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Original Paper

Human Antibodies Against the Polymorphic Epithelial Mucin in Ovarian Cancer Patients Recognise a Novel Sequence in the Tandem Repeat Region

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The humoral immune response to the polymorphic epithelial mucin (PEM) was studied by characterising the reactivity of human antibodies generated by EBV-immortalised B-cells from tumour-draining lymph nodes of ovarian cancer patients. All the human antibodies, selected in ELISA for their reactivity to the protein core tandem repeat sequence, reacted with PEM-expressing tumour cells. Aberrant glycosylation of the peptide core of the PEM molecule in cancer cells leads to the exposure of peptide epitopes that can be considered tumour specific. The epitope mapping of six human antibodies revealed that only one of them contained the PDTR sequence, shown to be the immunodominant epitope in the mouse. Four of the six human antibodies recognised a novel common immunogenic sequence (APPAH) in the tandem repeats. The binding of these human antibodies did not appear to be modulated by the length of the carbohydrate side chains, as shown by O-glycosylation inhibition studies. These results indicate that distinct sequences within the tandem repeat of PEM are target for a humoral immune response in humans. The presence of antibodies directed against different epitopes within the same antigenic region may modulate the antigen presentation process and the ongoing immune response. These data may help in clarifying the mechanisms of the immune response to PEM in cancer patients for the development of PEM-based immunotherapy.

Key words: polymorphic epithelial mucin, immune response, human antibodies, epitope mapping, tumour-associated antigens

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INTRODUCTION

POLYMORPHIC EPITHELIAL mucin (PEM), a high molecular weight transmembrane glycoprotein, is a potential target for active and passive immunotherapy of adenocarcinomas [1, 2]. The molecule, coded by the *MUC-1* gene, is overexpressed and aberrantly glycosylated in a variety of epithelial cancers [3, 4]. It is a type I protein composed of three main domains: a cytosolic region, a transmembrane domain and a large extracellular region [3]. The protein core of this last region is composed of numerous tandem repeats of a conserved 20 sequence [5]. As a result of altered glycosylation during malignant transformation, cryptic epitopes within the tandem repeat region of PEM are exposed and become immunogenic in the host [4, 6]. Several lines of evidence suggest the immunogenicity of this molecule in experimental models [7–9] and in humans [10–16].

A humoral immune response against this molecule has been demonstrated utilising Epstein–Barr virus (EBV)

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transformed B-cells from tumour-draining lymph nodes obtained from cancer patients [10]. Other investigators have detected both free and immunocomplexed anti-PEM antibodies in the sera of cancer patients [11–13]. A cellular immune response against an immunodominant part of the tandem repeat sequence in cancer patients has also been described [14]. The interesting feature of this $CD8^+$-mediated lysis is that it is not major histocompatibility complex (MHC) restricted, which could be explained by the simultaneous engagement of multiple T-cell receptor (TcR) with the repetitive tandem repeats [15, 16]. Another indication suggesting a possible role of this antigen in the development of immunity against cancer was shown in a recent report indicating a correlation between the protection against breast cancer observed in multiparous women and the development of a natural immunisation against *MUC-1* acquired during pregnancy [17]. The understanding of the type of immunity that is involved in the recognition of this self-antigen, that during malignancy is overexpressed showing altered cellular distribution and glycosylation, has important implications in the generation of PEM-based vaccines.

We report here the epitope mapping of the antigenic determinants within the tandem repeat of PEM recognised by human antibodies produced by B-cells from ovarian tumour-draining lymph nodes.

MATERIALS AND METHODS

Cell cultures

Established breast cancer cell lines MCF-7 and ZR-75-1 were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, Missouri, U.S.A.), while the ovarian cancer cell line OVCA-433 was grown in DMEM:F-12 HAM (Sigma) 1:1. All media were supplemented with 10% inactivated fetal calf serum (FCS) (Hyclone, Logan, Utah, U.S.A.), 50 μg/ml streptomycin (Flow Labs, Australia), and 50 UI/ml penicillin (Flow). Breast cancer cell line BT-20 was grown in DMEM supplemented with 15% inactivated FCS, 10 μg/ml insulin (Sigma) and streptomycin and penicillin as above.

Human B-cell lines producing anti-PEM antibodies were obtained after immortalisation of B-cells from tumour-draining lymph nodes by EBV infection. These were grown, screened and isotype-characterised as previously described [10]. In brief, single-cell suspensions of the specimens were produced within 3 h of surgery. Unseparated B-cells were immortalised using a semi-purified preparation of EBV obtained from the B 95-8 marmoset-transformed leucocyte EBV-producing cell line. These were cultured in RPMI-1640 (Sigma) culture medium supplemented with 10% inactivated fetal calf serum (Hyclone), 50 μg/ml streptomycin (Flow), 50 UI/ml penicillin (Flow), 2 mM glutamine (Hyclone), 1 mM pyruvate (Sigma), non-essential amino acids (Hyclone) diluted 1:100, and 1 μg/ml cyclosporine (kindly provided by Sandoz, Pharma Ltd). After 3 weeks, immortalised B-cells were seeded in 24- and 96-tissue culture plates and the supernatant was tested for human antibody production by an enzyme-linked immunosorbent assay (ELISA).

The enrichment of the antibody-secreting cell lines was achieved by frequent cloning of the selected clones. For each clone, a single batch of culture supernatant collected from a low density passage was used for all the experiments.

ELISA

Production of specific human antibodies was assessd by ELISA using the 24mer synthetic peptide (kindly provided by Dr J. Taylor-Papadimitriou, ICRF, London, U.K.) coupled or uncoupled to bovine serum albumin (BSA), incubated at 1 μg/ml in carbonate buffer for 2 h in 96-well polystyrene plates (Falcon). Alternatively, as negative control, an unrelated synthetic peptide (gonadotropine-releasing hormone, Sigma) was attached to the plates. Non-specific binding sites were blocked by incubation with 100 μl/well of PBS containing 5% BSA for 1 h at 37°C. After one wash with PBS containing 1% BSA and 0.05% Tween 20 (PBS–BSA–Tween), 50 μl/well of B-cell culture supernatant or, as positive control, HMFG-1 hybridoma supernatant, were added to the plates in duplicate and incubated for 2 h at room temperature (RT). After four washes with PBS–BSA–Tween, 100 μl/well of the affinity purified goat antihuman (IgG + A + M) biotinylated primary antibody (Sigma) diluted 1:500 and goat antimouse biotinylated secondary antibody (Zymed) were added and incubated for 1 h at RT. After four washes as described, 100 μl/well of an avidin/streptavidin peroxidase conjugate (Sigma) diluted 1:500 was added and incubated for 30 min at RT. After four washes, plates were developed with 100 μl/well of 0.5 mg/ml O-phenylendiamine dihydrochloride (Sigma) in phosphate–citrate buffer containing 0.03% hydrogen peroxide, incubating for 15 min at RT in the dark. Finally, the enzymatic reaction was blocked by adding 50 μl/well of 4 N H_2SO_4. Isotyping of the human antibodies was performed using the commercial kit BAK 1000 (Vector, Burlingame, California, U.S.A.). Optical density measurements were performed at 492 nm wavelength using the EAR 400 ELISA reader (SLT-Labinstruments, Austria).

Antibodies

Murine monoclonal antibodies (MAbs) HMFG-1 (IgG1) and SM3 (IgG1) were kindly provided by Dr J. Taylor-Papadimitriou (ICRF, London, U.K.) [6, 18]. MAb 436 (IgM) was generated in our laboratory as previously described [19]. The secondary antibodies used in this study were fluorescein-5-isothiocyanate (FITC) conjugated sheep $F(ab')_2$ fragment to mouse IgG/IgM (Cappel, Durham, North Carolina, U.S.A.) and FITC-conjugated goat $F(ab')_2$ fragment to human immunoglobulins (IgG, IgA, IgM) (Cappel). Purified human IgM (Sigma) was used as negative control in cytofluorimetric and epitope mapping experiments.

Epitope mapping

The sequence determination of the epitopes in the PEM tandem repeat recognised by the human antibodies was performed according to the procedure described by Geysen and associates [20] using the commercial kit SPOTs (Cambridge Research Biochemicals, Cambridge, U.K.). Octapeptides were simultaneously synthesised on a cellulose membrane provided with free amino functionalities in a matrix of small circular blue spots. Octamer peptides were synthesised, offset by one, from the first to the twentieth of

the tandem repeat. A twenty-first octamer corresponding to the first one was included as an internal control.

The membrane-bound peptides were analysed using an indirect colourimetric immunoassay based on the β-galactosidase activity. After blocking non-specific binding sites with an overnight incubation in blocking buffer (10% casein blocking agent (CRB), 0.15 M sucrose in Tris-buffered saline (TBS) containing 0.2% (vol/vol) Tween-20 (T-TBS)), the membrane was incubated for 3 h with antibody supernatant. After three washes in T-TBS, the membrane was incubated with the secondary antibody, affinity-purified biotin-conjugated goat antihuman polyvalent immunoglobulins (Sigma), at 1:500 dilution for 2 h at RT. After three washes in T-TBS, the membrane was incubated with streptavidin-β-galactosidase (Sigma), containing 1.5 U of β-galactosidase for each immunoassay for 1 h at RT. After

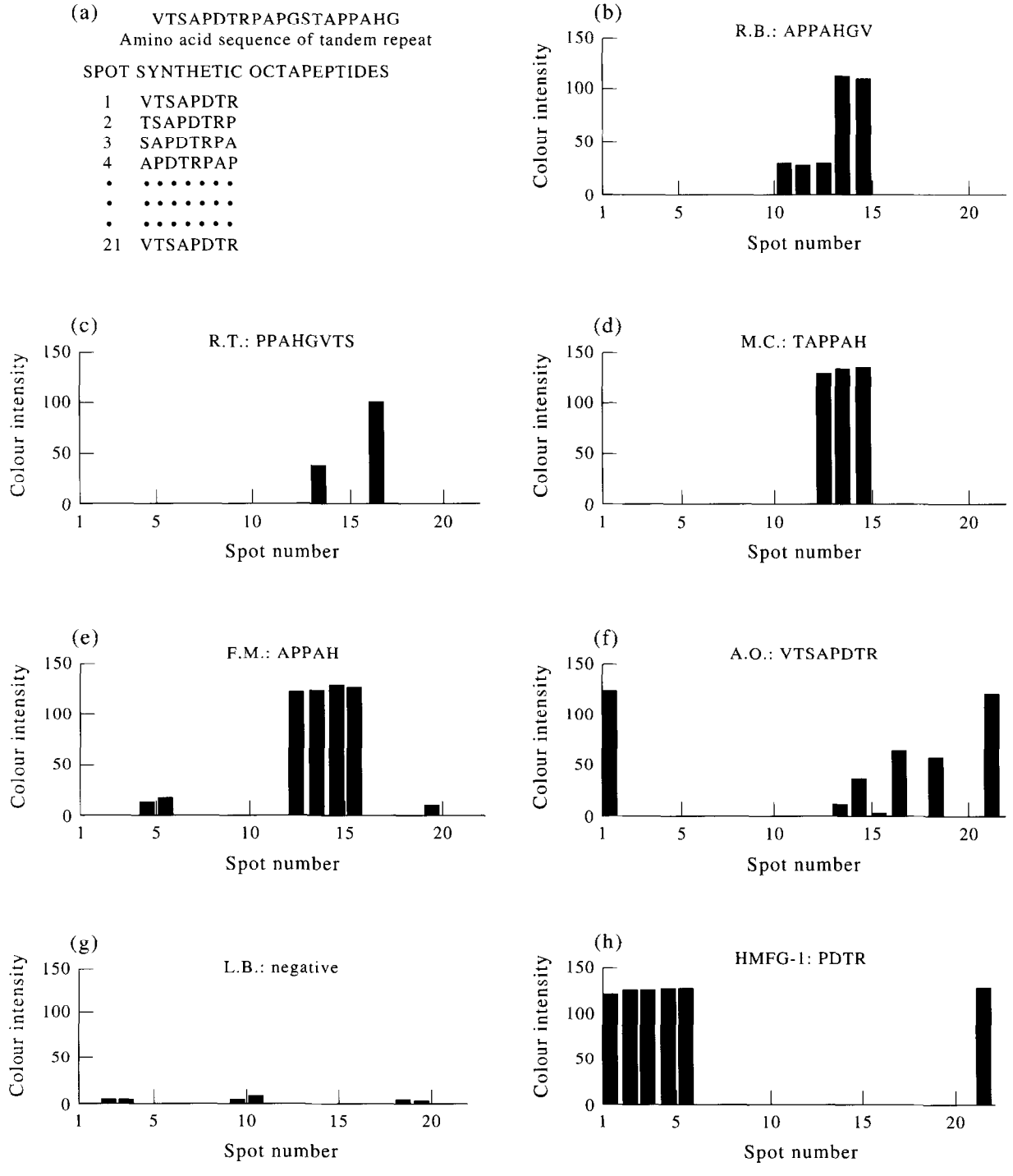

Figure 1. Histograms corresponding to the epitope mapping immunoblots obtained by computer evaluation of the colour intensity of the spots. The known epitope of MAb HMFG-1, used as positive control, is the sequence PDTR. The sequence of the octamers synthesised in each spot is shown in (a).

four washes as above, the membrane was developed with the cromogenic substrate (1 mM 5-bromo-4-chloro-3-indolyl-β-D-galactopiranoside (BCIG), 3 mM KCl, 1 mM $MgCl_2$ in phosphate-buffered saline (PBS)). As negative control, purified human IgM (Sigma) at 0.5 μg/ml was used instead of the isolated human antibodies. As positive control, one membrane was incubated with the HMFG-1 hybridoma supernatant [18, 21] for 1 h at RT. β-galactosidase conjugate sheep antimouse immunoglobulins antibody (CRB) was used as a secondary reagent. The development was performed as described for human antibodies. The octamers that reacted positively were visible as blue spots. The colour intensity was measured, normalised at 256 grey tones and converted in arbitrary units using the Video Digitizer Manager method by NIH image computer program. Spot reactivities above 65 arbitrary units were considered as positive. This cut-off value was applied since scattered spots were occasionally slightly coloured with intensity below 65.

Indirect immunofluorescence

Indirect immunofluorescence assay was performed on acetone/methanol fixed cytocentrifuged metastatic cells from ascites of two ovarian cancer patients after overnight incubation at 4°C with the human antibody (supernatant of B-cells culture), followed by an incubation with FITC-conjugated goat $F(ab')_2$ fragment to human immunoglobulins (IgG, IgA, IgM) (Cappel) at 1:40 dilution for 30 min at RT in the dark. The positive control was incubated with the murine MAb HMFG-1 supernatant whereas the negative control was performed by omitting the first antibody and using the secondary reagent only.

Cytofluorimetric assay

Cell lines were grown in tissue flasks up to 80% confluence, trypsinised, washed twice with medium containing 10% fetal calf serum and incubated (10^6/sample) for 3 h or overnight on a rotating plate with 200 μl human antibody at 4°C. As a negative control, a sample for each cell line tested was incubated with purified human IgM (Sigma) at 0.5 μg/ml. After three washes with medium containing serum and 0.02% sodium azide, the samples were resuspended in 100 μl of FITC-conjugated goat $F(ab')_2$ fragment to human immunoglobulins (IgG, IgA, IgM) (Cappel) at 1:50 dilution and incubated for 1 h at 4°C. After 3 washes in PBS, the cells were resuspended in 300 μl PBS with propidium iodide to exclude the dead cells and analysed by a Beckton Dickinson FACScan flow cytometer. As a positive control for immunofluorescence analysis, the cell lines were incubated with or without the MAbs SM3 and 436 and then with the FITC-conjugated sheep $F(ab')_2$ fragment to mouse IgG/IgM (Cappel) at 1:50 dilution in agreement with the previously described protocol.

Treatment of cells with benzyl-N-acetylgalactosamine

Cells were treated as previously described [22]. Briefly, monolayer cells at approximately 60–70% confluence were incubated for 48 h with or without 2 mM benzyl-*N*-acetyl-galactosamine (Sigma) in fresh culture medium. After 48 h of treatment, the cells were processed for the cytofluorimetric assay (see above). The efficiency of the glycosylation inhibition on the PEM molecule was evaluated using the MAbs SM3 and 436 in parallel samples.

RESULTS

Human antibodies isolated from the supernatant of EBV-transformed B-cell lines obtained from tumour-draining lymph nodes of six ovarian cancer patients were selected and screened for specificity against the tandem repeat region of PEM in ELISA and in immunohistochemistry with PEM-expressing cell lines [10].

The amino acids involved in the epitope recognised by these antibodies were assessed by an immunoenzymatic assay. Overlapping octamer peptides corresponding to the tandem repeat sequence of PEM (Figure 1a) were synthesised in defined circular areas on cellulose membranes. These membrane were then tested in three independent experiments with all the human antibodies and with the murine MAb HMFG-1. This antibody, recognising a known epitope (PDTR) within the tandem repeat of PEM [21], was used as control of the synthesised peptides. The immunoreactive coloured spots contain amino acids that are involved in the antibody binding. The colour intensity of each spot was measured and converted to arbitrary units by computer program. Figure 1b shows the histograms corresponding to one epitope mapping experiment for each human antibody and for the MAb HMFG-1.

Four of the antibodies bind to a region of the tandem repeat containing the PPAH sequence and which may include additional flanking amino acids. This PPAH motif is distinct from the PDTR sequence, which is known to be immunodominant in mice, and that is contained in the sequence recognised by the human antibody A.O. Immunoblotting performed with antibody A.O. results, in fact, in high positivity of spots 1 and 21. The human antibody L.B. was reactive with the 24mer tandem repeat peptide in ELISA, but no reactivity could be detected with the octamers. The definitive epitope for each human antibody was assigned on the basis of the common sequence that was reproduced in the different experiments and the results are summarised in Table 1.

Cytofluorimetric analysis on PEM-expressing cell lines of breast and ovarian histotypes (Figure 2) indicate a low and homogeneous reactivity of most of the antibodies tested with MCF-7 and ZR-75-1 breast carcinoma cell lines (Figure 2a,b). All the antibodies showed a better binding capacity with the ovarian cancer cell line OVCA-433 (Figures 2a and 3).

On the basis of antibody R.B. reactivity, a negative and a positive cell population could be distinguished in the T47-D

Table 1. Epitope sequences recognised by anti-PEM antibodies

Human antibodies	Epitope*
M.C.	GS**TAPPAH**GVTSAPDTRPAP
R.B.	GS**TAPPAH**GVTSAPDTRPAP
F.M.	GS**TAPPAH**GVTSAPDTRPAP
R.T.	GS**TAPPAH**GVTSAPDTRPAP
A.O.	GSTAPPAHG**VTSAPDTR**PAP
L.B.	NEGATIVE

*The definitive epitope for each human antibody was assigned on the basis of the common sequence that was reproduced in three separate experiments (bold characters). Underlined amino acids correspond to spot sequences that were also positive in some experiments.

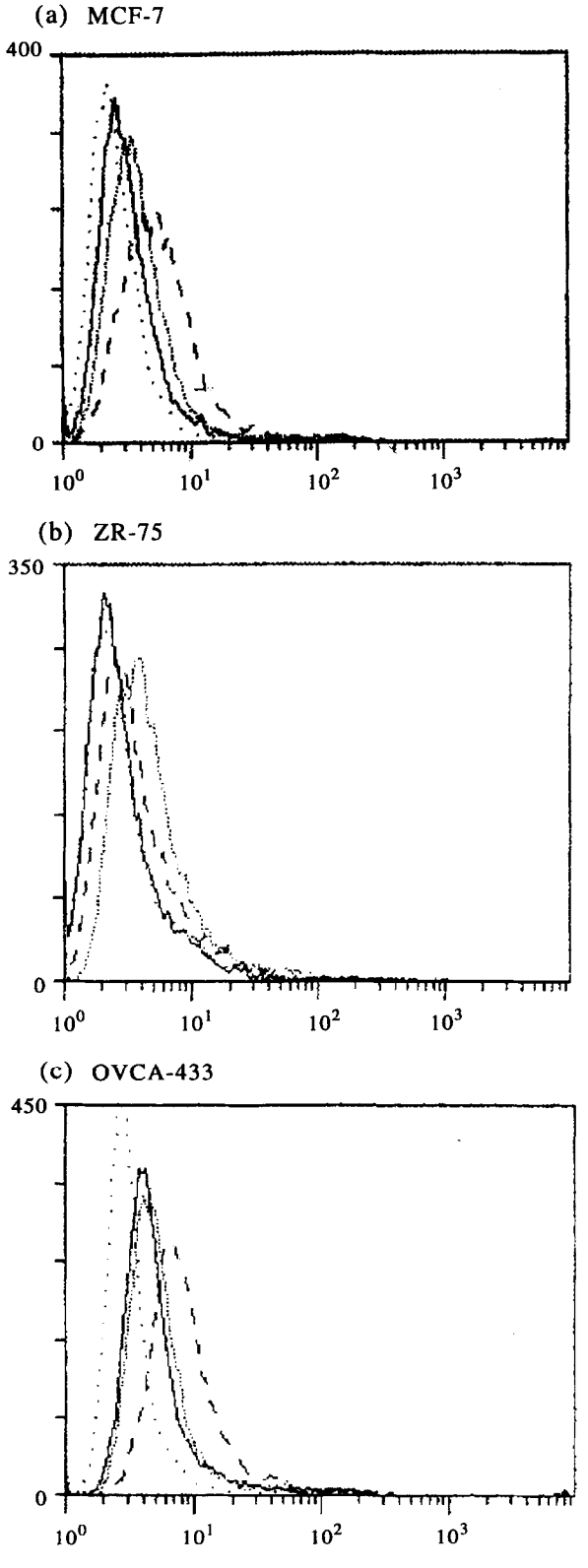

Figure 2. Reactivity of the human antibodies on carcinoma cell lines in FACS analysis. The breast carcinoma cell lines MCF-7 (a) and ZR-75 (b) and the ovarian carcinoma cell line OVCA-433 (c) were incubated with the human antibodies A.O. (——), F.M. (·····) and R.T. (– – –). Commercial purified human immunoglobulins (· · · ·) were used as negative control. The *x*-axis shows log fluorescence intensity; the *y*-axis shows the cell number.

breast cancer cell line, while no reactivity was observed with the BT-20 cell line (Figure 3).

Experiments were conducted to investigate the influence of the degree of glycosylation on antibody binding. Established cell lines expressing PEM were treated with benzyl-*N*-acetylgalactosamine, an O-linked glycosylation inhibitor that functions as a competitive inhibitor in the O-glycosylation pathway to block the elongation of the sugar side chain [23]. This treatment preceded cytofluorimetric analysis. Results show that benzyl-*N*-acetylgalactosamine treatment does not significantly change the degree of binding of the human antibodies to cancer cell lines at different levels of epitope expression. In Figure 3, results for the human antibody R.B. are shown. As a control of the effect of this treatment on the exposure of TR epitopes, the MAbs SM3 (PDTRP epitope) [22] and 436 (RPAP epitope) were used. As shown in Figure 3 the benzyl-*N*-acetylgalactosamine treatment often results in exposure of additional epitopes that become accessible to SM-3 and 436 antibody binding.

In order to evaluate the PPAH epitope expression directly on tumour specimens from cancer patients, cytocentrifuged metastatic ovarian cancer cells were tested in indirect immunofluorescence. The reactivity of the human antibodies R.B. and R.T. are shown in Figure 4a and b, respectively. The staining was intense, associated with the plasma membrane and limited to the tumour cells. Other cells present in the ascites were negative. The negative control, using the second antibody alone, and the positive control, using the MAb HMFG-1, are shown in Figure 4c,d.

DISCUSSION

During cellular transformation, PEM undergoes several modifications such as the level of expression, the degree of glycosylation and the pattern of cellular distribution. The modified molecule as expressed by epithelial tumour cells can, therefore, be considered a potential target for immunotherapy [24, 25]. The definition of the mechanisms necessary for a specific and successful immune response and the identification of the specific sequences involved in these processes are very important for the formulation of PEM-based vaccines [26].

Previously, we demonstrated a humoral immune response against the tandem repeat region of PEM in tumour-draining lymph nodes from an ovarian cancer patient [10]. Other investigators have subsequently supported this finding describing circulating human antibodies and immune complexes in the serum of tumour patients [11–13].

We report here the epitope mapping of human anti-PEM antibodies isolate from five different ovarian cancer patients. The analysis of the amino acid sequence involved in the antibody binding of four of the human antibodies revealed antigenic determinants within the tandem repeat protein core different from the one indicated as immunodominant in mice (PDTR) [27]. All of these antibodies in fact, include the PPAH sequence in their epitope. The human antibody A.O. recognises the sequence VTSAPDTR that contains the PDTR epitope. Another human antibody L.B. was isolated, but this antibody failed to bind, in the assay used, a specific octameric amino acid sequence, suggesting the need for a longer sequence for optimal binding.

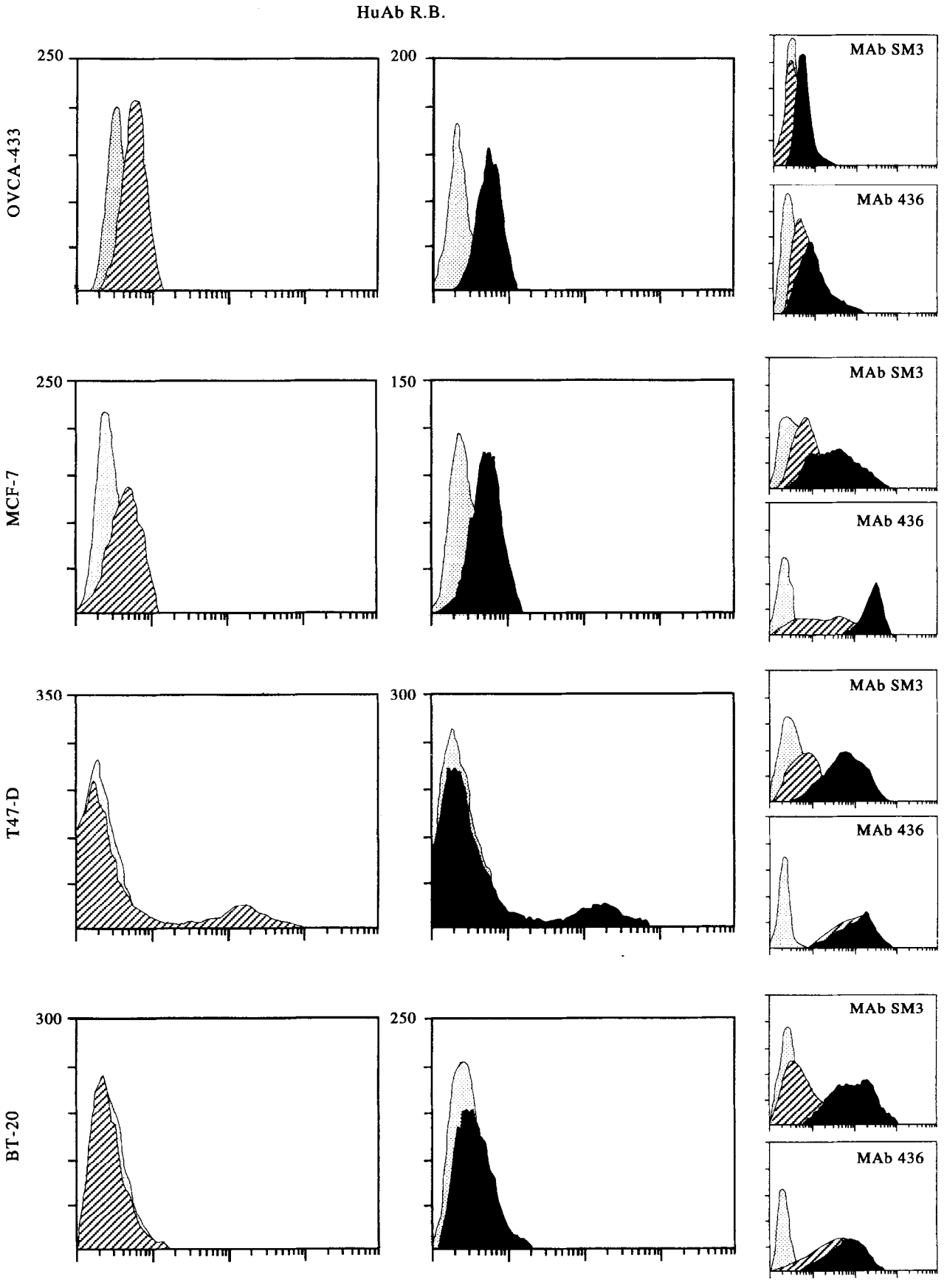

Figure 3. FACS analysis performed to evaluate the influence of O-glycosylation on the binding of human antibody R.B. and murine MAbs SM3 and 436 to the ovarian carcinoma cell line OVCA-433 and to the breast carcinoma cell lines MCF-7, T47-D and BT-20 treated without (hatched areas) or with (solid areas) an inhibitor of the O-glycosylation process. Shaded areas correspond to the reference sample using isotype/species matched immunoglobulin control.

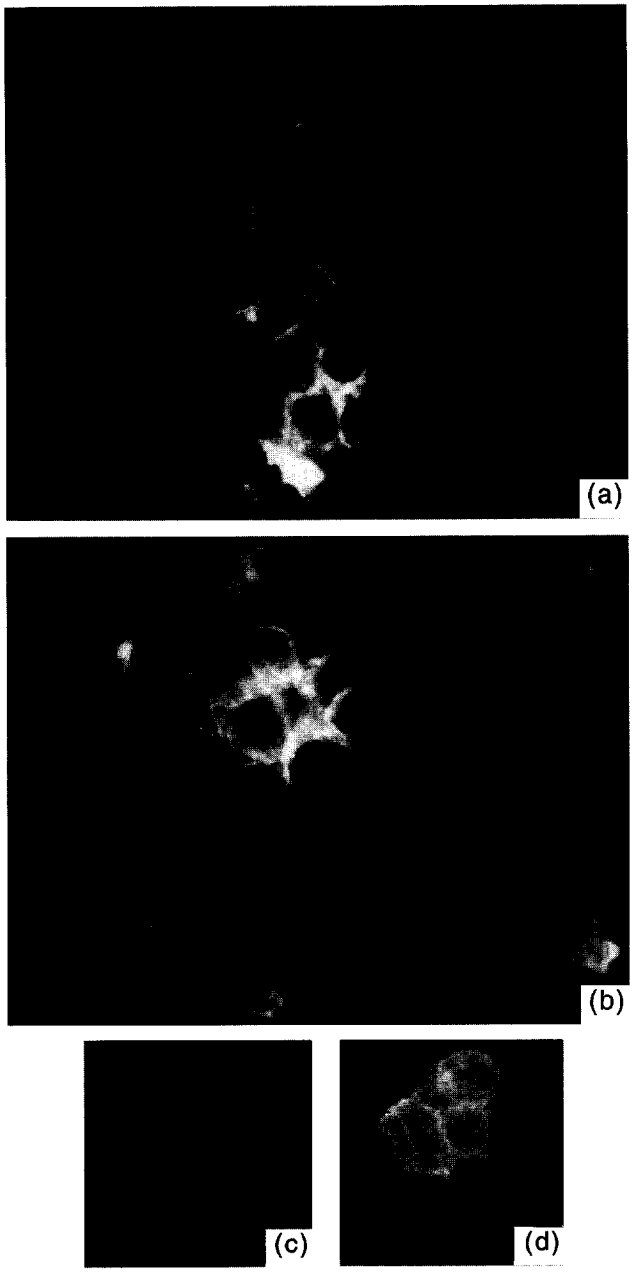

Figure 4. Indirect immunofluorescence performed on cytocentrifuged metastatic ascitic effusion from an ovarian cancer patient using the human antibodies R.B. (a) and R.T. (b) (magnification 1300×); (c) and (d) illustrate the negative and positive control (MAb HMFG-1).

Cytofluorimetric analysis showed that the human antibodies reacted differently with PEM-expressing cell lines, ranging from negative to weakly positive. However, the isolated human antibodies were still capable of recognising tumour cells in malignant effusions.

Experiments conducted to study the influence of O-glycosylation on epitope expession using the benzyl-*N*-acetylgalactosamine inhibitor showed that human antibody binding was not affected by this treatment. As previously demonstrated [22], the shortening of the sugar side chains affects the SM-3 epitope dramatically by increasing the binding of the antibody to the breast cancer cell lines. This modification is, however, very limited in the ovarian cancer cell line. Similarly, the expression of the RPAP epitope, which is recognised by MAb 436, can be increased (MCF-7) or remain unmodified (T47D, BT20 and OVCA433).

These results suggest that the human antibodies may recognise a naked amino acid region within the TR of PEM. Moreover, treatment with benzyl-*N*-acetylgalactosamine does not affect its expression, while the same treatment may profoundly influence the binding to adjacent sequences (PDTR and RPAP).

Recent *in vitro* studies on the PEM glycosylation process [27–29] in breast and pancreatic tumour extracts, have shown that only the two Thr residues flanking the APPAHGV sequence (**TAPPAHGVT**) are usually glycosylated while the Thr included in the PDTR is not. The accessibility to this latter sequence is, therefore, modulated by the length of the sugar chains branching from the other two Thr.

The finding that human antibodies, generated in response to a tumour-associated modified self-antigen, recognise one of the regions that appear to be covered by sugars, may be explained by several hypotheses. While it is possible that the antibodies can approach the peptide core from the opposite side to the attached carbohydrate, it is also likely that aberrant glycosylation involving the PPAH region can be only a rare and immunogenic event that occurs during tumour progression and that may be related to tissue histotype. It is interesting to note that MAbs against this sequence have only been generated using synthetic peptide-based immunogens [8].

Our results suggest that two distinct regions in the tandem repeat of PEM (PDTR and PPAH sequences) seem to be targets for a humoral immune response. These findings can be considered indicative of the type of humoral response elicited by PEM, since the antibodies were obtained from different patients and generated using B-cells from tumour-draining lymph nodes, an optimal source of lymphocytes primed against the tumour.

All the isolated human antibodies were of the IgM isotype. This is not surprising since IgM is the most frequently obtained isotype using EBV immortalisation. This can also explain the weak reactivity observed since IgM isotype is known to be a low affinity antibody. However, the presence of circulating IgM antibodies specific for tumour-associated antigens has also been described by other authors [30–35]; in particular, for PEM, this isotype has been associated with the tandemly repeated structure [11] on the molecule. The presence of PEM-containing immune complexes of the IgG class have also been described [12]. It would be quite important to determine which epitopes these IgG antibodies recognise.

The anti-PEM immune response described may not be completely representative of the effective immune response mounted *in vivo* against PEM. It is, in fact, possible that we selected human antibodies generated to cross-reactive epitopes. Nevertheless, the isolated human antibodies are all capable of successfully binding to tumour cells as well as circulating antigen, since PEM immunocomplexes have been described in breast cancer patients sera [13]. This can ultimately affect the degree of the immune response mounted by the host against the tumour.

The immunological significance of a humoral response against antigens differentially expressed by tumour cells is

quite controversial. In experimental models, antibody-dependent epitope masking has been suggested to explain the progressive growth of immunogenic tumours [36]. Alternatively, the presence of PEM immune complexes has been recently suggested to protect against disease progression [13]. Bound antibody has also been shown to modulate antigen processing [37, 38] and the T-cell response towards the antigen [39]. Therefore, the presence of human antibodies in cancer patients that bind distinct regions within the tandem repeat of PEM may route the antigen processing and ultimately influence the T-cell response versus this antigen. It is interesting to point out that very recently, the nine-mer peptide STAPPAHGV of the TR of PEM, which includes the epitope described in this paper, has been shown to bind strongly to the HLA-A11, and to a lesser extent also to A3, A2.1 and A1 molecules and to induce a primary and secondary T-cell response [40].

The results presented in this paper confirm that the TR of PEM is target for the immune response and suggest the possible role of different peptide squences for inducing the humoral response to this antigen in ovarian cancer patients. The definition of PEM sequences able to induce a specific immune response is necessary to formulate effective PEM-based immunogens.

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