear candidates for a possible NHL vaccination protocol were identified [23]. Since the routinely used immunotherapies for haematological malignancies use common lineage–specific haematological antigens [5] and since expression of CGAs in B-NHL has been shown to be limited [36], the appropriate cDNA source

Table 2 ATF-2 antibodies in sera from patients with NHL and healthy volunteers. *ND* not done

	DLBCL	Follicular lymphoma	Burkitt's lymphoma	T-cell lymphoma	Normal volunteers
ELISA	2/27	5/23	6/8	6/13	1/50
Immunoblotting	ND	ND	1/4	2/5	1/1

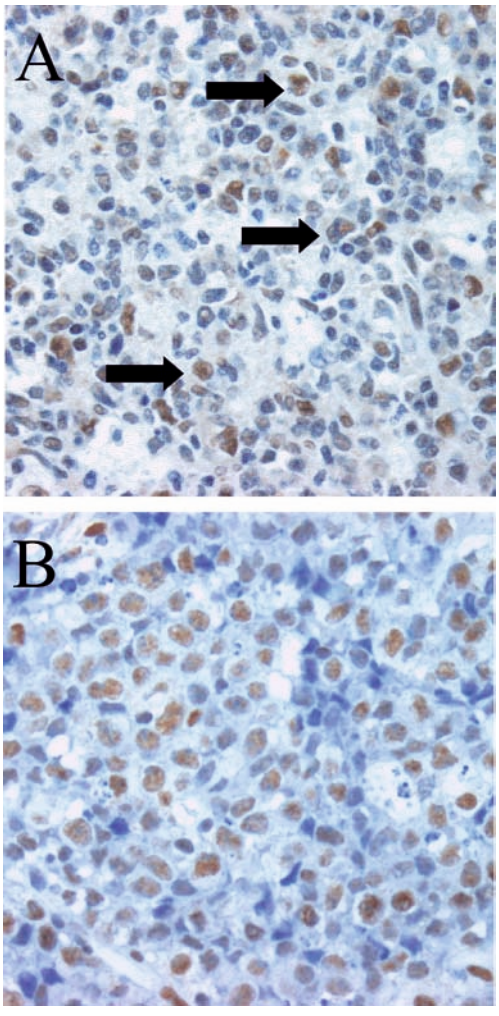

Fig. 2A, B Immunohistochemical staining for doubly phosphorylated ATF-2_{pp} (Thr69/71) in a tonsil biopsy sample (**A**) and a case of BL (**B**). A subpopulation of large centroblasts in germinal centres of tonsils (*black arrows*) expresses high levels of ATF-2_{pp} in contrast to most of the other germinal centre cells that express only low levels of the protein. In BL, most of the tumour cells express high levels of ATF-2_{pp}, with the exception of the apoptotic tumour cells

for identification of TAAs by SEREX in NHL appears to be lymphoma tissue [23]. We were not able to identify CGAs or haematological antigens in this study, which is in line with the findings of others [23].

Most of the previously identified SEREX-defined antigens are ubiquitously expressed [37]. This is consistent with our findings, where, except of the EST clone with unknown function from chromosome 6, all the detected antigens are known to be ubiquitously expressed in normal human tissues. Therefore none of these antigens appear to be obvious vaccine candidates for immunotherapy of cancer. However, it has been shown that coimmunisation with SEREX antigens and tumour antigens results in heightened resistance to tumour growth in mice [37]. Therefore, the repeated detection by SEREX of some of the antigens mentioned here might strengthen them as candidates for coimmunisation protocols in human [51]. It remains to be clarified, whether these coimmunisation antigens should be exclusive TAAs, which do not elicit humoral responses in healthy volunteers, or if it is possible to use autoantigens, as described [37]. It is in that respect of great interest that five of the seven antigens identified here, have been identified by the means of SEREX before.

To our knowledge, ATF-2 has not previously been detected by SEREX. It is a member of the basic region leucine zipper family of transcription factors, and together with the jun, fos and ATF subfamilies, it constitutes a component of the AP-1 complex [49, 53]. This transcription factor regulates gene expression in response to cell stress, growth factors [39], viral infections and inflammatory signals. Interestingly v-*myc* has been shown to activate and stabilize the phosphorylation of ATF-2 [34]. Despite of the known oncogenic potential of AP-1 transcription factors [53] and the known interaction of ATF-2 with both c-*myc* and EBV [1], little is known about the expression and phosphorylation of ATF-2 in NHL and leukaemia [33]. We therefore explored the expression pattern of ATF-2 and its activated, doubly phosphorylated isoform at the protein level in paraffin-embedded sections of six different BLs. Interestingly, most BL cells expressed high levels of the active isoform, with similar levels found only in subsets of centroblasts in reactive germinal centres. The total ATF-2 protein level was similar in BL cells and the majority of germinal centre B cells. The reason for enhanced phosphorylation of ATF-2 in BL cells and the possible effects of such changes remain to be determined. Recently, overexpression of c-*jun* and junB, to major components of AP-1, was found in Hodgkin's disease and shown to contribute to proliferation of HD cell lines [33]. Similar overexpression was not seen in other B-NHL entities, and ATF-2 expression was not analysed. The fact that oncogenic viruses such as HTLV-1 and EBV exert some of their effects through activation of ATF-2, and ATF-2 contributes to c-*jun*–mediated transformation in experimental systems, may warrant further studies as to the contribution of ATF-2 to oncogenisis in BL [1, 13, 53, 54]. Interestingly, humoral immune responses against ATF-2 could be detected in patients with BL and T-NHL. The occurrence of anti-ATF-2 antibodies in six out of eight sera from patients with BL, but only in 1 out of 50 sera from the healthy controls in this analysis, underlines the potential pathophysiological relevance of this transcription factor, since only a fraction of the serologically defined TAAs do not elicit serological responses in apparently healthy humans. Others have argued that a cancer-related serological profile of an autoantigen might result from tumour-associated post-translational modifications [40]. The reason for autoantibody production in these patients is not known. The high level of ATF-2 phosphorylation in BL cells points to a post-translational change that possibly enhances immunogenicity of the protein. Three patient sera elicited high signals in ELISA, but the immunoreactivity failed to be verified in

competition ELISA with the recombinant (unphosphorylated) ATF-2. Patient antibodies, which are specific against ATF-2_{pp}, might be the reason for this. However, one of these patients elicited many apparently unspecific bands in Western analysis, and we conclude that we did not succeed in removing all anti-*E. coli* antibodies for this patient, despite intensive preabsorption, as indicated, and parallel treatment of the sera. This emphasises the need for eukaryotic expression cloning systems which would make the identification of post-translationally modified antigens more likely [23, 30] and specific.

In this respect, it is tempting to speculate that phosporylation itself may have created a new epitope recognized by phosphopeptide-specific T cells in the patients. Such T cells have been described to be a constituent of the normal T-cell repertoire [3, 25]. If this was found to be the case, the high level of expression of such an epitope in lymphomas compared with normal tissues, might provide a window of opportunity for a vaccination strategy. The high degree of apoptotic cells associated with BL may also contribute to immunogenicity, since apoptotic tumour cells have been shown to trigger autoantibody production, including autoantibodies to snRNP components, antigens identified along with ATF-2 in the present screening [17, 20].

Given the important pathophysiological role of c-*myc* in BL, it is remarkable that in addition to ATF-2 we identified two further molecules, which are related to c-*myc*, i.e. Nm23-H2 and AHCY. NM23-H2 has, besides activating transcription of c-*myc* [42], been shown to be a downstream target of c-*myc* itself [48]. Inhibitors of AHCY have been tested in vitro for their possible utilisation as an antiviral drug [9] and as a drug in the treatment of leukaemia and lymphoma. In these experiments, AHCY inhibitors were shown to induce apoptosis, by possibly down-regulating AP-1, nm23-H2 and c-*myc* [26].

In conclusion, we analysed the autoantibody repertoire of BL patients and revealed an intriguing serological profile against ATF-2 in patients with BL compared with healthy controls. Our findings indicate that SEREX, besides identifying potential tumour vaccine candidates, has the ability of detecting antigens of pathophysiological interest.

Acknowledgements The authors would like to thank Inger Liv Nordli for excellent technical help and Rainer Siebert for verifying the BL diagnosis by FISH. This work was supported by the Norwegian Cancer Society and the Norwegian Research Council.

References

1. Adamson AL, Darr D, Holley-Guthrie E, Johnson RA, Mauser A, Swenson J, Kenney S (2000) Epstein-Barr virus immediate-early proteins BZLF1 and BRLF1 activate the ATF2 transcription factor by increasing the levels of phosphorylated p38 and c-Jun N-terminal kinases. J Virol 74:1224–1233
2. Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, Boldrick JC, Sabet H, Tran T, Yu X, Powell JI, Yang L, Marti GE, Moore T, Hudson J Jr, Lu L, Lewis DB, Tibshirani R, Sherlock G, Chan WC, Greiner TC, Weisenburger DD, Armitage JO, Warnke R, Staudt LM (2000) Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature 403:503–511
3. Andersen MH, Bonfill JE, Neisig A, Arsequell G, Sondergaard I, Valencia G, Neefjes J, Zeuthen J, Elliott T, Haurum JS (1999) Phosphorylated peptides can be transported by TAP molecules, presented by class I MHC molecules, and recognized by phosphopeptide-specific CTL. J Immunol 163:3812–3818
4. Bellan C, Lazzi S, De Falco G, Nyongo A, Giordano A, Leoncini L (2003) Burkitt's lymphoma: new insights into molecular pathogenesis. J Clin Pathol 56:188–192
5. Campbell P, Marcus R (2003) Monoclonal antibody therapy for lymphoma. Blood Rev 17:143–152
6. Chen G, Gharib TG, Wang H, Huang CC, Kuick R, Thomas DG, Shedden KA, Misek DE, Taylor JM, Giordano TJ, Kardia SL, Iannettoni MD, Yee J, Hogg PJ, Orringer MB, Hanash SM, Beer DG (2003) Protein profiles associated with survival in lung adenocarcinoma. Proc Natl Acad Sci U S A 100:13537–13542
7. Chiang PK, Gordon RK, Tal J, Zeng GC, Doctor BP, Pardhasaradhi K, McCann PP (1996) S-Adenosylmethionine and methylation. FASEB J 10:471–480
8. De Cesare D, Fimia GM, Sassone-Corsi P (1999) Signaling routes to CREM and CREB: plasticity in transcriptional activation. Trends Biochem Sci 24:281–285
9. De Clercq E (2002) Strategies in the design of antiviral drugs. Nat Rev Drug Discov 1:13–25
10. Duyndam MC, van Dam H, Smits PH, Verlaan M, van der Eb AJ, Zantema A (1999) The N-terminal transactivation domain of ATF2 is a target for the co-operative activation of the c-jun promoter by p300 and 12S E1A. Oncogene 18:2311–2321
11. Ehlken H, Schadendorf D, Eichmuller S (2004) Humoral immune response against melanoma antigens induced by vaccination with cytokine gene-modified autologous tumor cells. Int J Cancer 108:307–313
12. Eichmuller S, Usener D, Dummer R, Stein A, Thiel D, Schadendorf D (2001) Serological detection of cutaneous T-cell lymphoma-associated antigens. Proc Natl Acad Sci U S A 98:629–634
13. Eliopoulos AG, Gallagher NJ, Blake SM, Dawson CW, Young LS (1999) Activation of the p38 mitogen-activated protein kinase pathway by Epstein-Barr virus-encoded latent membrane protein 1 coregulates interleukin-6 and interleukin-8 production. J Biol Chem 274:16085–16096
14. Fossa A, Alsoe L, Crameri R, Funderud S, Gaudernack G, Smeland EB (2004) Serological cloning of cancer/testis antigens expressed in prostate cancer using cDNA phage surface display. Cancer Immunol Immunother 53:431–438
15. Fossa A, Siebert R, Aasheim HC, Maelandsmo GM, Berner A, Fossa SD, Paus E, Smeland EB, Gaudernack G (2000) Identification of nucleolar protein No55 as a tumour-associated autoantigen in patients with prostate cancer. Br J Cancer 83:743–749
16. Fuchs SY, Ronai Z (1999) Ubiquitination and degradation of ATF2 are dimerization dependent. Mol Cell Biol 19:3289–3298
17. Gensler TJ, Hottelet M, Zhang C, Schlossman S, Anderson P, Utz PJ (2001) Monoclonal antibodies derived from BALB/c mice immunized with apoptotic Jurkat T cells recognize known autoantigens. J Autoimmun 16:59–69
18. Godfried MB, Veenstra M, Sluis P, Boon K, Asperen R, Hermus MC, Schaik BD, Voute TP, Schwab M, Versteeg R, Caron HN (2002) The N-myc and c-myc downstream pathways include the chromosome 17q genes nm23-H1 and nm23-H2. Oncogene 21:2097–2101
19. Gupta S, Campbell D, Derijard B, Davis RJ (1995) Transcription factor ATF2 regulation by the JNK signal transduction pathway. Science 20(267):389–393

20. Hansen MH, Nielsen H, Ditzel HJ (2001) The tumor-infiltrating B cell response in medullary breast cancer is oligoclonal and directed against the autoantigen actin exposed on the surface of apoptotic cancer cells. Proc Natl Acad Sci U S A 98:12659–12664
21. Hartmann TB, Thiel D, Dummer R, Schadendorf D, Eichmuller S (2004) SEREX identification of new tumour-associated antigens in cutaneous T-cell lymphoma. Br J Dermatol 150:252–258
22. Hecht JL, Aster JC (2000) Molecular biology of Burkitt's lymphoma. J Clin Oncol 18:3707–3721
23. Huang S, Preuss KD, Xie X, Regitz E, Pfreundschuh M (2002) Analysis of the antibody repertoire of lymphoma patients. Cancer Immunol Immunother 51:655–662
24. Ji L, Arcinas M, Boxer LM (1995) The transcription factor, Nm23H2, binds to and activates the translocated c-myc allele in Burkitt's lymphoma. J Biol Chem 270:13392–13398
25. Kastrup IB, Andersen MH, Elliott T, Haurum JS (2001) MHC-restricted T cell responses against posttranslationally modified peptide antigens. Adv Immunol 78:267–289
26. Kim HS, Jeong SY, Lee JH, Kim BE, Kim JW, Jeong SW, Kim IK (2000) Induction of apoptosis in human leukemia cells by 3-deazaadenosine is mediated by caspase-3-like activity. Exp Mol Med 32:197–203
27. Klein G, Dombos L, Gothoskar B (1972) Sensitivity of Epstein-Barr virus (EBV) producer and non-producer human lymphoblastoid cell lines to superinfection with EB-virus. Int J Cancer 10:44–57
28. Krackhardt AM, Witzens M, Harig S, Hodi FS, Zauls AJ, Chessia M, Barrett P, Gribben JG (2002) Identification of tumor-associated antigens in chronic lymphocytic leukemia by SEREX. Blood 100:2123–2131
29. Lehmeier T, Raker V, Hermann H, Luhrmann R (1994) cDNA cloning of the Sm proteins D2 and D3 from human small nuclear ribonucleoproteins: evidence for a direct D1-D2 interaction. Proc Natl Acad Sci U S A 91:12317–12321
30. Li G, Miles A, Line A, Rees RC (2004) Identification of tumour antigens by serological analysis of cDNA expression cloning. Cancer Immunol Immunother 53:139–143
31. Line A, Slucka Z, Stengrevics A, Silina K, Li G, Rees RC (2002) Characterisation of tumour-associated antigens in colon cancer. Cancer Immunol Immunother 51:574–582
32. Martinez JA, Prevot S, Nordlinger B, Nguyen TM, Lacarriere Y, Munier A, Lascu I, Vaillant JC, Capeau J, Lacombe ML (1995) Overexpression of nm23-H1 and nm23-H2 genes in colorectal carcinomas and loss of nm23-H1 expression in advanced tumour stages. Gut 37:712–720
33. Mathas S, Hinz M, Anagnostopoulos I, Krappmann D, Lietz A, Jundt F, Bommert K, Mechta-Grigoriou F, Stein H, Dorken B, Scheidereit C (2002) Aberrantly expressed c-Jun and JunB are a hallmark of Hodgkin lymphoma cells, stimulate proliferation and synergize with NF-kappa B. EMBO J 21:4104–4113
34. Miethe J, Schwartz C, Wottrich K, Wenning D, Klempnauer KH (2001) Crosstalk between Myc and activating transcription factor 2 (ATF2): Myc prolongs the half-life and induces phosphorylation of ATF2. Oncogene 20:8116–8124
35. Montgomerie JZ, Gracy RW, Holshuh HJ, Keyser AJ, Bennett CJ, Schick DG (1997) The 28 K protein in urinary bladder, squamous metaplasia and urine is triosephosphate isomerase. Clin Biochem 30:613–618
36. Nemecek ER, Matthews DC (2002) Antibody-based therapy of human leukemia. Curr Opin Hematol 9:316–321
37. Nishikawa H, Tanida K, Ikeda H, Sakakura M, Miyahara Y, Aota T, Mukai K, Watanabe M, Kuribayashi K, Old LJ, Shiku H (2001) Role of SEREX-defined immunogenic wild-type cellular molecules in the development of tumor-specific immunity. Proc Natl Acad Sci U S A 98:14571–14576
38. Okabe M, Matsushima S, Morioka M, Kobayashi M, Abe S, Sakurada K, Kakinuma M, Miyazaki T (1987) Establishment and characterization of a cell line, TOM-1, derived from a patient with Philadelphia chromosome-positive acute lymphocytic leukemia. Blood 69:990–998
39. Ouwens DM, de Ruiter ND, van der Zon GC, Carter AP, Schouten J, van der Burgt C, Kooistra K, Bos JL, Maassen JA, van Dam H (2002) Growth factors can activate ATF2 via a two-step mechanism: phosphorylation of Thr71 through the Ras-MEK-ERK pathway and of Thr69 through RalGDS-Src-p38. EMBO J 21:3782–3793
40. Pfreundschuh M (2000) Exploitation of the B cell repertoire for the identification of human tumor antigens. Cancer Chemother Pharmacol 46 (3–7):S3-S7
41. Pieper R, Christian RE, Gonzales MI, Nishimura MI, Gupta G, Settlage RE, Shabanowitz J, Rosenberg SA, Hunt DF, Topalian SL (1999) Biochemical identification of a mutated human melanoma antigen recognized by CD4(+) T cells. J Exp Med 189:757–766
42. Postel EH, Berberich SJ, Flint SJ, Ferrone CA (1993) Human c-myc transcription factor PuF identified as nm23-H2 nucleoside diphosphate kinase, a candidate suppressor of tumor metastasis. Science 261:478–480
43. Preuss KD, Zwick C, Bormann C, Neumann F, Pfreundschuh M (2002) Analysis of the B-cell repertoire against antigens expressed by human neoplasms. Immunol Rev 188:43–50
44. Sahin U, Tureci O, Schmitt H, Cochlovius B, Johannes T, Schmits R, Stenner F, Luo G, Schobert I, Pfreundschuh M (1995) Human neoplasms elicit multiple specific immune responses in the autologous host. Proc Natl Acad Sci U S A 92:11810–11813
45. Scanlan MJ, Gure AO, Jungbluth AA, Old LJ, Chen YT (2002) Cancer/testis antigens: an expanding family of targets for cancer immunotherapy. Immunol Rev 188:22–32
46. Schaade L, Kleines M, Krone B, Hausding M, Walter R, Ritter K (2000) Enhanced transcription of the s-adenosylhomocysteine hydrolase gene precedes Epstein-Barr virus lytic gene activation in ganglioside-stimulated lymphoma cells. Med Microbiol Immunol (Berl) 189:13–18
47. Schmits R, Cochlovius B, Treitz G, Regitz E, Ketter R, Preuss KD, Romeike BF, Pfreundschuh M (2002) Analysis of the antibody repertoire of astrocytoma patients against antigens expressed by gliomas. Int J Cancer 98:73–77
48. Schuldiner O, Shor S, Benvenisty N (2002) A computerized database-scan to identify c-MYC targets. Gene 292:91–99
49. Shaulian E, Karin M (2002) AP-1 as a regulator of cell life and death. Nat Cell Biol 4:E131-E136
50. Sillekens PT, Beijer RP, Habets WJ, van Verooij WJ (1989) Molecular cloning of the cDNA for the human U2 snRNA-specific A' protein. Nucleic Acids Res 17:1893–1906
51. Stager S, Alexander J, Kirby AC, Botto M, Rooijen NV, Smith DF, Brombacher F, Kaye PM (2003) Natural antibodies and complement are endogenous adjuvants for vaccine-induced CD8(+) T-cell responses. Nat Med 9:1287–1292
52. Su AI, Cooke MP, Ching KA, Hakak Y, Walker JR, Wiltshire T, Orth AP, Vega RG, Sapinoso LM, Moqrich A, Patapoutian A, Hampton GM, Schultz PG, Hogenesch JB (2002) Large-scale analysis of the human and mouse transcriptomes. Proc Natl Acad Sci U S A 99:4465–4470
53. Van Dam H, Castellazzi M (2001) Distinct roles of Jun: Fos and Jun: ATF dimers in oncogenesis. Oncogene 20:2453–2464
54. Xu X, Heidenreich O, Kitajima I, McGuire K, Li Q, Su B, Nerenberg M (1996) Constitutively activated JNK is associated with HTLV-1 mediated tumorigenesis. Oncogene 13:135–142