Natural IgM Antibodies and Immunosurveillance Mechanisms against Epithelial Cancer Cells in Humans

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ABSTRACT

Malignancy is like a chronic disease, and the immune system is permanently involved in recognizing and eliminating the transformed cells. The human hybridoma technology offers the unique opportunity to study the mechanisms, structures, and targets involved in recognition and elimination of aberrant cells. Thousands of tumor-reactive human monoclonal antibodies were isolated by this technique from cancer patients and from healthy donors, and all of these antibodies were IgM antibodies; no IgG and IgA antibodies were found. Fourteen of these antibodies were selected for DNA sequence analysis, characterization of their binding patterns, and determination of their origin and genetics. All of the IgM antibodies studied expressed only few or no mutations at all (germ-line coded), bound to carbohydrates on modified tumor-specific receptors and induced apoptosis. The degree of cross-reactivity to other tumors correlated reciprocally with the number of mutations in coding regions. By using an anti-idiotypic antibody we were able to show that the IgM-producing cells were of CD5+ B-cell origin. The data presented here indicate that the innate immunity and natural IgM antibodies play an important role in immunosurveillance mechanisms against epithelial tumors in humans.

INTRODUCTION

During its lifetime each multicellular organism is permanently exposed to transformed cells, which arise spontaneously or by inducing factors. Even if not every transformed cell has the ability and potency for malignant behavior, the important question is not why malignant cells arise, but instead, why malignancy occurs so frequently (1). An early recognition and a rapid elimination of transformed cells by immune mechanisms is essential for keeping an organism free of tumor cells. The innate or natural immunity, a part of the immune system, which has for a long time been defined by immunologists as unspecific background immunity, seems to be of greater importance than has been thought (2). The importance of natural killer cells and natural antibodies for the defense against bacterial infections has been shown in detail, but only very little data on immunity against transformed cells, supporting a specific role of natural killer cells and natural antibodies for the defense against transformed cells (11–13, 15–19) both in vitro and in experimental in vivo systems (9, 11, 16). Clinical studies showed that SC-1 induced regression and apoptosis of primary stomach cancers without any toxic cross-reactivity with normal tissue (17).

The fully human germ-line coded monoclonal IgM antibody PAM-1 was isolated from a patient with a stomach carcinoma, too (20). PAM-1 binds to a membrane receptor present on almost all of the epithelial cancers of every type and origin. When evaluated on nonmalignant tissue, the only PAM-1-specific reactivity was an intracellular binding to proteins in the Golgi apparatus of the kidney (14). The PAM-1 receptor was purified from tumor cell membrane extracts and was found to be a M_130,000 integral membrane glycoprotein (14), homologous to cysteine-rich fibroblast growth factor receptor 1, which has thus far only been detected and described in Golgi of embryonic chicken cells and in Chinese hamster ovary cells (21). This post-transcriptionally modified receptor is expressed on almost all epithelial cancers of every type and origin, but not on healthy tissue. It is also present on precursor lesions found in: Helicobacter pylori-induced gastritis, intestinal metaplasia, and dysplasia of the stomach; ulcerative colitis-related dysplasia and adenomas of the colon; Barrett metaplasia and dysplasia of the esophagus; squamous metaplasia and dysplasia of the lung; and cervical intraepithelial neoplasia I-III (5, 14).

In this study we investigated the origin, reactivity patterns, and genetics of tumor-specific human monoclonal IgM antibodies isolated from cancer patients and healthy donors. We give evidence that innate immunity is not only responsible for the recognition and elimination of bacterial structures but also for the removal of transformed epithelial cells.

MATERIALS AND METHODS

Somatic Hybridization. Hybridomas were prepared and cultured as described previously (9). Briefly, lymph nodes or spleens obtained from lung-, pancreas-, or colon-carcinoma patients, or healthy donors during surgery were prepared by mechanical disruption of the tissue. The resulting single cell suspension was fused at a 2:1 ratio with the heteromyeloma HAB-1 with PEG1500, followed by subsequent distribution on 24-well plates. Hybridomas were cultured in RPMI 1640 containing 10% FCS and Hypoxanthin Aminopterin Thymidin (HAT) supplement (PAA, Pasching, Austria). After 4–6 weeks the supernatants were screened for antibody production in an ELISA. The average growth rate was ~80%, ~50% of the grown clones produced immu-
noglobulins. Positive clones were tested immunohistochecmically on autolo-
gous tumor tissue sections, and clones, which showed a positive reaction, were
subsequently recloned.

dNA Synthesis and Reverse Transcription-PCR. Total RNA was pre-
pared as described previously (22). dDNA synthesis from total RNA obtained
from hybridoma cell lines was performed with 5 g total RNA using Life
Technologies, Inc. (Eggenstein, Germany) Moloney murine leukemia virus
reverse transcriptase according to the manufacturer’s instructions. The ampli-
fication of VH and VL genes was carried out in a 25 ml volume with 1.75 mM
MgCl2, 0.4 pm primer, 200 mM of each dNTP, and 1 unit Taq polymerase (MBI
Fermentas, St. Leon-Rot, Germany). The PCR products were amplified using
the following cycle profiles: 95°C for 2 min, followed by 35 cycles of 94°C for
30 s, 65°C for 30 s (for VH and VH4 primers), 60°C for VH1, VH2, VH5,
VH6, and 52°C for VL primers, respectively, with a final extension at 72°C for
4 min.

Sequence Analysis. PCR products were purified by gel electrophoresis
through 2% agarose (Roht, Karlruhe, Germany) and extraction from gel using
a Jetseq gel extraction kit (Genomed, Bad Oeynhausen, Germany). Cloning of
PCR fragments was performed with pCR-Script Amp SK (+) cloning kit
(Stratagene, Heidelberg, Germany). Ten positive clones were sequenced using
the DyeDeoxy Termination Cycle Sequencing kit (Applied BioSystems Inc.,
Weiterstadt, Germany) and analyzed with an automated DNA sequencing ABI-
Prism373. Both strands were sequenced using T3 and T7 primers. The se-
quences were analyzed using DNASIS for Windows software and IMGT/V-
QUEST databases.

Glycosidase Assays. Detached and washed cells were resuspended in
RPMI 1640 containing 10% FCS and incubated for 1 h on ice, then counted,
and cytospins were prepared. After air-drying, cytospin preparations were
acetone-fixed (10 min), washed, and incubated with 20/mL O-glycosidase or
5 mU/mL N-glycosidase (Boehringer, Mannheim, Germany) for 4 h at 37°C.

Then, slides were washed and stained immunohistochemically.

Immunohistochemical Staining of Paraffin Sections. Paraffin-embedded
human tissues were sectioned (2), immunostained using a jarbogel extraction
kit (Genomed, Bad Oeynhausen, Germany). Cloning of PCR fragments was per
formed with pCR-Script Amp SK (+) cloning kit (Stratagene, Heidelberg,
Germany). Ten positive clones were sequenced using the DyeDeoxy Termination
Cycle Sequencing kit (Applied BioSystems Inc., Weiterstadt, Germany) and
analyzed with an automated DNA sequencing ABI-Prism373. Both strands were
sequenced using T3 and T7 primers. The sequences were analyzed using
DNASIS for Windows software and IMGT/V-QUEST databases.

Immunohistochemical Staining of Acetone-Fixed Cylots. The cytosplns were
blocked for 30 min with PBS/BSA (0.1%). After washing with Tris/NaCl
three times, the sections were incubated with the different antibodies or
mouse anticytokeratin 8 antibody diluted 1:50 with PBS/BSA (Dako, Ham-
bury, Germany) for 30 min as a positive control. Then they were washed three
times with Tris/NaCl, followed by incubation with secondary antibodies
(peroxidase-labeled rabbit antihuman or rabbit antimonochrome conjugate 1:50)
for 30 min. After washing three times with Tris/NaCl and incubation in PBS for
10 min, staining was performed with diaminobenzidine (0.05%)-hydrogen perox-
ide (0.02%) for 10 min at room temperature. The reaction was stopped under
running tap water, and the sections were counterstained with hematoxylin.

After mounting with glycerol-gelatin, the sections were analyzed using light
microscopy.

MTT1 Proliferation Assay. The MTT assay using the human stomach
adenocarcinoma cell line 23132/87 was performed as described (20). This
carcinoma cell line was derived from a freshly prepared primary culture of a
gastric tumor patient (23, 24). In general, for the tests early passages (<10)
were used to avoid cell culture artifacts. Briefly, trypsinized cells were diluted to
1 10^6 cells/ml in complete growth medium, and 50 g of cell suspension
was added to each well of a 96-well plate. Fifty g of the different antibodies
and controls diluted with complete growth medium was then added to the
wells, and the plates were incubated for 48 h at 37°C in a humidified incubator.
To demonstrate normal growth, the cells were supplemented with complete
growth medium (control 1). Unrelated human IgMs in the same concentration
(Chrompure IgM; Dianova) served as the negative control (control 2). For
measurement, 50 g of MTT solution (5 mg/ml) were added to each well, and
the plates were incubated for 30 min at 37°C. After incubation, the plates
were centrifuged at 800 g for 5 min followed by the removal of the MTT solution.
The stained cell pellet was dissolved in 150 g dimethylsulphoxide, and
absorption was measured at wavelengths of 540 nm and 690 nm.

Apoptosis Assay. The extent of antibody-induced apoptosis on human
stomach carcinoma cells (23132/87) was analyzed by the Cell Death Detection
ELISA kit (Roche, Mannheim, Germany). For this assay 1 10^6 tumor cells
were used and 96-well plates and incubated in the presence of the
different IgM antibodies for 48 h at 37°C and 7% CO2 in a humidified
incubator. To demonstrate normal growth, the cells were supplemented with
complete growth medium (control 1). Unrelated human IgMs in the same
concentration (Chrompure IgM; Dianova) served as the negative control (con-
rol 2). After incubation the cells were centrifuged for 10 min at 200 g, and
the supernatants were removed followed by an incubation with lysis buffer for
30 min at room temperature. After centrifugation the supernatants were
transferred into a streptavidin-coated microtiter plate, and immunoreagent was
added (mixture of 10% antihistone-biotin, 10% anti-DNA peroxidase (POD),
and 80% incubation buffer) and incubated for 2 h at room temperature on a
microtiter plate shaker at 250 rpm. After incubation, unbound components
were removed by washing with incubation buffer. POD was determined
photometrically with an ABTS substrate (1 ABTS tablet in 5-ml substrate
buffer). The antibody-induced apoptosis was measured at 405 nm against
ABTS solution as a blank (reference wavelength 740 nm).

Anti-Idiotype Antibody. BALB/c mice were immunized three times with
50 g purified SC-1 antibody, and after 14 days spleen cells were immortal-
ized by fusion to the mouse myeloma cells (Ag-8). Resulting monoclonal
antibodies were tested in an ELISA for binding on a panel of human IgM
antibodies, including SC-1. The murine monoclonal IgG antibody produced by
clon 6/22 was the only one, which was found to be specific for SC-1.

Immunofluorescence Staining. For the double staining assay, cryosec-
tions of human lymphatic tissue were incubated with the anti-idiotype anti-
body 6/22, a pan-B antibody and an anti-IgM antibody for 30 min at room
temperature, and subsequently incubated with a rabbit-antimouse secondary
antibody coupled with FITC or tetramethylrhodamine isothiocyanate for ad-
nitional 60 min at room temperature. After mounting with glycerol-gelatin, the
sections were analyzed using a fluorescence microscope.

RESULTS

IgM Antibodies from Tumor Patients. Lymphocytes from spleen, lymph
nodes, and the tumor itself obtained from >60 cancer patients with different
carcinomas served as sources for ~260 fusion experiments. No preselection regarding age and gender of patients,
tumor staging, and grading, was done. Comparing the lymphocyte sources, the best fusion efficacy was obtained with lymphocytes from spleens (Table 1). Altogether >18,000 growing triomas were investigated in an ELISA test for antibody production, and about half of them produced human immunoglobulins. Triomas from stomach, pancreas, lung, and breast cancer patients produced an excess of antibodies of IgM isotype, whereas triomas from colon and prostate cancer patients were more balanced with regard to the amount of IgG and IgA immunoglobulins.

The ELISA-positive clones were additionally tested for tumor-specific reactivity by immunohistochemistry on a panel of cancerous and noncancerous tissues. The results showed that all of the tumor-specific antibodies were of IgM isotype (for details see Table 1).

**Specificity of IgM.** The reaction patterns of the human monoclonal antibodies were investigated in more detail by immunohistochemistry on a broad panel of cryo- and paraffin-embedded primary carcinomas and normal tissues. The tissues were not preselected regarding age and gender of the patients. Three to 30 different cases of each tumor type were tested. The specimens were taken from different tumor stages and grades to analyze reaction patterns of the antibodies for a spectrum as broad as possible of different epithelial malignancies.

The tested antibodies showed a heterogeneous reactivity pattern with tumor tissues. The antibodies PAM-1, LM-1, PM-2, SAM-3, SAM-4, SAM-6, PM-1, and CM-1 reacted with most of the carcinomas tested in this study, whereas the reactivity of the antibodies SC-1, CM-2, and SAM-2 was restricted to specific carcinomas (for details see Table 2). Fig. 1 shows some examples of tumor-specific staining patterns of several antibodies. All of the tested antibodies showed a specific intensive staining of tumor cells, whereas the surrounding tissue was not stained. On normal tissues none of the antibodies exhibited any binding activity (data not shown).

**Targets of IgM.** In addition to the specificity analysis on tissues we performed some studies on the antibody reactivity. Previously published results indicated that the tumor-specific IgM antibodies SC-1 and PAM-1 bind to carbohydrate residues on the corresponding receptors, which are post-transcriptionally modified (13, 14). On the basis of these data the binding characteristics of selected IgM antibodies were analyzed. Cytospin preparations of tumor cells were incubated with O- and N-glycosidase, and after that tested by immunohistochemistry for binding of the IgM antibodies. It was found that four of the five antibodies tested bound to a carbohydrate residue on specific receptors. For example, as shown in Fig. 2, exemplary treatment of lung carcinoma cells with N-glycosidase led to a dramatic decrease in LM-1 antibody staining (Fig. 2D), whereas incubation with dephosphorylation buffer or digestion with O-glycosidase (data not shown) had no effect on the binding of the antibody LM-1.

**Genetics of IgM.** To investigate the genetic origin of these human monoclonal IgM antibodies, the variable gene regions of the immunoglobulin heavy (VH) and light chain (VL) genes were amplified, cloned, and sequenced. The sequences were compared with germ-line sequences of the corresponding germ-line genes to detect somatic mutations. The results are presented in Table 3, A and B. The degree...
of identity of the nucleotide sequences of the VH segment to those of the closest reported germ-line VH genes ranged from 92.3 to 100%, as summarized in Table 3A. The antibodies were expressed by different VH genes of the VH3, and VH5 gene family. Interestingly an accumulation of several families was observed. Most of the antibodies were expressed by VH genes of DP49 and DP47. The close homology of the VH regions to the germ-line genes and the low R:S ratio, which is an indicator for affinity maturation of antibodies, indicated that none of the antibodies studied was affinity maturated by somatic mutation due to antigen contact.

The degree of identity of the nucleotide sequences of the VL segment to those of the closest reported germ-line VL genes ranged from 94.8 to 100% (Table 3B). All but one of the antibodies used \(\lambda\)-light chain genes. The R:S ratio was again low. The antibodies were expressed by different VL genes of the VL1, VL2, VL3, and VL5 gene family. A preference of the VL3 gene family was observed, but other gene families were used, too.

**Functional Activity of IgM.** To examine the functional activity of the antibodies isolated from cancer patients _in vitro_, we used the colorimetric mitochondrial hydroxylase assay (MTT), which is a standard assay for proliferation (13).

In this assay primary cultures of human carcinoma cells were incubated with the IgM antibodies SAM-4, SAM-5, and SAM-6. After 48 h all of the tested antibodies inhibited cell proliferation, whereas in the controls with human unrelated IgM no inhibition of cell proliferation was observable (Fig. 3A).

For additional investigation of the inhibitory effect of the antibodies on tumor cell growth, the Cell Death Detection ELISA PLUS kit was used. This apoptosis assay enables a clear distinction between necrosis and specific antibody-induced apoptosis. The experiments clearly showed that each antibody induced apoptosis in carcinoma cells after 48 h of incubation (Fig. 3B).

**IgM Antibodies from Healthy Donors.** To prove that natural IgM antibodies with tumor specificity exist in humans, spleen cells from
Fig. 2. N-Glycosidase treatment of lung carcinoma cells. Cytospin preparations of the lung adenocarcinoma cell line Colo-699 were treated with N-Glycosidase and stained with anticytokeratin 8 and antibody LM-1. A, anticytokeratin 8 before treatment; B, antibody LM-1 before treatment; C, anticytokeratin 8 after treatment; and D, antibody LM-1 after treatment with N-glycosidase.

healthy donors were immortalized with the heteromyeloma HAB-1, and the resulting antibodies were tested for antitumor activity. From a small series of clones two IgM antibodies were selected, which showed a positive reaction with different tumor tissues but not with normal nontransformed cells. Figs. 4 and 5 show an example of the reactivity patterns of the antibodies NORM-1 and NORM-2 on several tumor tissues in comparison with staining data on normal tissues of the same organs. The antibodies isolated from healthy donors showed a specific staining of tumor cells, whereas the surrounding tissue and normal tissue was not stained.

Genetics of NORM-1 and NORM-2. To investigate the genetic origin of the human monoclonal IgM antibodies, which were isolated from healthy donors, the VH and VL genes were amplified, cloned, and sequenced. The sequences were compared with germ-line sequences in the IMGT/V-QUEST database to identify the most homologous germ-line genes and to detect somatic mutations. The results are presented in Table 4. The degree of identity of the nucleotide

Table 3. Sequence-analysis of human monoclonal IgM antibodies isolated from cancer patients

<table>
<thead>
<tr>
<th>Antibody</th>
<th>VH-family</th>
<th>Germ-line gene</th>
<th>Homology [%]</th>
<th>R.S FR</th>
<th>R.S CDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC-1</td>
<td>VH3</td>
<td>DP-49, (IgHV3-30/30.5*01)</td>
<td>97</td>
<td>4/2</td>
<td>2/0</td>
</tr>
<tr>
<td>PAM-1</td>
<td>VH3</td>
<td>DP-49, (IgHV3-30/30.5*01)</td>
<td>100</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>CM-1</td>
<td>VH3</td>
<td>DP-49, (IgHV3-30/30.5*01)</td>
<td>100</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>SAM-2</td>
<td>VH3</td>
<td>DP-49, (IgHV3-30/30.5*01)</td>
<td>92.3</td>
<td>2/2</td>
<td>5/7</td>
</tr>
<tr>
<td>SAM-5</td>
<td>VH3</td>
<td>DP-49, (IgHV3-30/30.5*01)</td>
<td>99.3</td>
<td>0/0</td>
<td>0/2</td>
</tr>
<tr>
<td>SAM-3</td>
<td>VH3</td>
<td>DP-49, (IgHV3-30/30.5*01)</td>
<td>100</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>SAM-6</td>
<td>VH3</td>
<td>(IgHV3-30/30.5*01)</td>
<td>100</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>SAM-4</td>
<td>VH3</td>
<td>DP-47 (IgHV3-23)</td>
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<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>PM-2</td>
<td>VH3</td>
<td>DP-47, (IgHV3-23*01)</td>
<td>100</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>PM-1</td>
<td>VH3</td>
<td>DP-47, (IgHV3-23*01)</td>
<td>100</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>SAM-1</td>
<td>VH3</td>
<td>DP-32, (IgHV3-33)</td>
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<td>0/0</td>
<td>0/1</td>
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<tr>
<td>CM-2</td>
<td>VH3</td>
<td>DP-73, (IgHV5-51*01)</td>
<td>97.2</td>
<td>2/1</td>
<td>3/2</td>
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</table>

<table>
<thead>
<tr>
<th>Antibody</th>
<th>VL-family</th>
<th>Germ-line gene</th>
<th>Homology [%]</th>
<th>R.S FR</th>
<th>R.S CDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC-1</td>
<td>λ</td>
<td>DPL-23, (IgLV3-1*01)</td>
<td>96.8</td>
<td>3/2</td>
<td>3/0</td>
</tr>
<tr>
<td>SAM-1</td>
<td>λ</td>
<td>DPL-23, (IgLV3-1*01)</td>
<td>100</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>SAM-2</td>
<td>λ</td>
<td>DPL-23, (IgLV3-1*01)</td>
<td>98.6</td>
<td>1/1</td>
<td>2/0</td>
</tr>
<tr>
<td>SAM-6</td>
<td>λ</td>
<td>DPL-23, (IgLV3-1*01)</td>
<td>99.6</td>
<td>1/0</td>
<td>0/0</td>
</tr>
<tr>
<td>PAM-1</td>
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<td>LV318, (IgLV3-21*03)</td>
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<td>3/0</td>
<td>0/1</td>
</tr>
<tr>
<td>PM-1</td>
<td>λ</td>
<td>(IgLV3-10*01)</td>
<td>99.0</td>
<td>2/1</td>
<td>0/0</td>
</tr>
<tr>
<td>CM-1</td>
<td>λ</td>
<td>(IgLV3-25*03)</td>
<td>99.3</td>
<td>1/1</td>
<td>0/0</td>
</tr>
<tr>
<td>SAM-4</td>
<td>λ</td>
<td>DPL-8, (IgLVL1-40*01)</td>
<td>98.6</td>
<td>3/0</td>
<td>0/1</td>
</tr>
<tr>
<td>SAM-5</td>
<td>λ</td>
<td>DPL-5, (IgLVL1-51*01)</td>
<td>94.8</td>
<td>6/3</td>
<td>4/2</td>
</tr>
<tr>
<td>CM-2</td>
<td>λ</td>
<td>DPL-11, (IgLVL2-14*01)</td>
<td>97.2</td>
<td>0/2</td>
<td>4/2</td>
</tr>
<tr>
<td>PM-2</td>
<td>λ</td>
<td>(IgLVL5-45*01)</td>
<td>98.2</td>
<td>3/2</td>
<td>0/0</td>
</tr>
<tr>
<td>SAM-3</td>
<td>λ</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>
sequences of the VH segment to those of the closest reported germ-line VH genes was 100%. VH genes of the VH3 gene family expressed the antibodies. The close homology of the VH regions to the germ-line genes and the low R:S ratio again indicated that none of these antibodies were affinity maturated by somatic mutation due to antigen contact. The degree of identity of the nucleotide sequences of the VL segment to their most homologous VL germ-line genes ranged from 99.3 to 99.6%, with both antibodies using \(\mu\)-light chain genes.

The R:S ratio was low again, and the mutations were restricted to the framework region.

**Functional Activity of NORM-1 and NORM-2.** To examine whether the antibodies isolated from healthy donors showed a functional activity in vitro, the MTT assay was used again. The tumor cells were incubated with antibody NORM-1 and NORM-2 for 48 h. Thereafter, the amount of antibody-induced inhibition of tumor cell proliferation was measured by photospectrometry. Fig. 6A shows that the antibodies isolated from healthy donors inhibited tumor cell proliferation after 48 h, whereas the controls with unrelated human IgM did not show any growth inhibition.

To additionally investigate the inhibitory effect of the antibodies NORM-1 and NORM-2 on tumor cell growth, the Cell Death Detection ELISA \(^\text{TM}\) kit was used. The results showed that each antibody induced apoptosis in carcinoma cells after 48 h of incubation (Fig. 6B).

**Origin of Natural IgM Antibodies.** Natural IgM antibodies are produced by CD5+ B cells (B1 cells), which belong to the innate immunity and first defense effectors against pathogenic organisms. These B1 cells represent a unique set of lymphocytes, which differ phylogenetically, phenotypically, and functionally from the conventional B2-lymphocytes.

We used the murine anti-SC-1 idiotypic antibody 6/22 to identify the B-cell clone carrying the SC-1 immunoglobulin receptor by im-
munofluorescence double-staining experiments. As illustrated in Fig. 7A, the SC-1-producing cell was CD5 positive as well as IgM positive. Similar stainings were done on different lymphatic material of other patients and healthy donors. Fig. 7B clearly shows that this CD5/SC-1-positive cell was also present in lymphoid tissue of other cancer patients and in addition in healthy persons. These data demonstrate that the SC-1 antibody is produced by a B1/CD5+ cell and is part of the natural immunity.

**Reactivity Pattern of Natural IgM Antibodies.** The reactivity pattern of the antibodies was investigated in detail by immunohistochemistry on a variety of paraffin- and cryo-embedded carcinomas and normal tissues. On normal tissues no specific bindings could be observed. The antibodies PAM-1, LM-1, PM-2, SAM-3, SAM-4, and SAM-6, for example, reacted with a lot of the tested carcinomas, whereas the reaction of antibody CM-2 was restricted to colon carcinomas, and the antibodies SC-1 and SAM-2 reacted only with stomach carcinomas (for details see Table 2).

The reactivity of all of the sequenced human monoclonal tumor-specific IgM antibodies was compared with the mutation rate. Interestingly, there was a striking correlation between the number of mutations and the reactivity pattern. Germ-line antibodies without mutations (e.g., PAM-1, PM-2, SAM-3, and SAM-4) always reacted with a broad spectrum of different tumors within their VH family, whereas the spectrum of reactivity decreased with mutational events (e.g., SC-1, CM-2, SAM-1, SAM-5, and SAM-2). Antibody PAM-1 (100% homology to germ-line gene) for example bound to almost all of the tumors tested, whereas antibody SC-1 (97% homology to germ-line gene) bound only to stomach cancer cells. This indicates that few mutational events determine the reactivity pattern of the antibodies. These observations are illustrated in Fig. 8. A remarkable reciprocal correlation was detected with regard to antibody reactivity and the amount of mutational events.

**DISCUSSION**

The conventional human hybridoma technology is an optimal approach to study the humoral immunity of humans against transformed cells. Immortalizing human lymphocytes by somatic hybridization generates human monoclonal antibodies, which will not only provide new therapeutic human antibodies in the fight against cancer, but will, even more importantly, also define important new targets on cancer tissues and give new information about the humoral immunity of humans against cancer. Our investigation and analysis of several thousands of tumor-reactive human monoclonal antibodies from cancer patients and healthy donors showed that all of the tumor-specific antibodies had several features in common.

All of the tumor-specific human monoclonal antibodies isolated from cancer patients, regardless of the tumor-type or the lymphocyte source, were of IgM isotype. No tumor-specific IgGs or IgAs and no affinity-maturated tumor-specific antibodies were isolated. All of the IgM antibodies were germ-line coded and belonged to distinct gene families. In addition, most of them bound to carbohydrates and were functionally active in vitro by inducing apoptosis. Furthermore, our data showed that tumor-specific antibodies were also present in healthy donors, and these antibodies were solely of one isotype, namely IgM, too. The reactivity pattern of all of these IgMs correlated reciprocally with their mutational status, i.e., with increasing mutation frequency the grade of reactivity to other tumors decreased. The origin of one of these IgM antibodies, SC-1, was determined by using an anti-idiotypic antibody, and we demonstrated that the human antibody SC-1 was produced by CD5+ lymphocytes and that these SC-1-producing cells were also present in lymphatic tissue of healthy donors.

Malignancy is like a chronic disease, and evolution had to develop a defense system that enabled organisms to detect and to remove transformed cells early and sufficiently. Components of the innate and the adapted immunity are responsible for the elimination of “nonself” cells. In invertebrates immunity is restricted to the innate system, and

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**Table 4 Sequence-analysis of human monoclonal IgM antibodies isolated from healthy donors**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>VH-family</th>
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<th>R.S CDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>NORM-1</td>
<td>VH3</td>
<td>DP-47, (IgHV3-23*01)</td>
<td>100</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>NORM-2</td>
<td>VH3</td>
<td>DP-77, (IgHV3-21*01)</td>
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<table>
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<th>Antibody</th>
<th>VL-family</th>
<th>Germ-line gene</th>
<th>Homology [%]</th>
<th>R.S FR</th>
<th>R.S CDR</th>
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<tr>
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<td>IgLV3-10*01</td>
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<tr>
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<td>IgLV1-40*01</td>
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<td>1/0</td>
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</table>

Fig. 6. Functional analysis of antibodies NORM-1 and NORM-2 in vitro. The consequences of antibody treatment on the proliferation of gastric carcinoma cell line 23132/87 were measured by (A) MTT proliferation assay and (B) Cell Death Detection ELISAPLUS. 

A. Inhibition of cell proliferation with antibodies NORM-1 and NORM-2 measured after 48 h. B. Antibody induced apoptosis detected by Cell Death Detection ELISAPLUS apoptosis assay after 48 h. Control 1, to demonstrate normal conditions, cells were supplemented with complete growth medium but without any antibody. Control 2, unrelated human IgM was added in a similar concentration.
vertebrates are equipped in addition with the adaptive, trainable immune system (25–28). The innate response is invariable and works by using a transmitted germ-line coded pool of specific receptors. The adapted response is derived from the first response, and its diversity is based on mutational events. The innate immunity (also referred to as “unspecific immunity”) consists of natural killer cells, dendritic and mast cells, macrophages, and natural IgM antibody producing B cells (29–40). This system can distinguish between self and nonself, and is responsible for the first specific immune response directed against bacteria, viruses, and malignant cells (40–42). This response is T-cell independent, which means that antigen presentation by T cells is not required (43).

The cells involved in innate immunity do not recognize specific single structures but instead specific patterns, which are conservative and expressed independently from mutational events (44). This recognition system guarantees that the immune response need not follow all of the mutational changes, but can focus on the detection of structures, which are most likely involved in primary cell stability and cell preservation mechanisms. The immune-competent cells fulfill that task by using specific sets of germ-line coded receptors, which belong to distinct protein families (44, 45). The acquired genetic variability of the innate immunoglobulin receptors is achieved by combinatorial association of germ-line immunoglobulin genes. Additional deletions, additions, and mistakes in recombination events guarantee a genetic and receptor variability, which is sufficient to cover a broad spectrum of antigens on pathogenic organisms and gives a sufficient protection without additional mutational adaptation (46–52). Thus, the innate immune recognition is entirely different from the diverse recognition of the adaptive immunity.

The most potent molecules of the immune system are pentameric IgM antibodies. Their unique structure is the prerequisite for the specific recognition and function of the innate immunity. The antibodies described here were selected in vitro for their antiproliferative activity. Most of the investigated human IgM antibodies isolated from cancer patients and from healthy donors induced apoptosis by binding to tumor-specific receptors (see also Refs. 4, 15). This effect was demonstrated on different established tumor cell lines (4) and on primary tumor cells isolated from cancer patients (9, 11, 23, 24). In addition, the human monoclonal IgM antibody SC-1 showed its apoptotic effect not only in vitro, but also in vivo, in animals as well as in patients when used in a clinical study of stomach cancer (13, 18). In contrast, former published IgM antibodies, which also bind speci-
We verified the hypothesis of the CD5+ origin of natural antibodies by using an anti-idiotypic antibody against the SC-1 IgM antibody. This anti-SC-1 idiotypic antibody was made in mice and is a specific SC-1 antibody. We used this murine antibody to identify the SC-1 immunoglobulin receptor-positive B-cell clone with immunofluorescence double-staining experiments, and we successfully demonstrated that the SC-1-producing cell is CD5 positive. In addition, similar immunoperoxidase stainings were done on lymphoid material of other patients and healthy donors, and the results showed that this CD5+/SC-1-positive cell was also present in lymphoid tissue of healthy persons. From those results we concluded that the SC-1 antibody is made by a CD5+ B cell (B1 cell) and that it is part of the natural immunity.

A good evidence for the theory of tumor-specific IgM antibodies being inherited natural antibodies is the existence of these antibodies in healthy persons. To prove that natural IgM antibodies with tumor specificity exist as an immunological reactivity platform in humans, spleen cells from “healthy” individuals were immortalized and the resulting antibodies tested for antitumor activity. Among a small series of selected clones two IgM antibodies could be identified, which showed a reactivity against different tumor tissues but not against nontransformed cells. Moreover, these antibodies showed similar genetic origins as the tumor-specific antibodies isolated from cancer patients. The sequence analysis showed that the antibodies isolated from healthy donors were members of the family of naturally occurring, nonaffinity matured antibodies, too. The in vitro functional analysis of the tested human monoclonal IgM antibodies isolated from healthy donors demonstrated that these antibodies, like the antibodies found in cancer patients, not only reduced cell proliferation, but also induced apoptosis in cancer cells. IgM antibodies with tumor reactivity were detected in sera of several healthy donors. But it was neither proven whether these antibodies were really natural antibodies nor was it excluded that these antibodies were autoantibodies (40, 45, 76–81). Without specificity and genetic analysis it is not possible to classify these observations. The data presented here indicate that the same group of naturally occurring IgM antibodies found both in healthy individuals and in cancer patients are responsible for the defense against malignant cells.

The reactivity of all of the sequenced human monoclonal tumor-specific IgM antibodies in this study was compared with the mutation rate. Interestingly, there was a striking correlation between the number of mutations and the reactivity pattern. Germ-line coded antibodies with no mutations (PAM-1, PM-2, SAM-3, and SAM-4) always reacted with a broad spectrum of different tumors within their VH-family, whereas the spectrum of reactivity decreased with mutational events (SC-1, CM-2, and SAM-5). Antibody PAM-1 (100% homology to germ-line gene) for example bound to nearly all of the tumors tested, whereas antibody SC-1 (97% homology to germ-line gene) bound to stomach cancer cells only. This indicates that few mutational events might determine the reactivity pattern, and the most important question is whether these mutations are the result of inheritance or of learning, or whether the natural immunity is trainable, like the adaptive.

In this article we give evidence that origin, reactivity patterns, and genetics of these truly tumor-specific antibodies are close to what we know about the defense against bacterial structures. Therefore, these data might indicate that the innate immunity is also responsible for immunosurveillance mechanisms against malignant epithelial cells.

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Natural IgM Antibodies and Immunosurveillance Mechanisms against Epithelial Cancer Cells in Humans

Stephanie Brändlein, Tina Pohle, Nele Ruoff, et al.


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