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Quality of Recombinant Protein Determines the Amount of Autoreactivity Detected against the Tumor-Associated Epithelial Cell Adhesion Molecule Antigen: Low Frequency of Antibodies against the Natural Protein

Oliver Schmetzer,[1]*† Gerhard Moldenhauer,‡ Rainer Riesenberg,§ José Ricardo Pires,‖ Peter Schlag,¶ and Antonio Pezzutto*†

The human epithelial cell adhesion molecule (EpCAM) is expressed on normal epithelial cells and is overexpressed in most carcinomas. EpCAM-targeted immunotherapy has been tried in several clinical studies. High titers of autoantibodies against EpCAM have been reported by different authors. We have generated large amounts of purified protein in S2 *Drosophila* cells (S2-EpCAM) with a purity of >96%. In contrast, the protein produced in baculovirus-dependent systems (baculo-EpCAM) that has been used in previous studies shows a purity of 79%. ^{1}H nuclear magnetic resonance spectrum of S2-EpCAM is typical of folded protein, whereas the baculo-EpCAM sample shows a spectrum corresponding to a partially unfolded protein. Using S2-EpCAM, denatured S2-EpCAM, and baculo-EpCAM, we measured EpCAM Abs of different isotypes in the serum of healthy controls and cancer patients. We found Ab titers against EpCAM in a much lower percentage of sera as published previously, and support the hypothesis that Ab reactivity in some published studies might be due to reactivity against denatured protein, to contaminating proteins in the baculovirus preparations, and to reactivity with BSA. Tetanus toxoid-reactive IgG Abs are present in 1000-fold higher titers compared with EpCAM-reactive Abs. Only IgA Abs were found in higher proportions and in higher concentrations than tetanus toxoid-specific Abs. Our study shows that EpCAM only rarely induces autoantibodies against native protein and emphasizes the importance of using extremely purified Ag preparations when evaluating Abs against tumor-associated Ags. *The Journal of Immunology,* 2005, 174: 942–952.

The epithelial cell adhesion molecule (EpCAM[2]; also termed CO17-1A, GA733-2, KS1-4, EGP, or KSA) has been originally identified as a tumor-associated Ag by the mAb CO17-1A (1). EpCAM is expressed in embryonic tissues and has important functions in the development of pancreas and liver (2–4). It functions as a homotypic cell-cell adhesion molecule and is abundantly expressed in human colon and to a lower extent by nearly all epithelial cells (3–7). In adenocarcinomas, EpCAM can be significantly overexpressed and is a potential target Ag for immunotherapy. Over 90% of all colorectal carcinomas overexpress EpCAM, and the expression is conserved during metastasis (8).

Natural humoral and cellular immune reactivity against EpCAM has been described in cancer patients (9–15), and extensive efforts have been made to develop therapeutic vaccines. Passive immunization with CO17-1A (Panorex) induced survival benefit compared with untreated patients in the clinical setting of minimal residual disease (16, 17). Active immunization has been conducted using anti-Id vaccines (18–20), recombinant protein (13, 21), and viral vectors (22), but has shown limited clinical efficacy so far.

Recombinant proteins contain both CD4 and CD8 epitopes and are potentially useful for vaccination, but their production in large amounts and high purity is not trivial. A frequently used system for the production of rEpCAM rely on the baculovirus infection system, but this yields only low amounts of protein (23). EpCAM production in tobacco plants has been recently described, but it also yields only low amounts of protein and is difficult to establish under good manufacturing practice conditions (24). Therefore, we have expressed EpCAM in S2 *Drosophila* cells (25), trying to establish a good manufacturing practice-suitable procedure. Recombinant protein generated in these cells is usually highly immunogenic due to the presence of mannose-type *N*-glycans, which enhance uptake by APCs and their activation, mediated by mannose receptors (26).

The S2 cells are of embryonic origin and can incorporate large amounts of plasmid DNA (27). They can be cultured in serum-free medium, avoiding contamination with serum proteins.

By optimizing cell culture conditions, transfection rate, and gene expression, we have established a method for obtaining large amounts of highly purified rEpCAM.

Autoantibodies against EpCAM have been demonstrated both in a relatively high percentage of patients with colorectal cancer and in some healthy controls (11, 15). Although the meaning of these autoantibodies is still a matter of debate, it is clear that accurate measurement of Ab titers is crucial for monitoring the immune

*Molecular Immunotherapy, Max Delbrück Centrum for Molecular Medicine, Berlin, †Department of Hematology and Oncology, Charité, Humboldt University, Berlin, ‡German Cancer Research Center, Division of Molecular Immunology (D050), Heidelberg, §Ludwig Maximilians University Muenchen, University Clinic Grosshadern, Department of Urology, Tumor Immunology Group, Munich, ¶Department of Surgical Oncology, Robert Rössle Klinik, Charité, Humboldt University, Berlin, Germany; and ‖Universidade Federal do Rio de Janeiro, Departamento de Bioquimica Medica, Rio de Janeiro, Brazil

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[1] Address correspondence and reprint requests to Dr. Oliver Schmetzer, Max Delbrück Centrum for Molecular Medicine, Robert-Rössle-Strasse 10, 12135 Berlin, Germany. E-mail address: oliver.schmetzer@gmx.net

[2] Abbreviations used in this paper: EpCAM, epithelial cell adhesion molecule; TT, tetanus toxoid; IEX, ion exchange; NMR, nuclear magnetic resonance; HSA, human serum albumin; DIG, digoxigenin.

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response during immunotherapy. Having planned vaccination with rEpCAM protein, we decided to optimize ELISA measurement of EpCAM autoantibodies. In a first series of blood samples, little or no reactivity against S2-EpCAM was detected, which prompted us to evaluate a large number of sera to clarify discrepancies with published results. To exclude individual variations in the humoral immune response because of the immunosuppressive effects of chemotherapy, we also measured tetanus toxoid (TT)-reactive Abs and compared these titers to the EpCAM-reactive Abs.

Our results indicate that patients with autoantibodies reacting with denatured or misfolded protein may lack Abs against the native form of the Ag. Most autoantibodies are detected by peptide scans or Western blots that would not detect conformational epitopes and may lead to the false assumption that Abs reacting with the native Ag are also present (28).

Materials and Methods

Sera from patients and healthy donors

Sera from 500 patients with carcinomas and from 60 healthy controls were stored in the vapor phase of liquid nitrogen until analyzed. The sera had been collected at Charité University Medicine, Berlin, and Ludwig Maximilians University, Munich, during the past years in the context of different projects. None of the patients received any immunotherapy.

EpCAM-reactive Abs

CO17-1A (Panorex) was obtained from GlaxoSmithKline. HEA125 (29) was produced in a Miniperm bioreactor (Vivascience) and purified by affinity chromatography over protein A-Sepharose CL-4B (Amersham Biosciences). The purity of eluted Ab was assessed by SDS-PAGE under reducing conditions and was >95%. Specificity was proven by indirect immunofluorescence using flow cytometry on living cells and positive staining of Colo205 and SW948 lysates in Western blots (data not shown).

Cell culture

Drosophila S2 cells (Invitrogen Life Technologies) were cultured at 28°C in HyQ SFX Serum (HyClone) supplemented with 10% FBS (FCS), Pen/Strep, gentamicin, pyruvate, and L-glutamine (Biochrom). The serum was only used during selection and expansion of the cells, but not in the protein expression step.

Generation of the rEpCAM from S2 cells

A truncated form of EpCAM was generated by PCR with Pfu-Polymerase (Stratagene) and the following primers: huEpCAM-trunc-5, 5′-AGA TCT ACG GCG ACT TTT GCC GCA GC-3′, and huEpCAM-trunc-3, 5′-TTC GAA TTT TAG ACC CTG CAT TGA GAA TTC-3′, from a cDNA library from human colon. The PCR conditions were 30 cycles of 45 s at 96°C, 45 s at 65°C, and 120 s at 72°C. The PCR product was separated on a 1% agarose gel and excised. The DNA was isolated with QIAEX (Qiagen) and ligated in a PCR Script vector (Stratagene). TOP10 *Escherichia coli* (Invitrogen Life Technologies) were transformed with the ligation product and plated with 100 μg/ml ampicillin. Plasmid minipreps (Qiagen) were analyzed by restriction digestion with *Bgl*II and *Bst*BI (MBI Fermentas) and were sequenced with the following primers: EpCAMs, 5′-agc gag tga gaa cct act gg-3′, and EpCAMas, 5′-acg cgt tgt gat ctc ctt ctg-3′. DNA from a mutation-free clone and from the pMT/BIP/V5-His vector (Invitrogen Life Technologies) was digested with *Bgl*II and *Bst*BI. The 0.75-kB EpCAM insert was ligated in the vector after gel extraction, and the product was used in a transformation of TOP10 *E. coli.* Miniprep DNA was analyzed by digestion with *Bgl*II and *Bst*BI. DNA from one positive clone and from TOP10 *E. coli*, transformed with pCoHygro (Invitrogen Life Technologies), was prepared endotoxin-free with a Maxi-Prep Endofree kit (Qiagen). The pMT-EpCAM vector was sequenced with the EpCAMs and EpCAMas primers and used to transfect S2 cells with Effectene (Qiagen): 2 μg of pMT-EpCAM DNA was mixed with 4 ng of pCoHygro DNA in 200 μl of EC buffer. After addition of 16 μl of Effectene enhancer and incubation for 10 min at room temperature, 100 μl of Effectene reagent was added. Following incubation for 10 min at room temperature, the mix was added to 0.5×10^6 S2 cells in 5 ml of medium. The cells were cultured in a 60-mm dish for 48 h. Selection was started with a concentration of 300 μg/ml hygromycin B (Invitrogen Life Technologies). After 10 days, the concentration was increased to 600 μg/ml hygromycin B and, after a further 10 days, to 1000 μg/ml. The cells were diluted 1/5 two times a week and cultured under these conditions for 12 mo. To induce the expression of the rEpCAM, the cells were washed with serum-free medium and injected in a Celline 1000 bioreactor (Integra Biosciences) at a concentration of 5×10^7/ml. Twenty-five milliliters were injected and 1 liter of serum-free medium was added to the medium compartment of the reactor. Copper sulfate was added to 5 mM after 1 wk. After another week, half of the cell suspension was harvested. This was repeated weekly with exchange of the medium in the medium compartment every 2 wk. The solution was centrifuged twice, and the supernatant was passed through a 0.2-μm filter unit (TPP).

Purification of the rEpCAM

The supernatant from the S2 cells was purified on a Ni-NTA column, packed with 300 ml Ni-NTA-Superflow (Qiagen). After binding, the column was washed with a gradient over 230 ml starting with 100% wash buffer (1.65 M NaCl, 10% glycerol, 0.05% Tween 20, 10 mM Na-phosphate (pH 7.4)) and ending with 96% wash buffer and 20 mM imidazol. Elution was started by applying 200 ml of a gradient, which further increases the imidazol concentration to 500 mM and decreases the amount of wash buffer to 0%. The elution was continued for 100 ml with 500 mM imidazol. For the ion exchange (IEX) chromatography, the protein was dialyzed against 15 mM NaCl and 1 mM Na-phosphate (pH 7.4) in a 6- to 8-kDa dialysis membrane (Spectrum). It was applied to a column with 75 ml of Q Sepharose FF (Amersham Biosciences), and the column was washed with 250 ml of water. Elution was done with a complex gradient, increasing the NaCl concentration over 150 ml to 150 mM. The concentration was further increased with a gradient over 300 ml to 750 mM. This concentration was applied for 200 ml.

Denaturation of the EpCAM protein

EpCAM protein was diluted to a concentration of 0.1 mg/ml in a denaturing buffer (4 M GuHCl, 14.3 mM 2-ME, 15 mM NaCl, 50 mM Tris-Cl (pH 7.4)) and incubated at 37°C for 20 min. The protein solution was dialyzed thereafter against PBS in a 6- to 8-kDa dialysis membrane (Spectrum).

Isolation of EpCAM from the baculovirus expression system

EpCAM produced in the baculovirus expression system was obtained from the MK-1 ELISA kit (Biovendor). According to the specification provided by the company, the standards of the ELISA kit were produced according to the original method described by Strassburg et al. (23).

Nuclear magnetic resonance (NMR) analysis

After dialysis against PBS in a 6- to 8-kDa dialysis membrane (Spectrum), the proteins were concentrated to 10 mg/ml in YM-3 Microcon columns (Millipore). After addition of 50 μl of D_2O to 500 μl of protein solution, the ^1H NMR spectrum was recorded in a Bruker DRX600. The watergate pulse sequence was used to suppress the water signal.

Lectin-binding assay

The lectin-binding assay was performed with the digoxigenin (DIG) Glycan Differentiation kit (Roche) according to the manufacturer's protocol. Briefly, the sample and the control proteins were dotted on a nitrocellulose membrane (Roth). After drying, the membrane was blocked with 2% dry milk powder (Roth) and 0.1% Tween 20 in PBS for 2 h at room temperature. Equal strips were then stained with the different lectin-DIG conjugates, followed by washing with PBS and incubated with the DIG-Fab-enzyme conjugate.

Western blotting

SDS-PAGEs were blotted on nitrocellulose membranes (Schleicher-Schüll) at 1 mA/cm^2 at 4°C for 3 h in a semidry blotting system. Detection of protein on the membrane was done by incubation with amido black solution (0.1% amido black, 25% 2-propanol, and 10% acetic acid) followed by rinsing with water. Blocking was conducted overnight at 4°C with blocking solution (0.5% BSA, 0.2% Tween 20, 5% dry milk powder, 0.1% sodium azide, and 5 mM EDTA in PBS (pH 7.4)). Patients' sera were diluted 1/20 in blocking solution, and 20 ml were used per membrane strip for an incubation overnight at 4°C. After three washes with TPBS (PBS supplemented with 0.1% Tween 20), 50 ml of TPBS with 5 μl each of anti-IgG1-, 2-, 3-, and 4-biotin Abs (BD Pharmingen) were added. The membranes were again incubated overnight and washed three times, and 50 ml of TPBS with 5 μl of avidin-HRP (BD Pharmingen) was added for 2 h at 4°C. Detection was done after four washes with the SuperSignal West Femto Maximum Sensitivity substrate (Pierce) by exposure of 1–5 s to an x-ray film.

ELISAs

Black FluoroNunc MaxiSorb Plates (Nunc) were coated with 250 ng of protein in 100 μl of PBS per well. After incubation overnight at 4°C, the plates were washed once with TPBS, and 400 μl of blocking buffer (2% high purity human serum albumin (HSA; Calbiochem; highest purity), 150 mM NaCl, 0.1% Tween 20, 10 mM Na-phosphate (pH 7.4)) was added to each well. The plates were again incubated overnight and washed once, and patients' sera were added in a geometric dilution in TPBS (1/10 to 1/80). Following overnight incubation, the plates were washed three times, and 100 μl of anti-Ig-biotin Ab (BD Pharmingen) at a concentration of 0.5 μg/ml was added. After 2 h at 4°C, the plates were washed three times, and 100 μl of 0.5 μg/ml streptavidin-AKP (BD Pharmingen) was added. Two hours later, the plates were washed again four times, and 200 μl of substrate solution (0.2 mM 4-methylumbelliferyl phosphate (Sigma-Aldrich), 0.05 M $NaCO_3$, 5 mM $MgCl_2$) was added. The plates were measured after 90 min at room temperature in a Victor II Spectrophotometer (Wallac) with an excitation filter at 365 nm and an emission filter at 450 nm.

The total amounts of Ab were calculated from a titration of purified anti-EpCAM Ab HEA125. The secondary Ab was a biotinylated anti-mouse-IgG1 Ab (BD Pharmingen).

Thirty samples could not be analyzed for IgG3 because BD Pharmingen stopped the production of the necessary Ab.

In an additional experiment, we blocked with BSA (fraction V; protease and nuclease free; Calbiochem) instead of HSA. The samples were considered positive when a fluorescence count >500,000 was reached. This is 3-fold the signal from the negative control (PBS).

Results

Purification of EpCAM protein from S2 cells

EpCAM protein was purified on a Ni-NTA column using an imidazole gradient for elution. The supernatant collected from the bioreactors showed two peaks. The first eluted at a concentration of ~50 mM imidazole and the second at a concentration of 190 mM. Analysis by PAGE showed several proteins with a molecular mass of 40–70 kDa within the first peak and two bands of 32 and 33 kDa in the second elution peak (Fig. 1*A*). The whole fractions of the first peak were discarded; fractions of the second peak were subjected to further purification on a Q Sepharose matrix. The elution resulted in two different protein peaks, whereas some protein did not bind to the matrix. The first peak eluted at 130 mM salt concentration and the second at 380 mM. In the first peak, we

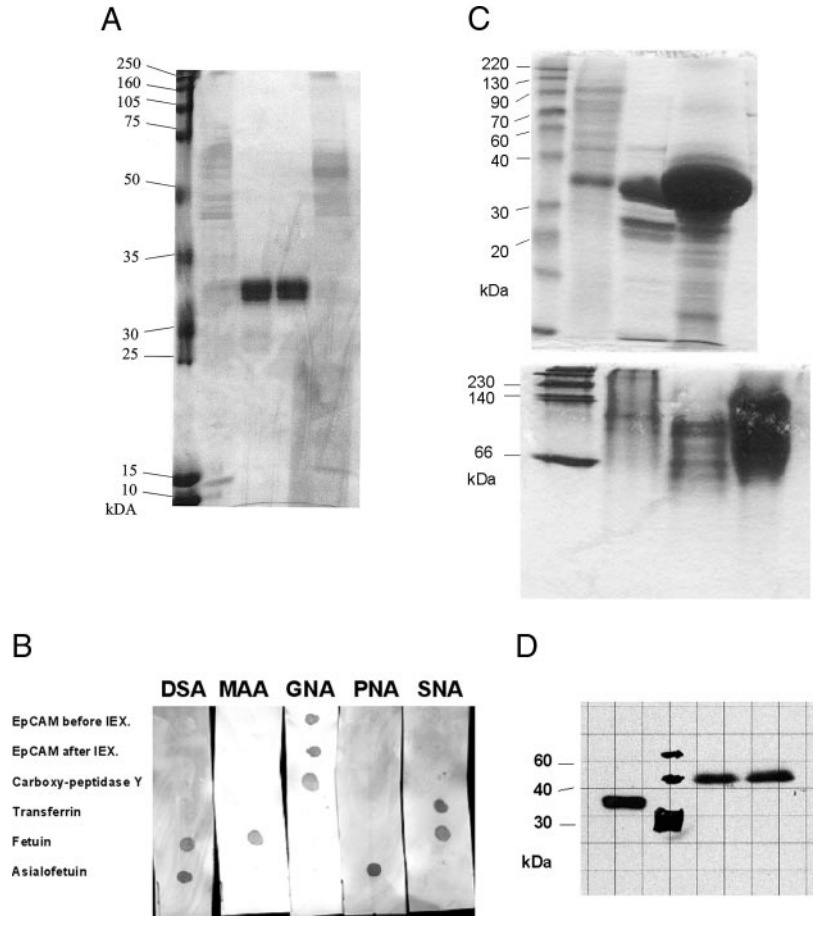

FIGURE 1. *A*, Analysis of rEpCAM protein from S2 cells. PAGE analysis of proteins from peaks eluted during purification. *Lane 1* shows marker proteins. The proteins eluting from the Ni-NTA column at low imidazole concentration are shown in *lane 2*, whereas *lane 3* shows the proteins eluting at higher imidazole concentration. *Lane 4* shows the protein from the IEX column at medium salt concentration. In *lane 5*, the proteins eluting at high salt concentration are shown. *B*, Lectin binding of the EpCAM protein and of control proteins (dot blot). GNA (*Galanthus nivalis* agglutinin) recognizes terminal mannose; SNA (*Sambucus nigra* agglutinin) recognizes sialic acid linked α(2–6) to galactose; MAA (*Maackia amurensis* agglutinin) recognizes sialic acid linked α(2–3) to galactose; PNA (peanut agglutinin) recognizes the core disaccharide galactose β(1–3)*N*-acetylgalactosamine; and DSA (*Datura stramonium* agglutinin) recognizes Galβ-(1–4)*N*-acetylglucosamine (GlcNAc) in complex and hybrid *N*-glycans, in *O*-glycans, and GlcNAc in *O*-glycans. EpCAM appears to contain mannose residues. *C*, Low (10-μg) and high (100-μg) concentrations of purified EpCAM protein were loaded on a PAGE under denaturing (*upper gel*) and native (*lower gel*) conditions. *Lane 1* shows a marker mixture. The removed impurities from IEX chromatography, low and high amounts of purified EpCAM are loaded in *lane 2*, *3*, and *4*, respectively. *D*, Western blot analysis of purified EpCAM with HEA125 as detection Ab. *Lane 1* shows the purified rEpCAM. A protein marker is shown in *lane 2*. *Lanes 3* and *4* show lysates from 293T cells that were infected with two different clones of recombinant adenovirus that carry a full-length *EpCAM* gene.

found again the two 32- and 33-kDa proteins, whereas the second peak yielded a band at ~60 kDa (Fig. 1*A*).

N-Terminal Edman Sequencing of the 32- and 33-kDa protein sample yielded the EpCAM sequence TFAAAQEECVCEN (data not shown). Binding of GNA-lectin in a dot blot showed *N*-glycosylation with mannose-type glycans (Fig. 1*B*).

Multimerization of EpCAM can occur at high protein concentration as shown by native PAGE (Fig. 1*C*) and gel filtration (data not shown). The protein was recognized by HEA125 in a Western blot (Fig. 1*D*) and by CO17-1A in ELISA (data not shown). Serum of animals (rabbits, mice) immunized with the protein stained strongly EpCAM-positive cell lines (Colo205) and EpCAM-cDNA-transfected cell lines (MCA205, B16/F10, and EL4) (data not shown). Spleen cells from mice immunized five times with purified EpCAM protein were used for cell fusion to generate new high-affinity mAbs. These recognize distinct epitopes of the EpCAM protein and can be used for tissue staining, Western blot, and ELISA (our manuscript in preparation).

The total amount of rEpCAM from a single bioreactor was 15–20 mg per harvest.

Comparison of the EpCAM protein purified from S2 cells and from baculovirus-infected cells

Although only the 32- and 33-kDa bands are detected in the eluates of columns loaded with native and denatured EpCAM protein collected from S2 cells supernatants (hereafter called S2-EpCAM), the protein from the commercial kit (hereafter called baculo-EpCAM) contained at least five more proteins with a molecular mass of ~28, 50, 70, 105, and 260 kDa, according to gel staining (Fig. 2*A*). The detection limit of the protein gel is ~0.5 μg of protein as determined by BSA standard dilution (data not shown). Because no bands other than 32–33 kDa were found by loading a total of 12 μg of S2-EpCAM protein, we calculated that the purity of the S2 protein should be >96% (also verified by HPLC analysis, data not shown). The five additional bands in the baculo-EpCAM preparation indicate most likely contaminating proteins with a total mass of at least 2.5 μg as indicated by the gel. The purity of EpCAM in this preparation therefore seems to be no better than 79%. We tried to extract protein from these bands for protein identification by mass spectrometry, but the amounts in the bands were too low. Fig. 2, *B* and *C*, show, respectively, the aliphatic and aromatic/amide regions of the ^{1}H NMR spectrum for S2-EpCAM (lower spectrum), baculo-EpCAM (middle spectrum), and denatured S2-EpCAM (upper spectrum). The ^{1}H NMR spectrum for S2-EpCAM shows comparatively sharp lines and good signal dispersion with several peaks <0.5 ppm and >9.0 ppm. This spectrum is very characteristic of a folded protein with a size of ~30 kDa. The spectrum of baculo-EpCAM otherwise shows very broad lines, suggesting the presence of molecules of higher molecular size, larger proteins or aggregates. In the spectrum of baculo-EpCAM, the dispersion of chemical shifts is also not evident and could indicate the presence of some unfolded protein. The spectrum of the denatured S2-EpCAM (generated by exhaustive reduction of S2-EpCAM) shows the smallest chemical shift dispersion, typical of unfolded proteins. Interestingly, baculo-EpCAM seems to be in an intermediate situation between S2-EpCAM and denatured S2-EpCAM.

Together, these results suggest that the S2-EpCAM consists of a mixture of molecules whereby most are in a native folding state compared with the baculovirus-derived protein, where at least some molecules have spectral features of a denatured protein.

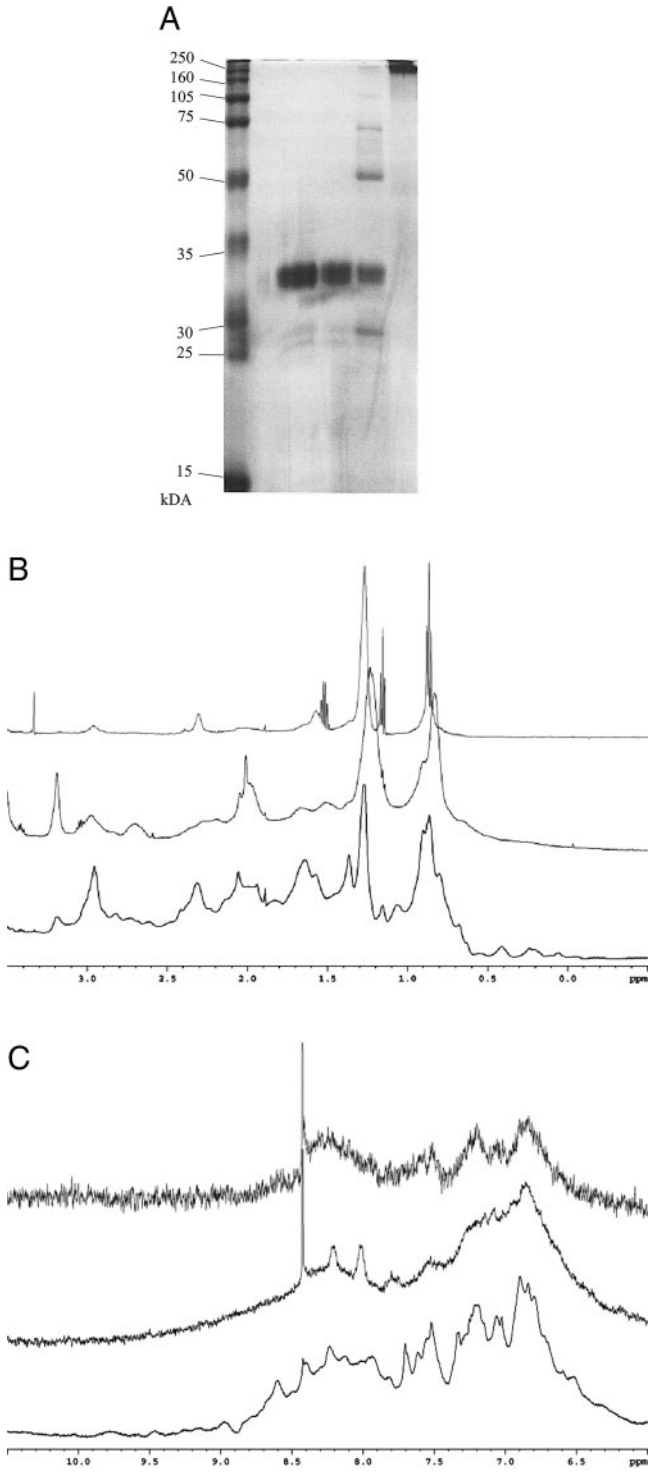

FIGURE 2. *A*, Comparison of EpCAM produced in S2 cells and in baculovirus-infected cells. PAGE analysis of the different test Ags. Marker mixture, native S2-EpCAM, denatured S2-EpCAM, baculo-EpCAM, and TT are shown in *lanes 1*, *3*, *4*, *5*, and *6*, respectively. *Lane 2* is empty. *B*, Aliphatic region of the NMR spectrum from denatured S2-EpCAM (*upper spectrum*), baculo-EpCAM (*middle spectrum*), and native S2-EpCAM (*lower spectrum*). *C*, Aromatic region of the NMR spectrum in the same order as in *B*.

Comparison of autoantibody levels detected with S2-EpCAM and baculo-EpCAM

One hundred fifteen randomly selected sera and five sera that had tested positive in ELISA with S2-EpCAM were further analyzed

for comparative reactivity with S2-EpCAM, denatured S2-EpCAM, baculo-EpCAM, and TT. Sera were considered positive for S2-EpCAM-reactive IgG Abs when the Ab concentration was >80 ng/ml. This amount (8-fold SD of the mean) was never found in 60 sera from healthy donors, as described below.

Abs against baculo-EpCAM were also found in healthy controls (data not shown) as described previously by others (30). As the Ab distribution shows, a threshold for distinguishing positive and negative samples cannot be defined.

All samples were positive for TT-reactive IgG Abs. Forty-five samples showed high amounts of IgG with concentrations exceeding 1 μg/ml. The IgG response consisted mainly of IgG1 and IgG4 Abs; IgG2 responses were often found in lower amounts. Only low levels of IgG3 Abs were detected (Fig. 3*A*). The mean level of IgG Abs against TT was ~1000 times higher compared with S2-EpCAM-reactive Abs. IgM Abs against TT were in the same range as IgM Abs against S2-EpCAM, whereas IgA Abs were more frequently found against EpCAM than against TT, with a mean 3-fold increase.

IgG1, IgG2, and IgG4 Abs against baculo-EpCAM were always present in much higher amounts compared with S2-EpCAM (Fig. 3, *B–G*). There was no correlation between IgG1, IgG2, and IgG4 Abs against S2-EpCAM and baculo-EpCAM. In contrast, similar reactivity was found for IgG3, IgM, and IgA Abs against S2-EpCAM and baculo-EpCAM. When the five selected sera that had tested positive for S2-EpCAM Abs in a preliminary screening were tested again with both reagents, all cases confirmed positive with S2-EpCAM but gave a much higher signal with baculo-EpCAM. No sera tested positive with the S2-EpCAM in the absence of reactivity with baculo-EpCAM.

In 120 tested samples, Abs reacting with denatured S2-EpCAM were detected at higher levels than against native S2-EpCAM with a mean 12-fold increase. Some samples even showed a 100 times higher Ab titer against the denatured preparation (data not shown).

There was no correlation between Ab presence and the age of the patients.

Immune reactivity against EpCAM and contaminating proteins

We further tried to demonstrate specific reactivity against EpCAM in both recombinant protein preparations and against the putative contaminating proteins in the baculo-EpCAM preparation by Western blotting. The staining of the membrane with amido black showed a thick band of EpCAM in the S2-EpCAM preparation, but the background was too high to detect the weak bands in the baculo-EpCAM preparation as expected because of the low sample load (not shown). For detection of Abs, we used five samples that had tested positive against baculo-EpCAM and/or denatured S2-EpCAM in ELISA. We detected immune reactivity against the S2-EpCAM in all samples (Fig. 4*A* and Table I). However, no reactivity against the baculo-EpCAM preparation could be found. The samples contained between 30 and 200 ng of Ab, reactive against denatured S2-EpCAM as determined by ELISA. They also contained 500–700 ng of Abs reactive to the baculo-EpCAM preparation.

We also tested sera from healthy controls for S2-EpCAM reactivity before and after IEX chromatography (Fig. 4*B*). Abs could be found in nearly all samples against the S2-EpCAM preparation without IEX chromatography. These Abs could not be detected after IEX chromatography.

Autoantibody levels detected with S2-EpCAM

In 60 sera from healthy donors, there were only very low levels of S2-EpCAM-reactive IgG Abs (Fig. 5, *A—D*, and Table II). The highest amounts were 8 ng/ml IgG1, 23 ng/ml IgG2, 11 ng/ml IgG3, and 1.8 ng/ml IgG4; however, sera of healthy individuals contained IgM and IgA Abs (Fig. 5, *E* and *F*, and Table II), the highest levels being 172 ng/ml IgM and 84 ng/ml IgA.

Samples were considered positive when the Ab amount exceeded the mean value of the control sera plus 8 SDs. Using this definition, all control sera were negative. Ab amounts higher than 8 ng/ml for IgG1, 27 ng/ml for IgG2, 20 ng/ml for IgG3, 3 ng/ml for IgG4, 371 ng/ml for IgM, and 133 ng/ml for IgA were considered positive according to this definition.

Four of 95 sera from colon cancer patients contained IgG, IgA, or IgM autoantibodies in ELISA using S2-EpCAM (Fig. 5, *A–D*, and Table II). One probe contained 15 ng/ml Ab of IgG1 isotype. One contained IgG2 isotype Abs (160 ng/ml). Abs of IgG4 isotype could be detected in another sample at a level of 41 ng/ml. One patient had IgA Abs: 0.3 μg/ml (Fig. 5*F* and Table II). Among 81 sera from patients with rectal cancer, one had IgG1 (14 ng/ml), two had IgG2 (160 and 221 ng/ml), and three had IgG4 Abs (4.5 and 47 ng/ml) (Fig. 5, *A—D*, and Table II). Three other sera contained IgA Abs (166, 284, and 363 ng/ml) (Fig. 5*F* and Table II). One of the sera was positive for both IgG4 and IgA, whereas all of the others were positive for only one Ab isotype.

Three of 39 sera from patients with gastric cancer had higher Ab amounts compared with the control sera (Fig. 5, *A–D*, Table II). One had IgG1, IgG2, IgG4, and IgA Abs: 79, 121, 22, and 207 ng/ml, respectively. Another patient was positive for IgG4 and IgA (4 and 203 ng/ml, respectively) The third patient had IgG2 Abs only (30 ng/ml). Of 261 sera from patients with breast cancer, six were positive for IgG1 (37, 29, 24, 13, 12, and 10 ng/ml), five were positive for IgG2 (310, 206, 65, 57, and 54 ng/ml), one for IgG3 (23 ng/ml), eight for IgG4 (58, 31, 26, 18, 15, two times 5 and 4 ng/ml), and four for IgA (275, 266, 258, and 172 ng/ml) (Fig. 5 and Table II). Among 32 sera obtained from patients with prostate carcinoma, one tested positive for IgG1 (146 ng/ml) and three for IgA (355, 225, and 158 ng/ml) (Fig. 5 and Table II).

In another experiment, we measured 107 sera from patients with colorectal, stomach, or breast cancer whereby the ELISA plates were blocked with BSA (Table III). All sera had Abs of the IgM isotype. Sixty-one percent of the samples were positive for IgA. IgG was found in 50% of the sera from patients with gastric carcinoma, in 36% of the sera from patients with breast cancer, in 21% of sera from patients with colon cancer, and in 19% of patients with rectal carcinoma.

All samples were measured as duplicates with a SD <5%; all four dilutions showed the expected reduction in the signal strength. All positive sera and 30 randomly selected negative sera were tested again in at least one completely independent experiment with similar results. No correlation with the age of the patients was found in all tested sera. Although clinical data were available only for a minority of the sera, there was no indication of a possible correlation with the disease stage.

Discussion

The presence of autoantibodies against the tumor-associated EpCAM Ag in both cancer patients and normal individuals has been reported widely in the literature. Several immunotherapeutic approaches targeting EpCAM have been developed over the past 20 years. One of the arguments for targeting this Ag despite its wide distribution in the body is the assumption that a certain degree of naturally occurring autoreactivity in cancer patients does not lead to autoimmune organ damage. Autoreactivity against EpCAM is probably representative for a whole range of tumor-associated autoantigens.

In all papers described so far, rEpCAM protein produced in baculovirus systems has been used for detection of autoantibodies.

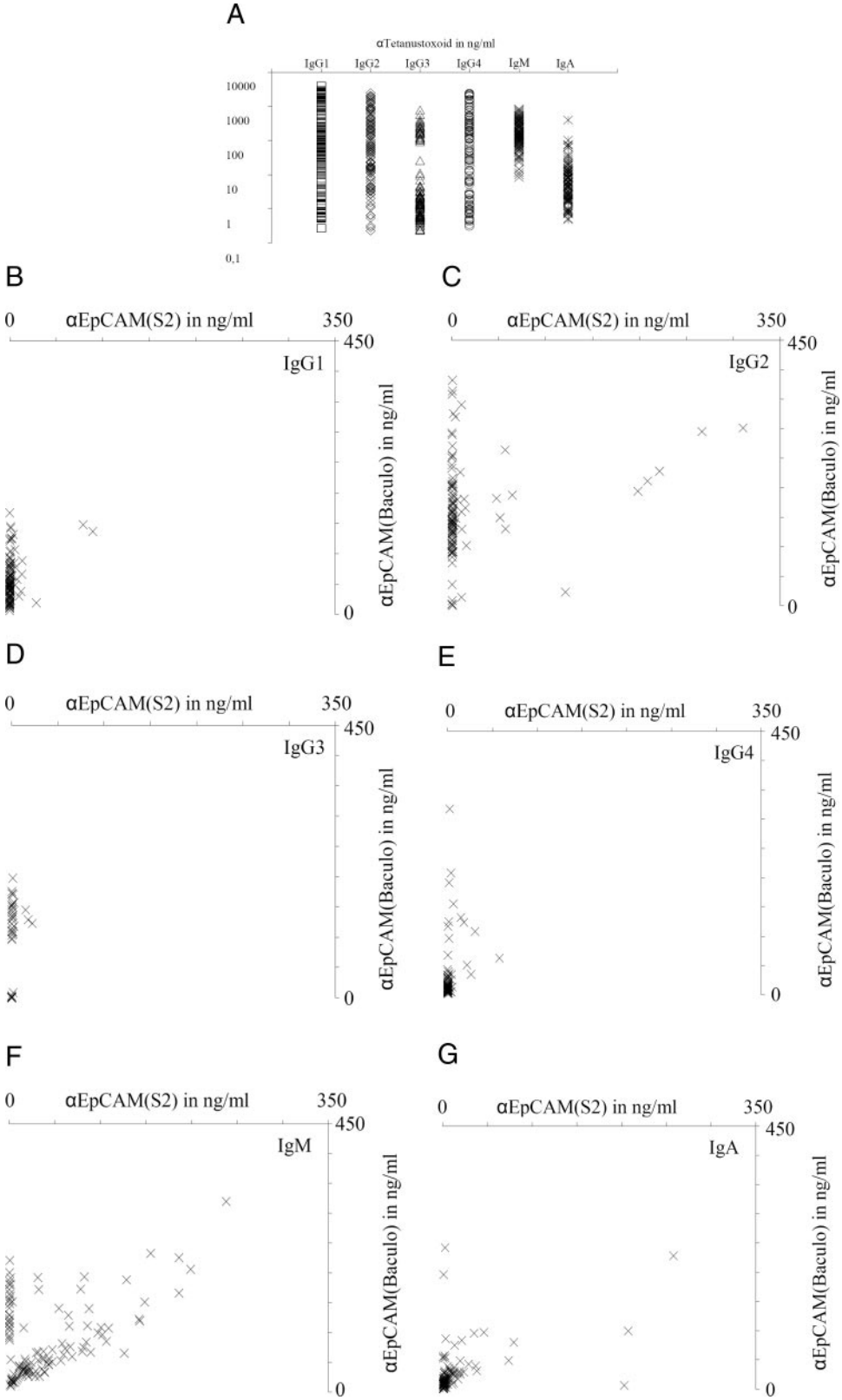

FIGURE 3. *A*, Analysis of the isotypes and amounts of TT-reactive Abs. IgG1 (□), IgG2 (◇), IgG3 (△), IgG4 (○), IgM (×), and IgA (∗) against TT from 120 samples are shown. *B—G*, Comparison of EpCAM-(S2)-reactive and EpCAM-(baculo)-reactive IgG sera. Shown are 115 randomly selected and 5 previously positive tested sera, subtype-specifically analyzed for EpCAM-reactive IgG1, IgG2, IgG3, IgG4, IgM, and IgA autoantibodies.

Even commercial kits have been developed for detection of circulating EpCAM protein in the blood.

Because we envisaged tumor vaccination against EpCAM, highly purified recombinant protein was produced in our lab in S2 *Drosophila* cells. The purity of our protein is significantly higher than the purity of baculovirus EpCAM purified from a commercial preparation (>96 vs ~80%) and was achieved by several optimization steps in the procedure.

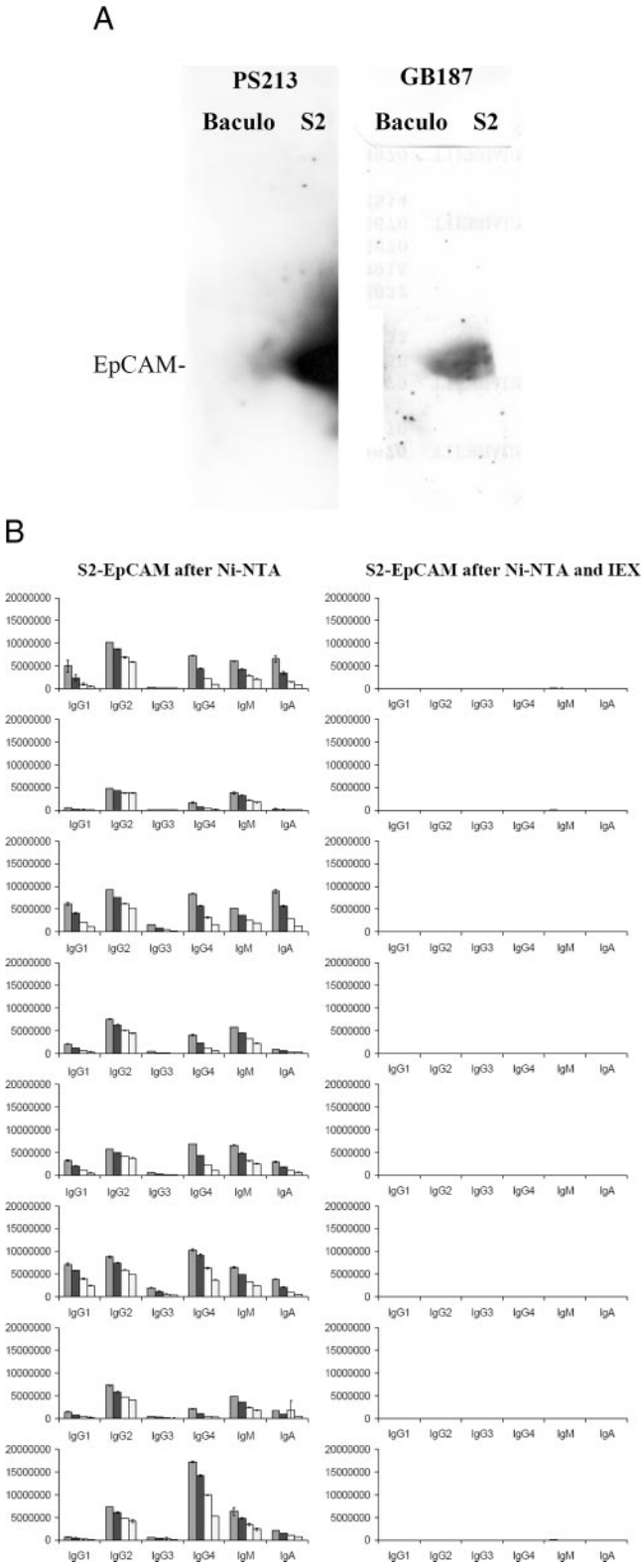

FIGURE 4. *A*, Western blots with patients' sera as primary Ab with either baculo-EpCAM or S2-EpCAM in two lanes on the membrane are shown. *B*, ELISA data from nine healthy controls are shown using EpCAM obtained before and after IEX as test Ag.

Most importantly, our protein appears to be correctly folded according to NMR spectroscopy compared with the baculovirus-produced EpCAM where presence of unfolded or misfolded protein can be assumed.

Analysis by lectin binding indicated that S2-EpCAM is highly mannosylated. N-terminal Edman sequencing verified the purity of the S2-EpCAM protein and showed that removal of the BIP signal peptide was completed in at least 95% of the protein. It also showed that the two 32- and 33-kDa proteins obtained have the same N termini and most likely represent the identical protein, differing only by posttranslational modification, which is in line with our glycosylation data.

Our long selection procedure on hygromycin resulted in an extremely high protein expression by S2 cells; further optimizations of protein expression were achieved by adapting the cells to grow at high concentration in the bioreactors and by optimizing the induction with copper sulfate. This resulted in EpCAM protein concentrations in the supernatant of up to 0.8 mg/ml compared with previous studies where usually 0.5–20 μg/ml recombinant protein was achieved (27, 31, 32).

Comparison of EpCAM purified from S2 cells and baculovirus-derived EpCAM showed major differences. The impurities in the baculovirus preparation become evident only when high amounts of protein are analyzed by PAGE. We detected five different proteins in the baculovirus preparation, which cannot be detected with lower sample loads. These additional bands were either not detected or not described in previous publications. The 70-kDa protein has been discussed as a dimer of EpCAM (23), but the band is visible to the same extent in the original publication, with both reducing and nonreducing conditions. Although it cannot be excluded that dimerization of EpCAM might occur by a mechanism other than disulfide bond formation, it is rather possible that this band represents a contaminating protein. In publications describing the biochemical properties of EpCAM, but not in most publications describing autoantibody reactivity, the baculovirus-derived protein was further purified by at least one further purification step, e.g., by HPLC (33, 34).

Contaminating proteins can produce false-positive results in Ab detection assays. As an example, low titers of Abs can be present in FCS: they can be immobilized and enriched considerably on affinity columns. These Abs or the corresponding immune complexes can elute as contaminating proteins. Furthermore, heterophilic anti-animal Ig Abs (35, 36) can be present in patients' sera and react with BSA or contaminants. The risk of enrichment for contaminating proteins is considerably increased when the concentration of the target protein in the supernatant is low, as is usually the case in baculovirus systems. Although we can purify ~150 mg of protein from 300 ml of supernatant, the protein concentration in the baculovirus system is around 1 mg/L (23).

Moreover, recombinant proteins from baculovirus-infected cells are released upon lysis of the cells by viral infection and not by secretion via the Golgi network, eventually preventing the naturally occurring removal of misfolded protein. This is particularly important for a protein like EpCAM that with its 12 cysteine residues can form many wrong disulfide bridge configurations.

We compared detection of EpCAM-reactive autoantibodies using the baculovirus-derived and the highly purified S2-derived EpCAM protein. Most sera were completely negative if tested with S2-EpCAM, with minimal amounts of Abs being detected in a few sera. In contrast, testing the same sera with baculo-EpCAM showed higher amounts of Abs. This reactivity might be directed against contaminating proteins or misfolded EpCAM protein. To test this, S2-EpCAM was subjected to denaturation and tested in the assay: indeed, 12 times higher amounts of Abs reacted with the denatured EpCAM compared with native S2-EpCAM.

Five sera that tested positive using S2-EpCAM were also positive with baculo-EpCAM as well, indicating that they did recognize EpCAM in both preparations. The recognition of denatured

Table I. *Results from ELISA and Western blotting*[a]

	Patient				
	PS213	KB199	HJ192	GB187	BS205
Anti-EpCAM (S2-nativ) IgG (ng/ml)	3.78	4.32	4.14	30.70	11.91
Anti-EpCAM (S2-denat) IgG (ng/ml)	200.00	66.43	34.83	258.74	55.55
Anti-EpCAM (baculo) IgG (ng/ml)	596.90	725.96	625.31	611.87	616.26
Anti-TT IgG (ng/ml)	702.46	659.47	572.22	396.10	2486.20
Reactivity against S2-EpCAM in WB	+++	++	+	++	++
Reactivity against other proteins in WB	−	−	−	−	−

[a] WB, Western blot.

EpCAM in the S2-EpCAM preparation could also been shown in all tested and ELISA-positive samples by Western blotting, with a good correlation of signal strength and ELISA data. We could not find any reactivity against EpCAM or against the contaminating proteins in the baculo-EpCAM preparation by Western blotting. The Abs reacting against this preparation could recognize nonlinear epitopes, which are destroyed in the SDS gel. Another possibility is the presence of small peptides, large protein aggregates, or nonprotein contaminations that are not separated by the gel. The NMR data suggest the presence of such misfolded large protein aggregates.

The importance of further purification of the test Ag could be shown by performing the ELISA with EpCAM obtained directly after Ni-NTA chromatography and after further purification with IEX chromatography. Only highly purified EpCAM protein showed no reactivity in healthy controls. It is important to mention here that the IEX chromatography removed obviously impurities that could not been seen in SDS-PAGE.

To exclude a falsely low EpCAM reactivity due to selection of patients that have been immunosuppressed by chemotherapy treatment, TT-reactive Abs were also measured in all of the samples. No correlation between EpCAM reactivity and TT reactivity could be found the TT-specific humoral response being ~1000 times higher compared with the EpCAM-specific response. Moreover, TT Abs consisted always of a mixture of several different IgG isotypes, whereas only Abs of a single isotype were found among EpCAM-reactive autoantibodies. These results suggest that the humoral immune response of the patients was not severely compromised by either the tumor itself or the chemotherapy treatment and emphasize the different potency of protective humoral responses against foreign pathogenic Ags with respect to self Ags in patients lacking clinical evidence for autoimmunity.

Among 176 sera from colorectal cancer patients in stage Dukes C or D, 4.5% contained S2-EpCAM-reactive IgGs. One publication describes a frequency of 16% EpCAM-reactive Abs in Dukes C patients and of 32% in Dukes D patients (11). In addition to the false-positive events due to contaminating or misfolded proteins, differences in recruitment of patients and different treatment of control sera could explain the higher frequency in this study. Yet, we do not have a clear explanation for the lack of reactivity of normal sera in this study.

Abs against denatured EpCAM are frequently found: it is possible that due to the presence of proteases and to a certain degree of necrosis and tissue disruption in rapidly growing cancer tissue, denatured protein is generated, which might be more immunogenic than its normal counterpart. Overexpression of the protein, which is typically found in many cancer cells, might also lead to the generation of a higher proportion of misfolded EpCAM, which in turn might be released by damaged cells or undergo increased proteasomal degradation (37). This degradation might also be responsible for the generation of epitopes that could be recognized by T cells, as suggested by our finding that in about one-third of colon cancer patients, T cell reacting against one or more MHC class II binding EpCAM epitopes can be demonstrated.[3] Reactivity against denatured proteins might be at least partially responsible for the reported presence of autoantibodies against many tumor-associated Ags such as cancer-testis Ags. Indeed, in most—if not all—of these studies, denatured protein was used to detect autoantibodies.

This does not necessarily lessen the meaning of these findings, but it may have different implications compared with Abs against correctly folded proteins. Abs against linear epitopes might reflect an epiphenomenon of protein turnover (including formation of misfolded protein and cell death), but are not likely to directly cause cellular damage. This would rather be a possibility with Abs against naturally folded proteins, particularly if expressed on the cell surface. As reviewed recently by Mahler et al. (28) for autoimmunity, disease-causing autoantibodies such as receptor-stimulating Abs against conformational epitopes of the TSH receptor are only found in Graves disease patients and not in normal individuals (38) and anti-glomerular basal membrane Abs that cause Goodpasture disease only react with three-dimensional conformational B epitopes of glomerular basal membrane protein (39). On the contrary, in cancer patients anti-p53 Abs react against a linear peptide that is cryptic in the native p53 and accessible only on the denatured or mutant protein (40).

In a few publications, similar low frequencies of EpCAM autoantibodies as in our study were reported for colorectal cancer patients (18).

Besides reactivity against denatured Ag, all tested sera showed some reactivity to the commonly used BSA. This reactivity is mainly due to IgM Abs, but in many samples, also BSA-reactive IgGs were detected. For this reason we used only highly purified HSA, which resulted in lack of unspecific reactivity. In all studies that measured Abs against EpCAM, BSA was used for blocking, and therefore some degree of BSA reactivity can be assumed. This could also provide an explanation for the high background and the flat profile of the serum dilution curves in some of these studies. Indeed, several publications have shown that BSA-reactive IgG Abs are present in various amounts in 20–100% of sera from different age groups (41–44).

A significant higher amount of EpCAM-reactive IgM was never found in patients with colorectal carcinoma. In healthy controls

[3] O. Schmetzer, M. Schnitzler, G. Richter, W. Zheng, A. Nicolau, H. Koop, B. Hoppe, P. Schlag, F. Sinigaglia, and A. Pezzuttó. Colon cancer patients have circulating T cells that specifically recognize several MHC class II peptides of the tumor-associated EpCAM antigen. *Submitted for publication.*

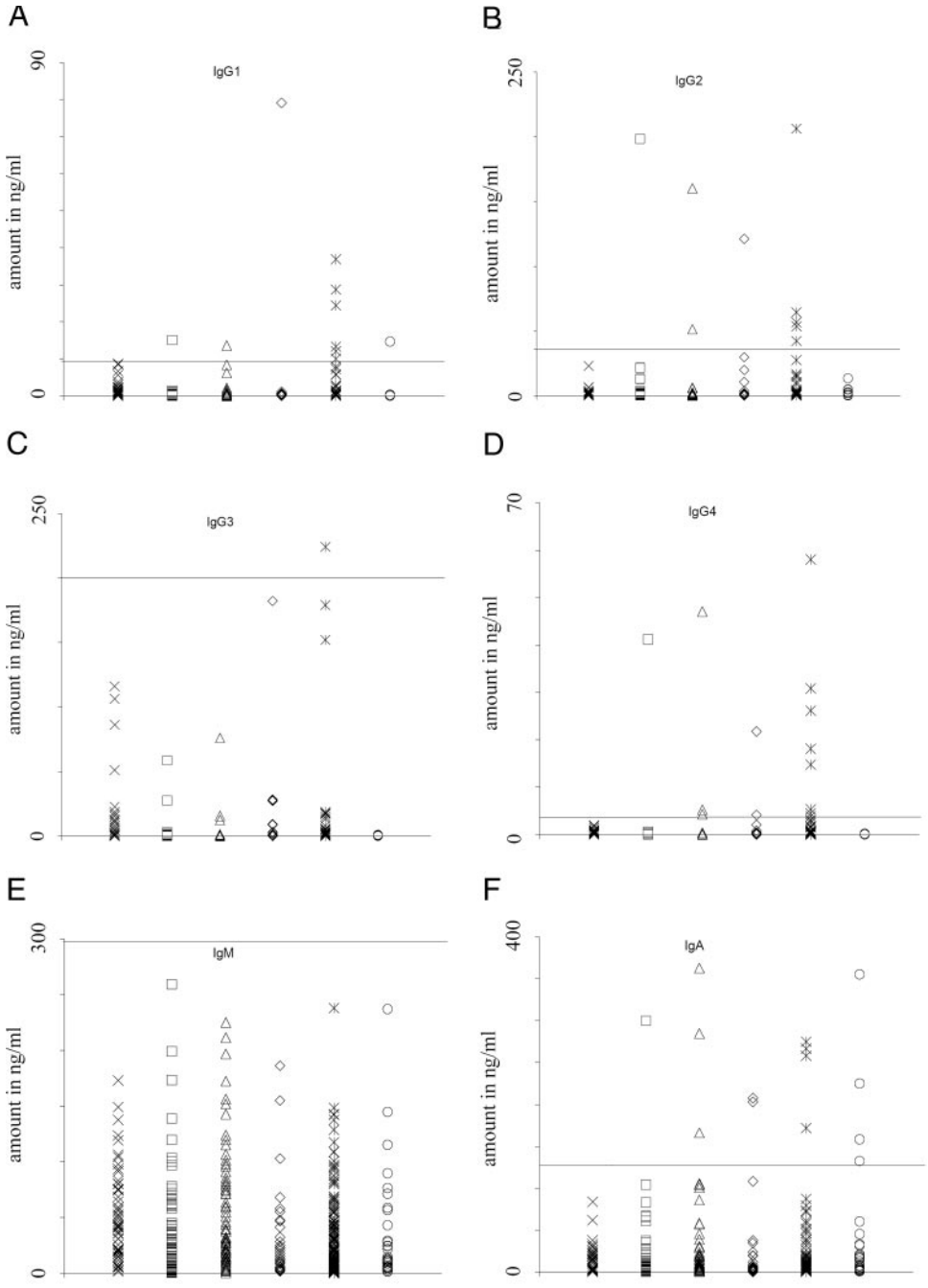

FIGURE 5. Naturally occurring EpCAM-reactive Abs in patients and healthy controls. Shown are 60 sera from healthy control (×), 95 sera from patients with colon carcinoma (□), 81 sera from patients with rectum carcinoma (△), 39 sera from patients with stomach carcinoma (◇), 261 sera from patients with breast cancer (*), and 32 sera from patients with prostate carcinoma (○). The threshold (horizontal line) for positivity has been set at the mean of the sera plus 8 SDs. *A*, EpCAM-reactive IgG1 Abs. *B*, EpCAM-reactive IgG2 Abs. *C*, EpCAM-reactive IgG3 Abs. *D*, EpCAM-reactive IgG4 Abs. *E*, EpCAM-reactive IgM Abs. *F*, EpCAM-reactive IgA Abs.

Table II. *Ab responses (HSA blocking)*[a]

Tumor Type	Number of Samples (*n*)	IgG1 Positive (% (No.))	IgG2 Positive (% (No.))	IgG3 Positive (% (No.))	IgG4 Positive (% (No.))	Total IgG-Positive Samples (% (No.))	Samples Positive for Two or More IgG Isotypes (% (No.))	IgM Positive (% (No.))	IgA Positive (% (No.))
Healthy controls	60	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Colon CA	95	1.1 (1)	1.1 (1)	0.0 (0)	1.1 (1)	3.2 (3)	0.0 (0)	0.0 (0)	1.1 (1)
Rectum CA	81	1.2 (1)	2.5 (2)	0.0 (0)	3.7 (3)	6.2 (5)	1.2 (1)	0.0 (0)	3.7 (3)
Stomach CA	39	2.6 (1)	5.1 (2)	0.0 (0)	5.1 (2)	7.7 (3)	2.6 (1)	0.0 (0)	5.1 (2)
Mamma CA	261	2.3 (6)	1.9 (5)	0.4 (1)	3.1 (8)	6.9 (18)	0.8 (2)	0.0 (0)	1.5 (4)
Prostate CA	32	3.1 (1)	0.0 (0)	0.0 (0)	0.0 (0)	3.1 (1)	0.0 (0)	0.0 (0)	9.4 (3)

[a] CA, Cancer.

Table III. *Ab responses (BSA blocking)*[a]

Tumor Type	Number of Samples *n*	IgG1 Positive (% (No.))	IgG2 Positive (% (No.))	IgG3 Positive (% (No.))	IgG4 Positive (% (No.))	Total IgG-Positive Samples (% (No.))	Samples Positive for Two or More IgG-Isotypes (% (No.))	IgM Positive (% (No.))	IgA Positive (% (No.))
Colon-CA	33	6.1 (2)	15.2 (5)	0.0 (0)	15.2 (5)	21.2 (7)	9.1 (3)	100.0 (33)	48.5 (16)
Rectum-CA	21	0.0 (0)	19.0 (4)	0.0 (0)	0.0 (0)	19.0 (4)	0.0 (0)	100.0 (21)	66.7 (14)
Stomach-CA	8	0.0 (0)	50.0 (4)	0.0 (0)	12.5 (1)	50.0 (4)	12.5 (1)	100.0 (8)	75.0 (6)
Mamma-CA	45	4.4 (2)	33.3 (15)	6.7 (3)	6.7 (3)	35.6 (16)	6.7 (3)	100.0 (45)	64.4 (29)

[a] CA, Cancer.

also similar high amounts of IgM were detectable, which may correspond to autoantibodies of low specificity. Such autoantibodies have been described widely (45).

EpCAM-reactive IgA autoantibodies have never been described previously. We found approximately three times higher amounts of IgA against EpCAM compared with TT. This could be due to the different localization of the Ag. Although TT-reactive Abs are often induced by vaccine injection in muscle tissue, EpCAM is abundantly expressed in the gut, where high concentrations of TGF-β are needed to support the growth of epithelial stem cells. Because TGF-β facilitates IgA class switch (43, 44), B lymphocytes resident in the gut may be induced to preferentially secrete IgA by the local TGF-β-rich environment.

EpCAM-reactive Abs of the IgG3 subclass were present in minimal amounts in only one of 120 samples. This low frequency could be explained by the higher complement-activating capacity of this Ig isotype (46, 47): one could assume that the class switch to IgG3 is more tightly regulated compared with other isotypes to prevent autoimmune tissue damage.

We could find very low levels of EpCAM-reactive Abs in healthy donors. This is consistent with recent findings (30). Because of the high sensitivity of our test, some normal individuals appeared to have EpCAM autoantibodies in low titers if we used 3 SDs as a threshold. We felt that using 8 SDs provided a safe boundary for keeping most of healthy individuals negative and most of the patients who appear to have circulating autoantibodies positive. Arbitrary thresholds have to be set for many autoantibodies such as rheumatoid factors or cold agglutinins, which are present in many normal individuals in low concentrations.

Because of the high expression of EpCAM in several fetal organs, one would expect the existence of central tolerance for this Ag (2, 4, 7). Also, the high expression of EpCAM on nearly all epithelial cells should render T cells that may have escaped the thymic selection anergic and induce a state of peripheral tolerance. Moreover, oral tolerance may be induced by the long-term shedding of dead epithelial cells in the gut. In the thymus, negative selection of CD8 cells is more accurate than for CD4 cells. However, $CD4^+$ T cell responses against EpCAM have been demonstrated (48). We could also define specific MHC-II binding EpCAM epitopes that appear to be recognized by a small percentage of cancer patients.[3] The presence of IgG Abs against EpCAM in some patients indeed fits well to CD4-mediated Ig-class switching.

A few clinical trials have been conducted with the aim of inducing EpCAM-specific immune responses using viral vectors or anti-idiotypic Abs (22, 49–52). There was none or only a minimal benefit to the patient in studies with anti-idiotypic vaccines, although the vaccine induced strong humoral and cellular immune response against the injected Ab. Autoimmunity was never observed.

Our results indicate that Ab reactivity against tumor Ags as claimed by many publications must be cautiously interpreted and clearly depend on the purity of the Ag used for Ab detection. The immune response that, indeed, some cancer patients appear to mount against EpCAM seems to be weak, and the effectivity of strategies aiming at increasing this response might be severely limited by a state of immune tolerance. Breaking tolerance against this tumor-associated Ag might indeed provide antitumor activity but at the price of autoimmunity.

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