Influence of antigen on the development of MALT lymphoma

Dido Lenze, Erika Berg, Rudolf Volkmer-Engert, Armin A. Weiser, Axel Greiner, Constanze Knörr-Wittmann, Ioannis Anagnostopoulos, Harald Stein, and Michael Hummel

Mucosa-associated lymphoid tissue (MALT) B-cell lymphomas develop in the context of autoimmune or chronic inflammations like *Helicobacter pylori*–induced gastritis. Remission of most gastric MALT lymphomas after eradication of *H pylori* links tumor cell proliferation to antigen-induced inflammation and the need for antigenic contact. Furthermore, the tumor cells correspond to antigen-activated memory B cells. To investigate the reactivity of the tumor immunoglobulins we employed in vitro–generated antibodies identical to those produced by MALT lymphoma cells. The immunoglobulin rearrangements of 7 MALT lymphomas were amplified, cloned, and expressed as single-chain fragment variable (scFv) antibodies. Antigen specificity of these 7 scFvs was analyzed by immunohistochemical staining of various normal, reactive, and malignant human tissues. Also, an expression library comprising approximately 30 000 proteins from human fetal brains (protein filter) and a peptide library were screened. One scFv stained a subpopulation of tonsillar plasma cells in immunohistochemical studies. On protein filters this scFv recognized the plasma cell–related protein Ufc1. Peptide library screening identified 9 peptides as binding partners of an additional scFv. The majority of MALT lymphoma immunoglobulins studied, however, showed no reactivity against antigens, indicating that the tumor immunoglobulins do not play a significant role in stimulation and proliferation of the MALT lymphoma tumor cells. (Blood. 2006;107:1141-1148)

Introduction

Mucosal surfaces and skin are primary entry sites of potential pathogens (antigens) into the human body. To provide a first line of defense, secondary lymphoid tissues called mucosa-associated lymphoid tissues (MALTs) develop in these organs if antigenic challenge occurs. Apart from infections with external pathogens, the generation of MALT can also be induced by autoimmune disorders.[1-3] Ongoing exposure of the lymphoid cells of the MALT to a foreign or self-antigen (chronic inflammation) triggers their perpetual proliferation in order to eliminate the antigen, which is accompanied by an increased risk of acquisition of genetic defects. Such genetic defects often induce the deregulation of apoptosis and, at the end of a multistage process, might lead to malignant cells, unable to control cell growth. A well-documented example of tumor development on the basis of chronic inflammation is gastric MALT B-cell lymphoma. These tumors have been found to arise in the context of *Helicobacter pylori*–induced gastritis.[4,5] Eradication of *H pylori* by antibiotic treatment led to a complete remission of the associated gastric MALT lymphomas in most cases.[6,7] Based on this observation the concept evolved that MALT lymphoma development is associated with an antigen-induced inflammation, and that the tumor B cells require, at least in an initial phase, antigen contact for their proliferation. In later stages the lymphomas cease to respond to antibiotic therapy, having apparently lost their dependence on antigenic stimulation. This change is attributed to a further acquisition of genetic aberrations and associated oncogenic activations.[8-11]

This theory was supported by the identification of the MALT lymphoma tumor cells as B cells that express functional immunoglobulin molecules and carry rearranged and somatically mutated immunoglobulin genes.[12,13] These features point toward their derivation from antigen-activated memory B cells. In addition, intraclonal variations of the immunoglobulin genes, caused by ongoing somatic mutations[14,15] and/or replacement of a part of the variable heavy (VH) segment (receptor revision),[16] have been reported. These findings further substantiated the idea that antigen activation plays a role in MALT lymphoma development.

However, the question remains whether this activation occurs directly via the immunoglobulins of the tumor B cells or through interaction with antigen-activated T cells. In vitro, gastric MALT lymphoma cells cocultured with *H pylori* require *H pylori*–activated T cells for survival and proliferation.[17,18] On the other hand, the immunoglobulins of some MALT lymphomas were found to have a rheumatoid factor reactivity,[19] and binding of tumor cell immunoglobulins to various structures of normal human tissues (follicular dendritic cells, venules, epithelial cells, and connective tissue) has been described.[20,21] In these latter studies the immunoglobulins of the tumor cells were produced as monoclonal antibodies by heterohybridoma technology and used in immunohistochemical stainings. A shortcoming of these experiments was that the concordance of the specificity determining immunoglobulin

From the Institute of Pathology, Campus Benjamin Franklin, Charité-Universitätsmedizin Berlin, Germany; Institute of Medical Immunology, Campus Mitte, Charité-Universitätsmedizin Berlin, Germany; and the Institute of Pathology, University of Würzburg, Germany.

Submitted April 28, 2005; accepted September 23, 2005. Prepublished online as *Blood* First Edition Paper, October 4, 2005; DOI 10.1182/blood-2005-04-1722.

Supported by the Deutsche Krebshilfe.

D.L. performed research, designed research, analyzed data, and wrote the paper; E.B. performed research; R.V.-E. and A.A.W. contributed analytical tools and analyzed data; I.A., A.G., and C.K.-W. contributed vital new reagents or analytical tools; H.S. designed research and made significant contributions to discussion; and M.H. designed research, analyzed data, and made significant contributions to discussion.

Reprints: Michael Hummel, Institute of Pathology, Campus Benjamin Franklin, Charité-Universitätsmedizin Berlin, Berlin, Germany; e-mail: michael.hummel@charite.de.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

gene rearrangements of the hybridoma antibodies with that of the original tumor immunoglobulins was not demonstrated.

To overcome this drawback we used a different approach to elucidate the role of an interaction of the immunoglobulins of the tumor cells with (auto)antigens for lymphomagenesis. For this purpose, the clonal immunoglobulin gene rearrangements of 7 MALT lymphomas were identified, amplified, cloned, and expressed in insect cells as single-chain fragment variable (scFv) antibodies. The antigen specificity of the 7 tumor scFv's was analyzed by immunohistochemical stainings of a wide range of normal human tissues, of the MALT lymphomas they originated from, and of *H pylori*–positive gastric biopsies. The reactivity against *H pylori* proteins and potential superantigenic structures represented by the bacterium was further examined by Western blot analysis. In parallel, an expression library of approximately 30 000 human fetal brain proteins and an extensive peptide library were screened. Our results demonstrate that the MALT tumor cell immunoglobulins, despite their functional expression, are not directed against specific antigens in most instances.

Patients, materials, and methods

Patient material

All tumors were diagnosed as low-grade MALT lymphomas (World Health Organization [WHO] classification[22]). Four cases (patients 1-4) were of gastric origin, and the lymphomas of patients 5, 6, and 7 were localized in the thyroid, lung, and salivary gland, respectively. Patients 1, 2, 5, 6, and 7 received no treatment before biopsy; patient 4 had a subtotal colectomy (colon diverticulosis) 8 years before the gastric biopsy. For patient 3, no data about previous treatments were available.

Cell lines

Karpas 299 cells and DG 75 cells were cultured in RPMI 1640 with GlutaMAX (Invitrogen, Carlsbad, CA) without phenol red and 10% fetal calf serum (FCS). Ber-H2 hybridoma cells[23] were cultured in 45% RPMI 1640/45% Dulbecco modified Eagle medium (DMEM) with GlutaMAX, 1 g/L glucose, and 10% FCS (all Invitrogen).

Preparation of nucleic acids for polymerase chain reaction

Genomic DNA and total RNA were isolated from frozen tissue sections and hybridoma cells according to the manufacturer's protocols (QIAamp DNA Mini Kit and RNeasy Mini Kit; Qiagen, Hilden, Germany). For reverse transcription–polymerase chain reaction (RT-PCR), 1 μg total RNA was transcribed into cDNA (GeneAmp RNA PCR Core Kit; Perkin Elmer, Shelton, CT).

Identification of the tumor immunoglobulin gene segments

To identify the rearranged segments of the MALT lymphoma immunoglobulin genes, a nested PCR was performed for the heavy-chain genes (*IGH*) as described previously.[24] The variable kappa and lambda light-chain genes (*IGL*) were amplified according to the BIOMED-2 protocol.[25] The resulting predominant PCR products, representing the clonal Ig gene rearrangements, were directly sequenced (Big Dye Terminator Cycle Sequencing Kit and DNA Sequencing Automat 377; Applied Biosystems, Weiterstadt, Germany). The sequences obtained were compared with each other and with our own and published databases (International ImMunoGeneTics database[26]) to determine the corresponding germ-line segments and the number and location of somatic mutations. Furthermore, the dominant IgH and IgL amplificates of each case were cloned (TOPO TA Cloning Kit; Invitrogen) and several clones (Quantum Prep Plasmid Miniprep Kit; Bio-Rad, Hercules, CA) were also sequenced and analyzed by database comparison. The IgH and IgL sequences that were identical in the majority of clones and to the rearrangement obtained from direct sequencing were used to produce the scFv of the respective case.

The *IGH* and *IGL* genes were also amplified by RT-PCR, and the resulting PCR products were sequenced and compared with the sequences obtained from the whole-tissue sections.

Calculation of antigen selection

Antigen selection of the *IGH* sequences was assessed by the multinomial model[51] as well as by the ratio of replacement (R) to silent (S) mutations in the complementarity determining regions (CDRs) and the framework regions (FRs). Sequences were evaluated as antigen-selected when R/S CDR was greater than 2.5 and R/S FR was less than 1.5.

GeneScan analysis

GeneScan analysis (ABI 310; Applied Biosystems) was performed to demonstrate the presence of clonal variable diversity joining (VDJ) rearrangements and to determine their size. For this purpose, reamplification was carried out with a fluorescence-labeled primer specific for the heavy-chain joining segments (JH). Labeled PCR products were separated on a sequencing gel and evaluated by the GeneScan software 672.

Amplification and cloning of the complete tumor immunoglobulin genes

Tumor rearrangement–specific amplifications were conducted with primers (20 pM each) located at the outmost 5′ and 3′ ends of the *IGH* and *IGL* genes. Amplification (0.2 mM each dNTP [Amersham Bioscience, Arlington Heights, IL]; 2 mM $MgSO_4$; 2.5 U PfuTurbo Polymerase [Stratagene, La Jolla, CA]) was done in a PTC-200 Cycler (MJ Research,) at 96°C for 2 minutes, 5 × (96°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds), 35 × (96°C for 30 seconds and 72°C for 1 minute), and 72°C for 10 minutes (Table 1).

PCR products were used for cloning of the respective immunoglobulin genes as scFv's. For this purpose the *IGH* and *IGL* genes of each tumor case and of the Ber-H2 hybridoma cell line (control scFv) were cloned into the vector pIT2 (from Dr Ian Tomlinson, Medical Research Council Laboratory of Molecular Biology, Cambridge, United Kingdom) that contains the coding sequence for the $(G_4S)_3$ peptide linker for joining of the heavy and light chains. For expression in *Drosophila S2* cells, each of these scFv modules was subsequently cloned into the vector pMT-BiP/V5-HisC (Invitrogen). This vector contains the coding sequences for a His-tag and a V5-epitope.

Amplification and cloning of the Ber-H2 hybridoma immunoglobulin genes (control scFv)

To amplify the complete mouse *IGH* and *IGL* genes of the Ber-H2 hybridoma cell line, a nested PCR was performed (0.2 mM each dNTP [Amersham Bioscience]; 1.0 mM $MgCl_2$, primary amplification; 1.5 mM $MgCl_2$, reamplification; AmpliTaq buffer II and 2 U AmpliTaq, all from Applied Biosystems). Cycling conditions were 96°C for 2 minutes, 35 × (96°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds); 72°C for 7 minutes for the first amplification and 96°C for 2 minutes, 5 × (96°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds); 30 × (96°C for 15 seconds, 72°C for 60 seconds); and 72°C for 7 minutes for reamplification (Table 2).

The resulting PCR products were used for cloning of the respective immunoglobulin genes as scFv antibodies as described for the tumor immunoglobulin genes.

Expression and purification of scFv's

Stable transfectants of *Drosophila S2* cells expressing the respective tumor immunoglobulin genes or the immunoglobulin genes of the Ber-H2 hybridoma as scFv's were created according to the manufacturer's instructions (Drosophila expression system; Invitrogen). Secretory recombinant protein expression was induced and after 5 days the scFv-containing culture supernatant was separated from the cells by centrifugation and filtered.

ScFv's were purified from the supernatants by immobilized metal ion affinity chromatography (BioLogic FPLC system; Bio-Rad) using nickel-charged chelating sepharose Fast Flow (Amersham Bioscience, United Kingdom).

Table 1. Primer sequences for amplification of the complete tumor immunoglobulin genes

Primer	Sequence 5′-3′	Application
DFDP47	GAGTCGACCATGGGAGGTGCAGCTGTTGGAGTCTGG	Rearrangement-specific VH primer for patients 1, 4
DFVH3	GAGTCGACCATGGGAGGTGCAGCTGGTGGAGTCTGG	Rearrangement-specific VH primer for patients 2, 3
DFDP71	TAGAGACCCATGGCAGGTGCAGCTGCAGTCG	Rearrangement-specific VH primer for patient 5
DFDP75/10	CAGTAGTCCATGGCAGGTGCAGCTGGTGCAGTC	Rearrangement-specific VH primer for patients 6, 7
DFJH3	CAGTAGACTCGAGGAAGAGACGGTGACCATTGTCCCTTGG	Rearrangement-specific JH primer for patient 3
DFX2JH4b	GAGTCGACTCGAGGAGGAGACGGTGACCAGGGTTCC	Rearrangement-specific JH primer for patients 1, 2, 4
DFJH4d	GACAGCACTCGAGGAGGAGACGGTGACCAGGG	Rearrangement-specific JH primer for patient 6
DFJH6b	CAGTAGACTCGAGGAGACGGTGACCGTGGTC	Rearrangement-specific JH primer for patient 5
DFDPK20/22	AGCTGAGGTCGACGGAAATTGTGTTGACGCAGTCTCCAG	Rearrangement-specific VK primer for patients 1, 7
DFDPK21	AGCTGAGGTCGACGGAAATAGTGATGACGCAGTCTCC	Rearrangement-specific VK primer for patient 3
DFSVK4	CTCAGCTGTCGACGGACATCGTGATGACCCAGTCTC	Rearrangement-specific VK primer for patient 2
DFS2DPK8	AGCTGAGGTCGACGGACATCCAGTTGACCCAGTCTCC	Rearrangement-specific VK primer for patient 4
DFJK1.2	GAGTCGAGCGGCCGCTTTGATTTCCACCTTGGTCCCTTG	Rearrangement-specific JK primer for patients 1-3, 7
DFJK4.2	GAGTCGAGCGGCCGCTTTGATCTCCACCTTGGTCCC	Rearrangement-specific JK primer for patient 4
DF2b2	CTCTACAGTCGACGCAGTCTGCCCTGACTCAGCCTGC	Rearrangement-specific VL primer for patient 5
DF31	GTCTACTGTCGACGTCTTCTGAGCTGACTCAGGACC	Rearrangement-specific VL primer for patient 6
DFJL3	TTGTATTGCGGCCGCTAGGACGGTCAGCTTGGTC	Rearrangement-specific JL primer for patient 5
DFJL1	ATTGACTGCGGCCGCTAGGACGGTGACCTTGGTC	Rearrangement-specific JL primer for patient 6

VH indicates variable segments of the heavy-chain gene; VL, variable segment of the lambda light chain gene; VK, variable segment of the kappa light chain gene; JH, joining segment of the kappa light chain gene; JK, joining segment of the kappa light chain gene; and JL, joining segment of the lambda light chain gene.

Biotinylation of scFv's

Purified scFv molecules were biotinylated using the EZ-Link Sulfo-NHS-LC Biotinylation Kit (Perbio, Rockford, IL) according to the manufacturer's instructions. The biotin incorporation was determined by the HABA method (Perbio).

Fluorescence-activated cell-sorter analysis

Karpas 299 (CD30-positive anaplastic large-cell lymphoma [ALCL] cell line) and DG75 cells (CD30-negative mantle cell lymphoma cell line) were incubated with biotinylated anti-CD30 Ber-H2 scFv and biotinylated monoclonal Ber-H2 IgG1 antibody, respectively. As controls, each cell line was also incubated with biotinylated IgG1 and a biotinylated scFv not binding to CD30. The cells were then incubated with phycoerythrin-conjugated streptavidin and analyzed in a FACSort (Becton Dickinson, Franklin Lakes, NJ).

Preparation of *H pylori* cell lysates

H pylori colonies were taken up with a sterile cotton swab and resuspended in lysis buffer (50 mM Tris-buffer [pH 7.8], 150 mM NaCl, 1% Nonidet P40, and 1 × Complete Protease Inhibitor [Roche Diagnostics, Basel, Switzerland]). After 3 freeze-thaw cycles the insoluble matter was removed by centrifugation and the soluble protein fraction was used for Western blotting.

Quantity and quality assessment of proteins

Protein quantity was determined by Bradford assay (Bio-Rad). The quality was evaluated on a Bioanalyzer 2100 using the Protein 50 Assay (Agilent Technologies, Palo Alto, CA).

Immunohistochemical staining

Paraffin sections were deparaffinized and antigenicity was retrieved by cooking in citrate buffer (100 mM, 2 minutes). Frozen sections were treated with acetone prior to staining. Endogenous peroxidase was blocked with 3% H_2O_2 (paraffin sections) or glucose oxidase buffer (frozen sections; 20 mM β-D-glucose, 2 mM sodium azide, and 800 U glucose oxidase in phosphate-buffered saline [PBS]). Endogenous biotin was blocked with an avidin-biotin blocking system (DakoCytomation, Copenhagen, Denmark) and the slides were then incubated with the respective scFv's (1 μg/mL). Signals were amplified with the Catalyzed Signal Amplification System (CSA; DakoCytomation) and detected by a chromogenic horseradish peroxidase (HRP) substrate (SIGMA FAST 3,3′-diaminobenzidin tablets; Sigma-Aldrich, Seelze, Germany). For double stainings, paraffin sections were cooked again after the first development and incubated with antibodies against IgA (1:20 000), IgD (1:200), IgE (1:400), IgG (1:1000), and IgM (1:400; all from DakoCytomation) or IRF4 (1:20; Santa Cruz Biotechnology, Santa Cruz, CA). Antibody binding was visualized with the APAAP Mouse ChemMate Detection Kit (DakoCytomation), producing a red precipitate that could be distinguished from the brown precipitate of the first staining.

Western blot

Proteins were separated on a discontinuous SDS-PAGE as well as under native conditions on 4% to 20% Novex Tris-Glycine gels (Invitrogen) and transferred to Hybond-P membrane (Amersham Bioscience). Primary antibodies (1 μg/mL biotinylated scFv's or 1:1000 rabbit polyclonal anti–*H pylori* antibody; DakoCytomation) were applied followed by incubation with secondary antibodies (streptavidin-conjugated HRP [PerkinElmer] or 1:1000 anti–rabbit HRP conjugate [Amersham Bioscience, UK]). Binding of antibody was detected by a chemiluminescence detection system (ECL; Amersham Bioscience, UK).

Staining of protein filters

High-density protein arrays consisting of 27 648 proteins derived from a human fetal brain expression library[52] were purchased from the RZPD (Deutsches Ressourcenzentrum für Genomforschung, Berlin, Germany). The filters were incubated with the respective scFv's (0.5 μg/mL) followed

Table 2. Primer sequences for amplification of the Ber-H2 hybridoma immunoglobulin genes

Primer	Sequence 5′-3′	Application
BVH	GTGATACCTACTATCCTTCTGTCC	Forward primer for the first amplification of the Ber-H2 *IGH* gene
BVH	ACCTGCAGAGACAATGACC	Reverse primer for the first amplification of the Ber-H2 *IGH* gene
BVH/p	GACTCGACCATGGCAGGTCCAGCTTCAC	Forward primer for the reamplification of the Ber-H2 *IGH* gene
BVH557	GACTCGACTCGAGGCAGAGACAGTGAC	Reverse primer for the reamplification of the Ber-H2 *IGH* gene
BVL	TGCTGATGGGAACATTGTAA	Forward primer for the first amplification of the Ber-H2 *IGK* gene
BVL	ACGTTTTATTTCCAGCTTGG	Reverse primer for the first amplification of the Ber-H2 *IGK* gene
BVL387	GACTCGAGTCGACGAACATTGTAATGA	Forward primer for the reamplification of the Ber-H2 *IGK* gene
BVL707	GACTCGAGCGGCCGCTTTTATTTCCAGC	Reverse primer for the reamplification of the Ber-H2 *IGK* gene

IGH indicates immunoglobulin heavy chain gene; IGK, immunoglobulin kappa light chain gene.

by application of mouse anti-V5 antibody (1:5000; Invitrogen) and anti–mouse HRP conjugate (1:2000; Amersham Bioscience, UK). Bound scFv's were made visible by a chemiluminescence detection system. The resulting signals were assigned to the respective cDNA clones by comparison with a matrix. Positive clones were sequenced and compared to the nonredundant sequence database of the National Center for Biotechnology Information. The translated sequence of the cDNA clones was also compared to the protein families database of alignments and hidden Markov models.[52]

Synthesis and staining of peptide arrays

A sequence library of 5520 different 15-mer peptides was generated randomly.[28] The peptides were then synthesized on cellulose-amino-hydroxypropyl-ether (CAPE) membranes[29] by the SPOT synthesis technique[30] using a semiautomated SPOT synthesizer (Abimed, Langenfeld, Germany). The membranes were initially incubated with the secondary antibody (Penta-His-HRP, 1:2000; Qiagen,) to identify nonspecific signals. After application of HRP substrate (SuperSignal West Dura Substrate; Perbio, Hilden, Germany) the resulting chemiluminescence was visualized on a Lumi Imager (Roche Diagnostics). The membranes were then incubated with scFv (10 μg/mL) followed by incubation with the anti-His antibody and signal detection as described for the identification of unspecific signals. Interpretation of the signals and mapping to the corresponding peptide sequences was carried out with the Genespotter software (MicroDiscovery, Berlin, Germany). The sequences of the peptides identified as binding partners were submitted to databases (see above).

Results

Analysis of the MALT lymphoma immunoglobulin genes

Germ-line segments with highest homology to the tumor immunoglobulin gene sequences were identified (Table 3). Rearranged *IGH* and *IGL* genes of all cases, except for the light chain of patient 2, displayed somatic mutations. To determine selection by antigen, 2 different methods were used (ratio of replacement to silent mutations and multinomial model). These calculations led to deviating results. Only 1 patient was found to be antigen selected after calculation of the ratio of replacement to silent mutations, whereas the multinomial model led to the identification of 4 patients with signs of antigen selection (Table 3). Sequencing of the cloned *IGH* and *IGL* amplification products revealed intraclonal variations in patients 3, 4, 5, and 7. Most of these variations had no consequence for the amino acid sequence. However, in each patient one predominant gene rearrangement with a stable mutation pattern was clearly identified and used for the generation of the scFv's.

The rearranged Ig gene sequences of each patient amplified from the whole-tissue DNA extracts were identical to those obtained by RT-PCR, thus verifying expression of these rearrangements by the MALT lymphoma tumor cells.

Cloning and expression of the tumor immunoglobulin genes as scFvs

The complete variable *IGH* and *IGL* genes of the 7 MALT lymphomas were amplified, cloned, expressed as scFv in *Drosophila* S2 cells, and purified by affinity chromatography via the His-tag. Gel analysis revealed scFv sizes from 30 to 38 kDa, corresponding to the different lengths of the respective tumor immunoglobulin gene rearrangements.

Establishing the Ber-H2 control scFv

In addition to the tumor immunoglobulins the hybridoma antibody Ber-H2 (anti-CD30) was synthesized as a control scFv. The Ber-H2 antibody recognizes the CD30 antigen, overexpressed on the tumor cells of classical Hodgkin lymphoma and ALCL and also, to a much lesser extent, expressed on activated normal lymphoid cells.[23,31]

The antigen-binding capacity of the biotinylated Ber-H2 scFv to Karpas299 cells (CD30-positive) and DG 75 cells (CD30-negative) was compared by fluorescence-activated cell-sorter (FACS) measurements to that of the Ber-H2 monoclonal antibody and controls. A selective binding of the Ber-H2 scFv to CD30-positive cells but not to CD30-negative cells could be demonstrated. The avidity of the Ber-H2 scFv was only a little lower than that of the monoclonal Ber-H2 antibody.

Immunohistochemical staining of normal human tissue

To identify self-antigens potentially involved in MALT lymphoma pathogenesis, sections from 21 different formalin-fixed and paraffin-embedded normal human tissues and sections from 14 different frozen normal human tissues were screened for reactivity to the 7 MALT lymphoma–derived scFv's (Table 4). All tissues were also incubated without antibody and with the anti-CD30 Ber-H2 control scFv. The Ber-H2 scFv stained, as expected, CD30-positive cells in tissues with activated lymphoid cells (tonsil, small intestine). With most of the tumor-derived scFv's merely nonspecific staining of various cell types (eg, granulocytes or glandular or muscle cells), also present in the "no antibody" and Ber-H2 scFv controls, could be observed (Table 4). A specific staining was only visible with the tumor scFv derived from patient 1 (Figure 1). This scFv recognized cells with plasmacellular morphology in formalin-fixed and paraffin-embedded tonsillar sections not detected by the Ber-H2 control scFv or by the other tumor scFv's. To further characterize the labeled cells, double immunohistochemical stainings were conducted using the scFv derived from patient 1 and an antibody against the plasma cell–specific antigen IRF4 (interferon regulating factor 4; Figure 2A). All cells positive for the scFv of patient 1 were also positive for IRF4, identifying the cells recognized by the patient-1 scFv as plasma cells. To determine whether the staining of the patient-1 scFv was restricted to a certain plasma cell isotype, additional

Table 3. Overview of the germ-line segments used for the immunoglobulin gene rearrangements and of the somatic mutations in the 7 MALT lymphomas

Patient	VH segment	VH family	JH segment	Intraclonal diversity VH	Antigen selection*	VL segment	VL family	JL segment	Intraclonal diversity VL
1	VH3-23	VH 3	JH 4	No	Yes/Yes	IGKV3-20	VK 3	JK 1	No
2	VH3-07	VH 3	JH 4	No	No/NA	IGKV4-01	VK 4	JK 1	No
3	VH3-07	VH 3	JH 3	Yes	Yes/NA	IGKV3-15	VK 3	JK 1	No
4	VH3-23	VH 3	JH 4	Yes	Yes/No	IGKV1-09	VK 1	JK 4	No
5	VH4-59	VH 4	JH 6	Yes	No/NA	IGLV2-23	VL 2	JL 3	Yes
6	VH1-02	VH 1	JH 4	No	Yes/No	IGLV3-19	VL 3	JL 1	No
7	VH1-69	VH 1	JH 6	Yes	No/No	IGKV3-20	VK 3	JK 1	Yes

VH indicates variable segment of the heavy chain; JH, joining segment of the heavy chain; VL, variable segment of the light chain; JL joining segment of the light chain; and NA, number of mutations too low for calculation of antigen selection.

*Based on the multinomial model and/based on the ratio of replacement to silent mutations.

Table 4. Overview of the 23 normal human tissues (paraffin-embedded and/or frozen sections) stained with the tumor scFv's and controls, and the resulting staining patterns

Tissue	Staining pattern	
	Ber-H2 scFv	Patient 1 scFv
Tonsil*	Lymphoid blasts	Plasma cell subpopulation
Spleen†	—	—
Salivary gland‡	—	—
Esophagus‡	—	—
Stomach§	—	—
Small intestine‖	Lymphoid blasts	—
Duodenum‡	—	—
Colon¶	—	—
Appendix‡	—	—
Kidney	—	—
Adrenal gland‡	—	—
Testis	—	—
Prostate‡	—	—
Uterus‡	—	—
Ovary‡	—	—
Mammary#	—	—
Placenta#	—	—
Thyroid**	—	—
Nerve‡	—	—
Liver	—	—
Lung	—	—
Skeletal muscle‡	—	—
Skin	—	—

The paraffin-embedded tissues were assembled as a tissue array (Biochip; Prof A. C. Feller, Institute of Pathology, Campus Lübeck, Universitatsklinikum Schleswig Holstein, Germany); in addition, single sections of tonsil, spleen, small intestine, and thyroid were stained. "Nonspecific staining" refers to signals detectable in the negative control (no antibody) and with the Ber-H2 control scFv, respectively.

No specific signals were found for patients 2 through 7 or for the negative control.

— Indicates no specific signals.

*Nonspecific staining of single plasma cells or granulocytes in surrounding muscle.

†Nonspecific staining of single lymphatic cells.

‡Stained as paraffin-embedded tissue only.

§Nonspecific staining of single glandular cells and of single plasma cells and granulocytes in the surrounding muscle.

‖Nonspecific staining of single plasma cells in the glandular tissue and of single plasma cells and granulocytes in the surrounding muscle.

¶Nonspecific staining of single glandular cells and of single plasma cells in the glandular tissue.

#Stained as frozen tissue only.

**Nonspecific staining of thyroglobulin.

double immunohistochemical stainings with the scFv derived from patient 1 and antibodies specific for these distinct subtypes (IgA, -D, -E, -G, and -M) were performed. As can be seen from Figure 2B and C, the patient-1 scFv clearly stained IgG- as well as IgA-positive plasma cells. Double immunohistochemical staining of IgD-, IgE-, and IgM-positive cells together with the patient-1 scFv could not be identified. These results show that the scFv derived from patient 1 recognizes a subpopulation of plasma cells that does not belong to a certain isotype.

Immunohistochemical staining of MALT lymphoma tissues

Tissue sections of 4 of the 7 MALT lymphomas used to generate the scFv's of this study were used to identify potential antigens specifically present in the tumor. Formalin-fixed and paraffin-embedded sections as well as frozen sections were stained using all 7 tumor scFv's, the Ber-H2 control scFv, and a "no antibody" control. The anti-CD30 Ber-H2 control scFv as expected visualized CD30-positive lymphoid cells. In contrast, none of the tumor scFv's produced specific signals. Again, in most sections a nonspecific staining of single cells (eg, granulocytes or glandular or muscle cells) was seen.

Reactivity against *H pylori* and superantigenic structures

Reactivity of the MALT lymphoma–derived scFv's against *H pylori* or potential superantigenic structures represented by the bacteria was assessed by Western blot analysis of native and denatured protein extracts of *H pylori* and by immunohistochemical stainings of formalin-fixed and paraffin-embedded sections of 5 different *H pylori*–positive gastric biopsies. With an anti–*H pylori* antibody, the presence of the bacterium could be demonstrated in all samples examined. Neither the Ber-H2 control scFv nor any tumor scFv showed any reactivity with *H pylori*–derived antigens in these experiments.

Screening of protein filters

The protein filters screened in this study comprised 2 PVDF membranes containing 27 648 proteins from human fetal brains.[27] The low degree of differentiation of this tissue results in the expression of a wide range of proteins and makes the filters an ideal tool to identify potential cellular binding partners of the tumor scFv's. The filters were exemplarily incubated with the scFv's of patients 1, 5, and 6. These scFv's were chosen because the MALT lymphomas they descend from originate from distinct organs (stomach, lung, and thyroid) and to further examine the reactivity of the patient-1 scFv observed by immunohistochemistry. Compared with the control incubations (anti-V5 antibody and anti–mouse HRP antibody alone), only the scFv of patient 1 showed specific binding to 2 proteins. Sequencing of the corresponding cDNA clones revealed that both clones harbored identical sequences, strongly arguing for the specificity of the binding of the patient-1 scFv. Comparison of the consensus sequence with

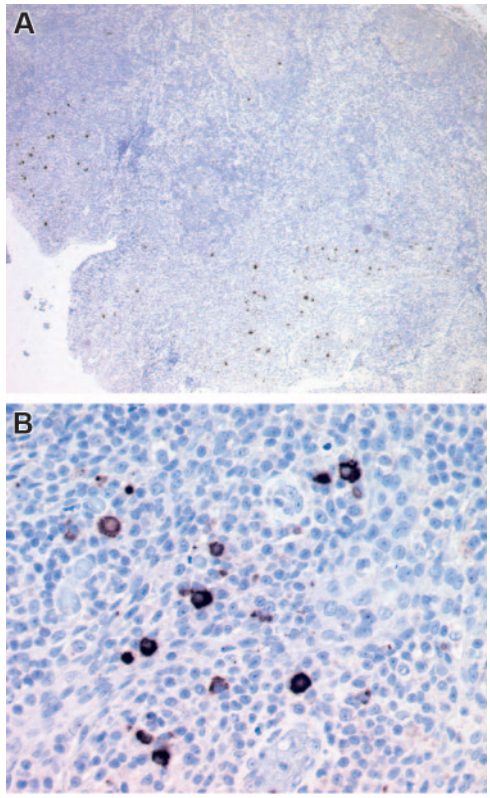

Figure 1. Immunohistochemical staining of human tonsil with the patient-1 scFv. Overview (A) and detail (B) of the distribution pattern and morphology of the cells stained by the patient-1 scFv (CSA amplification system; Dakocytomation). Images were obtained with an Olympus AX70 microscope (Olympus, Tokyo, Japan) equipped with a JVC KY-F70U camera (JVC, Kyoto, Japan) using Diskus v4.28 software (Hilgers, Königswinter, Germany). Images were captured with (A) a 4 ×/0.16 NA objective (total magnification, × 6.4) and (B) a 20 ×/0.7 NA objective (total magnification, × 140).

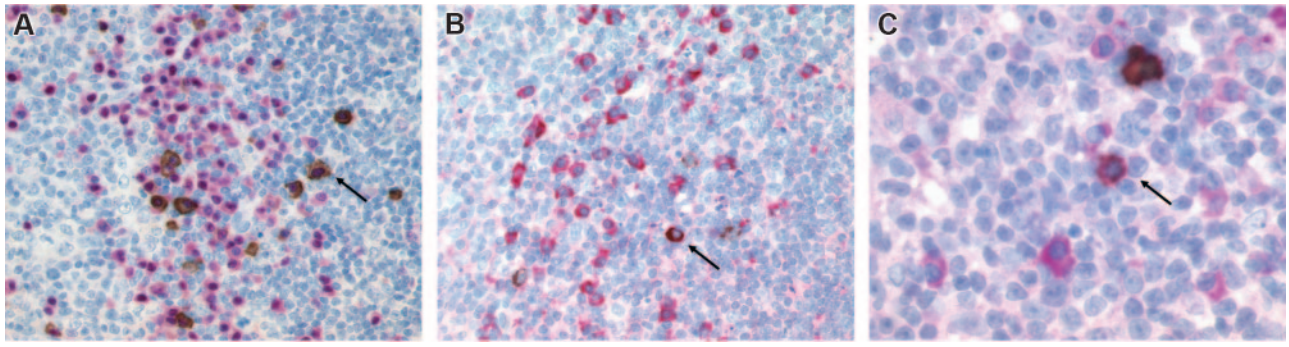

Figure 2: Immunohistochemical double stainings of human tonsil with the patient-1 scFv. Double stainings with the patient-1 scFv (brown) and antibodies against (A) the plasma cell–specific transcription factor IRF4 (red), (B) the immunoglobulin subclass G (red), and (C) the immunoglobulin subclass A (red). Arrows denote double-stained cells. Images were obtained with an Olympus AX70 microscope equipped with a JVC KY-F70U camera using Diskus v4.28 software. Objectives used were as follows: panels A and B, 20 ×/0.7 NA (total magnification, × 140); panel C, 40 ×/0.95 NA (total magnification, × 380).

nucleotide and protein databases discovered 100% homology to the gene coding for the Ufm1-conjugating enzyme *(UFC1)*.[32]

Screening of a peptide array

The peptide array consists of 5520 15-mer peptides of random sequence, yet the actual number of potential epitopes is much higher, since an epitope on average comprises 6 to 8 amino acids and each peptide therefore represents several overlapping epitopes. The randomly generated peptide sequences of this assay also allowed for screening of an extremely broad range of epitopes, including antigens other than autoantigens. Two identical arrays were synthesized and exemplarily incubated with the scFv of patient 4. Signals present in both experiments, but not detectable in the control incubations (anti-His HRP antibody alone) were evaluated as specific and mapped to the respective peptide sequences. Nine peptides were thereby identified as specific binding partners of the patient-4 scFv (Table 5). With the exception that 8 of the 9 peptides share tyrosine as their first amino acid, no sequence homologies could be detected among them. Also, no similarity to the translated sequence of the cDNA clone from the protein filter experiments was found. Submission of the peptide sequences to databases yielded no conclusive results.

Discussion

Several findings regarding clinical[1-7] and cellular biological[14,15,33] aspects of MALT lymphomas led to the concept that an antigenic stimulus marks the beginning of MALT lymphomagenesis, and that its sustained presence is necessary for maintenance of the tumor, at least in the early phase of tumor development.

From previous reports[19,20,34-36] it was concluded that the tumor immunoglobulins bind to autoantigens (eg, rheumatoid factor, follicular dendritic cells, epithelial cells, and connective tissue) and that this self-reactivity marks the beginning of the events that lead to MALT lymphoma formation. In most of these studies the immunoglobulins of the tumor cells were produced as monoclonal antibodies by heterohybridoma technology and used in immunohistochemical stainings of various normal human tissues. A drawback of these studies was that the identity of the hybridoma antibodies with the original tumor immunoglobulins was not demonstrated by comparison of the characteristic Ig gene rearrangements of the monoclonal antibodies and the tumor cells.

In order to examine the reactivity of the tumor immunoglobulins with (auto)antigens and the role of these interactions for tumor genesis, we synthesized the MALT lymphoma immunoglobulins as recombinant scFv's. This approach ensured that the specificity of the antibodies used in our study corresponds to that of the MALT lymphoma tumor cells. Furthermore, scFv's are an established tool for immunologic and immunotherapeutic studies. Their application to several laboratory methods[37,38] showed an identical affinity and a lower avidity (due to their monovalency) when compared with monoclonal antibodies. We could verify this observation with our anti-CD30 control scFv (Ber-H2 scFv), which showed specific but weaker binding to the CD30 antigen by FACS analysis and in immunohistochemical stainings of tonsils compared with the monoclonal Ber-H2 antibody. To compensate for the lower avidity in the immunohistochemical stainings we established a very sensitive amplification system. With this method the sensitivity was substantially increased and thus enabled demonstration of the CD30 antigen by the Ber-H2 scFv that equaled that of the Ber-H2 monoclonal antibody. In this way a sensitive and specific detection of the unknown antigens of the tumor scFv's by the immunohistochemical stainings was assured. The other assays employed (protein filter, peptide arrays) are inherently very sensitive due to their high antigen density, and an enhancement of the detection was not necessary. Recently, scFv's generated from the immunoglobulin genes of clonally expanded B cells from cerebrospinal fluid of patients with multiple sclerosis have been used successfully in immunohistochemical stainings to identify the former unknown specificity of these B cells.[39] This further argues for the suitability of our scFv-based approach for the detection of antigens potentially involved in MALT lymphomagenesis.

For expression of the cloned immunoglobulin genes as scFv antibodies *Drosophila S2* cells were chosen. Insect cell-expression systems have been shown to produce functional recombinant antibodies,[40-44] and they are able to perform a wide range of posttranslational modifications.[45]

Table 5. Sequences of the peptides recognized by the scFv of patient 4 on the peptide array

Peptide no.	Peptide sequence
1	YSGWYSLHTRMSMLV
2	YWFKMNHNMSPFILM
3	PMVFTHKIRMINHAS
4	YFHSMSKDSRGRNHT
5	YQNTSHWGTRWRSVL
6	YYYQRFNAMILAMFF
7	YTLRFAYKRDPFRER
8	YRKWDYERFYVDPDG
9	YKWRTELYWNTWGFT

The analysis of the rearranged Ig gene sequences of the 7 MALT lymphomas (Table 3) revealed that the VH segments used in 6 of the 7 rearrangements (*VH1*-02, *VH1*-69, *VH3-07*, and *VH3-23*) are frequently used by autoantibodies.[46-49] This indicates that the tumor immunoglobulins might indeed be directed against self-antigens. The Ig genes of all patients displayed somatic mutations consistent with the derivation of the tumor cells from memory B cells. To examine the antigen selection of the Ig sequences, 2 widely accepted methods were used. Both calculations yielded differing results (one patient vs 4 patients). This discrepancy demonstrates that the determination of antigen selection by mathematical models does not cover all aspects of a complex biological process such as somatic hypermutation, and has only limited significance. Thus, we could not clearly assign a selection by antigen to our sequences as done in other studies.[12,14,15] In 4 of the 7 patients (patients 3, 4, 5, and 7) intraclonal variations, resulting from ongoing somatic mutations and/or from receptor revision,[16] were present in the immunoglobulin genes. These immunoglobulin gene modifications are physiologically introduced during the germinal center reaction and lead, after antigen selection, to high-affinity memory B cells. However, MALT lymphomas do not display features of germinal center–derived lymphomas, and antigen-presenting cells are usually missing. We therefore suggest that the ongoing mutational process in MALT lymphomas is presumably the result of an erroneous activation of the immunoglobulin mutation machinery. This notion is well in line with our observation that different methods for the mathematical estimation of antigen selection demonstrate discrepant results.

One approach to study the specificity of the tumor scFv's was the immunohistochemical staining of a broad range of normal and pathologic human tissues. This method was chosen to detect protein and nonprotein (auto)antigens. The analysis revealed no specific binding of the tumor scFv's to structures in normal human tissues, a finding in contrast to previous reports.[20,21,34-36] Only background signals, also present in the negative control or with the Ber-H2 control scFv, were observed in some tissues. An exception was the staining of cells with plasma cell morphology in tonsillar sections by the scFv of patient 1. Double immunohistochemical stainings with this scFv and an antibody against the transcription factor IRF4[50] confirmed the plasma cell identity of the cells marked by the scFv of patient 1. However, only a fraction of plasma cells was labeled, leaving the majority unstained by the scFv of patient 1. The labeled plasma cells belong to different immunoglobulin subclasses as shown in further double immunohistochemical stainings (Figure 2). Reactivity of a tumor immunoglobulin against a subclass independent antigen on plasma cells has also been reported by Greiner at al.[21] In the tissue sections of 4 MALT lymphomas (patients 1-4), no reactivity of any of the 7 tumor scFv's could be found, arguing against a potential antigenic stimulus in the tumor vicinity. Screening of sections of *H pylori*–positive gastric biopsies and of native and denatured *H pylori* lysates by Western blot also revealed no reactivity of the tumor scFv's. Thus, *H pylori* does not appear to be a direct antigenic stimulus for the tumor cells. This is in agreement with previous observations.[21] The screening of our scFv's against *H pylori* also aimed at the identification of superantigenic structures that might be shared by several antigens. Such a common epitope could help to provide a link between the development of gastric and nongastric MALT lymphomas. In summary, of the 7 tumor scFv's only the scFv of patient 1 showed binding to antigen in the immunohistochemical stainings of a wide range of formalin-fixed and frozen tissues.

Another approach to determine potential binding partners of the tumor scFv's was the screening of the protein filter set. As in the immunohistochemical stainings, specific signals were only detected by the scFv of patient 1. The corresponding cDNA was identified as coding for the Ufm1-conjugating enzyme (Ufc1).[32] Its mRNA is reported to be ubiquitously expressed in human tissues; however, a significantly higher expression level has been observed in plasma cell lines (D.L. and M.H., unpublished data, October 2003). This is in agreement with the binding of the scFv of patient 1 to plasma cell antigens as shown by immunohistochemistry. That no binding of this scFv to Ufc1 protein was found in immunohistochemical stainings of human tissues, despite the ubiquitous expression pattern of Ufc1 mRNA, might be ascribed to a cellular protein concentration too low for immunohistochemical detection.

Further experiments were conducted with a peptide array. Nine potential binding partners (Table 5) were identified but no significant homologies between them or to other proteins were observed. This might be due to the fact that the peptide array used in this study is designed for detection of linear antigen epitopes, encoded by the primary sequence of a protein. In such epitopes not all amino acids are equally involved in antibody binding. For the peptides bound by the patient-4 scFv this means only some amino acids are essential for binding to the scFv, whereas others could be exchanged by amino acids with similar physicochemical properties in the native epitope. This could be an explanation for the lack of homology of the 9 peptides with database entries. In summary, the peptide array provided no conclusion in regards to a potential binding partner of the scFv.

Although numerous evidences point at an interaction of the immunoglobulins of MALT lymphoma tumor cells with antigens, this hypothesis was not confirmed by the results presented here. Only 2 of the 7 scFv's examined showed binding to potential antigens, and a role of these proteins for MALT lymphoma development could not be determined. In contrast, previous studies detected binding partners for the tumor immunoglobulins in almost all patients examined.[20,21,35,36] The discrepant results might be explained by the use of hybridoma antibodies in these studies that did not unequivocally represent the tumor immunoglobulins and thus led to an overestimation of the reactivity of the MALT lymphoma immunoglobulins with (auto)antigens. In a recent study the immunoglobulins of some gastric and salivary gland MALT lymphomas were shown to bind to a hitherto undescribed target, the Fc portion of IgG (rheumatoid factor).[19] However, such reactivity was not found in our scFv's despite the application of highly sensitive techniques.

The fact that, in our study, the majority of the tumor cell immunoglobulins displayed no binding to antigen suggests that the tumor Ig's do not play a significant role in stimulation and proliferation of the MALT lymphoma tumor cells. Instead, activation of the tumor B cells might be mediated by other receptors participating in antigen-induced signaling. This is in line with the in vitro finding that tumor-infiltrating T cells are important for proliferation of MALT lymphoma tumor cells in the presence of *H pylori*.[17,18]

References

1. Jonsson V, Wiik A, Hou-Jensen K, et al. Autoimmunity and extranodal lymphocytic infiltrates in lymphoproliferative disorders. J Intern Med. 1999; 245:277-286.
2. Pileri S, Piccaluga PP, De Vivo A, et al. Malignant lymphomas of the gastro-intestinal tract: a reappraisal on the basis of the newly proposed Revised European American Lymphoma Classification. Ital J Gastroenterol. 1994;26:405-418.
3. Isaacson PG. Pathogenesis and early lesions in extranodal lymphoma. Toxicol Lett. 1993;67:237-247.
4. Wotherspoon AC, Ortiz-Hidalgo C, Falzon MR, Isaacson PG. Helicobacter pylori-associated gastritis and primary B-cell gastric lymphoma. Lancet. 1991;338:1175-1176.
5. Nakamura S, Yao T, Aoyagi K, et al. Helicobacter pylori and primary gastric lymphoma: a histo-

pathologic and immunohistochemical analysis of 237 patients. Cancer. 1997;79:3-11.

6. Wotherspoon AC, Doglioni C, Diss TC, et al. Regression of primary low-grade B-cell gastric lymphoma of mucosa-associated lymphoid tissue type after eradication of Helicobacter pylori. Lancet. 1993;342:575-577.
7. Montalban C, Santon A, Boixeda D, et al. Treatment of low grade gastric mucosa-associated lymphoid tissue lymphoma in stage I with Helicobacter pylori eradication: long-term results after sequential histologic and molecular follow-up. Haematologica. 2001;86:609-617.
8. Wotherspoon AC, Pan LX, Diss TC, Isaacson PG. Cytogenetic study of B-cell lymphoma of mucosa-associated lymphoid tissue. Cancer Genet Cytogenet. 1992;58:35-38.
9. Dierlamm J, Michaux L, Wlodarska I, et al. Trisomy 3 in marginal zone B-cell lymphoma: a study based on cytogenetic analysis and fluorescence in situ hybridization. Br J Haematol. 1996; 93:242-249.
10. Horsman D, Gascoyne R, Klasa R, Coupland R. t(11;18)(q21;q21.1): a recurring translocation in lymphomas of mucosa-associated lymphoid tissue (MALT)? Genes Chromosomes Cancer. 1992;4:183-187.
11. Streubel B, Lamprecht A, Dierlamm J, et al. T(14; 18)(q32;q21) involving IGH and MALT1 is a frequent chromosomal aberration in MALT lymphoma. Blood. 2003;101:2335-2339.
12. Qin Y, Greiner A, Trunk MJ, et al. Somatic hypermutation in low-grade mucosa-associated lymphoid tissue-type B-cell lymphoma. Blood. 1995; 86:3528-3534.
13. Bertoni F, Cazzaniga G, Bosshard G, et al. Immunoglobulin heavy chain diversity genes rearrangement pattern indicates that MALT-type gastric lymphoma B cells have undergone an antigen selection process. Br J Haematol. 1997;97:830-836.
14. Du M, Diss TC, Xu C, et al. Ongoing mutation in MALT lymphoma immunoglobulin gene suggests that antigen stimulation plays a role in the clonal expansion. Leukemia. 1996;10:1190-1197.
15. Qin Y, Greiner A, Hallas C, Haedicke W, Muller-Hermelink HK. Intraclonal offspring expansion of gastric low-grade MALT-type lymphoma: evidence for the role of antigen-driven high-affinity mutation in lymphomagenesis. Lab Invest. 1997; 76:477-485.
16. Lenze D, Greiner A, Knorr C, et al. Receptor revision of immunoglobulin heavy chain genes in human MALT lymphomas. Mol Pathol. 2003;56:249-255.
17. Hussell T, Isaacson PG, Crabtree JE, Spencer J. The response of cells from low-grade B-cell gastric lymphomas of mucosa-associated lymphoid tissue to Helicobacter pylori. Lancet. 1993;342: 571-574.
18. Hussell T, Isaacson PG, Crabtree JE, Spencer J. Helicobacter pylori-specific tumour-infiltrating T cells provide contact dependent help for the growth of malignant B cells in low-grade gastric lymphoma of mucosa-associated lymphoid tissue. J Pathol. 1996;178:122-127.
19. Bende RJ, Aarts WM, Riedl RG, et al. Among B cell non-Hodgkin's lymphomas, MALT lymphomas express a unique antibody repertoire with frequent rheumatoid factor reactivity. J Exp Med. 2005;201:1229-1241.
20. Hussell T, Isaacson PG, Crabtree JE, Dogan A, Spencer J. Immunoglobulin specificity of low grade B cell gastrointestinal lymphoma of mucosa-associated lymphoid tissue (MALT) type. Am J Pathol. 1993;142:285-292.
21. Greiner A, Marx A, Heesemann J, et al. Idiotype identity in a MALT-type lymphoma and B cells in Helicobacter pylori associated chronic gastritis. Lab Invest. 1994;70:572-578.
22. World Health Organization Classification of Tumours: Pathology and Genetics of Tumours of Haematopoetic and Lymphoid Tissues. IARC Press; Lyon, France: 2001.
23. Schwarting R, Gerdes J, Durkop H, et al. BER-H2: a new anti-Ki-1 (CD30) monoclonal antibody directed at a formol-resistant epitope. Blood. 1989;74:1678-1689.
24. Marafioti T, Hummel M, Anagnostopoulos I, et al. Origin of nodular lymphocyte-predominant Hodgkin's disease from a clonal expansion of highly mutated germinal-center B cells. N Engl J Med. 1997;337:453-458.
25. van Dongen JJ, Langerak AW, Brüggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98–3936. Leukemia. 2003;17:2257-2317.
26. Lossos IS, Tibshirani R, Narasimhan B, Levy R. The inference of antigen selection on Ig genes. J Immunol. 2000;165:5122-5126.
27. Bussow K, Cahill D, Nietfeld W, et al. A method for global protein expression and antibody screening on high-density filters of an arrayed cDNA library. Nucleic Acids Res. 1998;26:5007-5008.
28. Reineke U, Kramer A, Schneider-Mergener J. Epitope mapping with synthetic peptides prepared by SPOT synthesis. In: Konterman R, Dübel S, eds. Antibody Engineering (Springer Lab Manual). Berlin: Springer Verlag; 2001:443-459.
29. Volkmer-Engert R, Hoffmann B, Schneider-Mergener J. Stable attachment of the HMB-linker to continuous cellulose membranes for parallel solid phase spot synthesis. Tetrahedron Lett. 1997;38: 1029-1032.
30. Frank R. Spot-synthesis: an easy technique for the positionally adressable, parallel chemical synthesis on a membrane support. Tetrahedron. 1992;48:9217-9232.
31. Durkop H, Anagnostopoulos I, Bulfone-Paus S, Stein H. Expression of several members of the TNF-ligand and receptor family on tonsillar lymphoid B cells. Br J Haematol. 1997;98:863-868.
32. Komatsu M, Chiba T, Tatsumi K, et al. A novel protein-conjugating system for Ufm1, a ubiquitin-fold modifier. EMBO J. 2004;23:1977-1986.
33. Nakamura S, Aoyagi K, Furuse M, et al. B-cell monoclonality precedes the development of gastric MALT lymphoma in Helicobacter pylori-associated chronic gastritis. Am J Pathol. 1998;152: 1271-1279.
34. Greiner A, Qin Y, Knorr C, et al. Autoantigen receptors in extranodal non-Hodgkin B-cell lymphomas. Verh Dtsch Ges Pathol. 1996;80:160-168.
35. Greiner A, Marx A, Schmausser B, Muller-Hermelink HK. The pivotal role of the immunoglobulin receptor of tumor cells from B cell lymphomas of mucosa associated lymphoid tissue (MALT). Adv Exp Med Biol. 1994;355:189-193.
36. Greiner A, Knorr C, Qin Y, et al. CD40 ligand and autoantigen are involved in the pathogenesis of low-grade B-cell lymphomas of mucosa-associated lymphoid tissue. Dev Immunol. 1998;6:187-195.
37. Kim DJ, Chung JH, Ryu YS, et al. Production and characterisation of a recombinant scFv reactive with human gastrointestinal carcinomas. Br J Cancer. 2002;87:405-413.
38. Tungpradabkul S, Sandee D, Puthong S, Laohathai K. Construction of scFv derived from a tumor-associated monoclonal antibody having tumoricidal activity on human hepatocellular carcinoma. Mol Immunol. 2005;42:713-719.
39. Zhang Y, Da RR, Guo W, et al. Axon reactive B cells clonally expanded in the cerebrospinal fluid of patients with multiple sclerosis. J Clin Immunol. 2005;25:254-264.
40. Reavy B, Ziegler A, Diplexcito J, et al. Expression of functional recombinant antibody molecules in insect cell expression systems. Protein Expr Purif. 2000;18:221-228.
41. Mahiouz DL, Aichinger G, Haskard DO, George AJT. Expression of recombinant anti-E-selectin single-chain Fv antibody fragments in stably transfected insect cell lines. J Immunol Methods. 1998;212:149-160.
42. Lemeulle C, Chardes T, Montavon C, et al. Anti-digoxin scFv fragments expressed in bacteria and in insect cells have different antigen binding properties. FEBS Lett. 1998;423:159-166.
43. Bei R, Schlom J, Kashmiri SV. Baculovirus expression of a functional single-chain immunoglobulin and its IL-2 fusion protein. J Immunol Methods. 1995;186:245-255.
44. Hasemann CA, Capra JD. High-level production of a functional immunoglobulin heterodimer in a baculovirus expression system. Proc Natl Acad Sci U S A. 1990;87:3942-3946.
45. Murphy CI, Piwnica-Worms H. Overview of the baculovirus expression system. In: Current Protocols in Protein Science. John Wiley and Sons Inc.; 1995:.
46. Sanz I, Casali P, Thomas JW, Notkins AL, Capra JD. Nucleotide sequences of eight human natural autoantibody VH regions reveals apparent restricted use of VH families. J Immunol. 1989;142: 4054-4061.
47. Sanz I, Dang H, Takei M, Talal N, Capra JD. VH sequence of a human anti-Sm autoantibody: evidence that autoantibodies can be unmutated copies of germline genes. J Immunol. 1989;142:883-887.
48. Lecerf JM, Chen Y, Richalet-Secordel P, Wang X, Stollar BD. Autoreactivity of human VH domains from cDNA libraries: analysis with a bacterial expression system. J Immunol. 1998;161:1274-1283.
49. Aguilera I, Melero J, Nunez-Roldan A, Sanchez B. Molecular structure of eight human autoreactive monoclonal antibodies. Immunology. 2001; 102:273-280.
50. Falini B, Fizzotti M, Pucciarini A, et al. A monoclonal antibody (MUM1p) detects expression of the MUM1/IRF4 protein in a subset of germinal center B cells, plasma cells, and activated T cells. Blood. 2000;95:2084-2092.
51. ImMunoGenetics. The International Immunogenetics Information System. http://imgt.cines.fr: 8104/home.html. Accessed December 14, 2005.
52. Lossos IS, Tibshirani R, Narasimhan B, Levy R. Calculation of antigen selection pressure on immunoglobulin genes. http://www-stat.stanford.edu/immunoglobulin. Accessed December 14, 2005.
53. Wellcome Trust Sanger Institute. Protein Families Database of Alignments and HMMs. http://www.sanger.ac.uk/Software/Pfam. Accessed December 14, 2005.

2006 107: 1141-1148
doi:10.1182/blood-2005-04-1722 originally published online October 4, 2005

Influence of antigen on the development of MALT lymphoma

Dido Lenze, Erika Berg, Rudolf Volkmer-Engert, Armin A. Weiser, Axel Greiner, Constanze Knörr-Wittmann, Ioannis Anagnostopoulos, Harald Stein and Michael Hummel

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.