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ORIGINAL ARTICLE

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Cellular and humoral immune responses to MUC1 mucin and tandem-repeat peptides in ovarian cancer patients and controls

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Abstract The objective of this study was to demonstrate the presence of proliferative T cell responses to human polymorphic epithelial mucin (MUC1) and its tandem-repeat peptides in peripheral blood mononuclear cells (PBMC) from ovarian cancer patients and from controls and to correlate these cellular responses to a humoral response to MUC1. PBMC were obtained from 6 healthy women, from 13 women in the third trimester of pregnancy and from 21 ovarian cancer patients. Only 1 of the 6 healthy women showed a weak primary proliferative response (stimulation index, SI < 2) to a 20-mer MUC1 tandem-repeat peptide in the presence of interleukin-2 (IL-2). In PBMC from 5/13 pregnant women (38%) a weak response could be induced by the 20-mer and/or 60-mer tandem-repeat peptides (SI ≤ 3.0) and in PBMC from 8/15 ovarian cancer patients (53%) 20-mer and/or 60-mer MUC1 tandem-repeat peptides induced primary responses (SI ≤ 5.4). MUC1 mucin purified from a breast tumor cell line and/or from urine of a healthy donor had a relatively strong stimulating effect (SI ≤ 19) on PBMC from 4 of 16 ovarian cancer patients (25%). In contrast, in PBMC of 9 ovarian cancer patients stimulated by the addition of a *Candida albicans* extract, MUC1 mucin strongly inhibited proliferation. This inhibition could partially be abrogated by the addition of IL-2. MUC1 (CA 15.3 assay) and free circulating MUC1 IgG and IgM antibodies (PEM.CIg assay) were determined in the plasma of all subjects. The MUC1 and the free circulating MUC1 IgG antibody plasma levels were significantly higher in the ovarian cancer patients than in the healthy women. Although no significant correlations were found between MUC1 mucin, MUC1 Ab plasma levels and the individual proliferative responses to the MUC1 antigens, an association may exist between them, since all three are significantly higher in the ovarian cancer patients than in the healthy women.

Key words Cellular response · Humoral response · Ovarian cancer · MUC1 · Immunotherapy

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Introduction

Existing therapeutic modalities have had a limited impact on survival of ovarian cancer patients, indicating a need for new approaches to the treatment of the disease. In the long run, immunotherapy of ovarian cancer may be more successful. One possible approach is the use of monoclonal antibodies against tumour-associated antigens [26, 35], another the use of tumour-associated antigens such as polymorphic epithelial mucin (PEM, MUC1) as a vaccine and target for cellular and humoral immune responses.

MUC1 is a transmembrane glycoprotein expressed on the apical surface of normal glandular epithelial cells [42]. The extracellular domain consists mainly of a variable number of tandem repeats [13] and has a cytoplasmic tail of 69 amino acids [32]. In the vast majority of human adenocarcinomas this protein is over-expressed and poorly glycosylated [9], exposing an immunodominant repetitive amino acid sequence. The overproduction and secretion of MUC1 are correlated to the progression of ovarian [6, 40], breast [38] and colon [33] carcinoma. In breast cancer patients, MUC1 serum levels are used to monitor therapy and for early detection of recurrences [8, 17].

MUC1-specific cytotoxic T lymphocytes have been demonstrated in tumour-draining lymph nodes of ovarian [22] and breast [23] cancer patients but few in vitro studies have been performed to demonstrate proliferative responses to MUC1 in peripheral blood mononuclear cells (PBMC) of patients with adenocar-

cinoma. Agrawal et al. [1] found proliferative responses to MUC1 tandem-repeat fragments in PBMC of multiparous and pregnant women. This finding indicates that autoimmune reactivities to MUC1 may be evoked during pregnancy, which even may protect against tumour recurrence, as described for a breast cancer patient [25]. This substantiates the epidemiological evidence that multiparity can protect against breast cancer [27]. Patients with ovarian, breast and pancreatic adenocarcinomas can develop cytotoxic T cells that are specific for the MUC1 tandem repeat [20, 21, 23, 24]. Recognition of MUC1 by T lymphocytes may be due to exposure of the immunogenic PDTRP region of the tandem repeat, which normally is not exposed because of extensive *O*-glycosylation of the peptide core. The cytotoxic action of these T cells was described to be MHC-nonrestricted and due to crosslinkage to underglycosylated MUC1 tandem repeats on tumor cells [5]. More recently, an MHC-I-restricted immune response was described in mice [4, 5] and humans [6, 11, 21]. Not only cellular but also humoral responses to MUC1 are found in ovarian [15, 37], breast, colon and pancreatic carcinomas [15, 29, 31]. Moreover, the presence of immune complexed MUC1 in breast cancer patients is related to a favourable disease outcome [31]. These findings point to MUC1 and its repeat peptide as a target for immunotherapy of carcinomas.

MUC1 may have different immunomodulating effects since, in two recent reports, immunosuppressive actions of MUC1 on activated human T cells have been described [2, 14]. It has also been reported that MUC1 is a ligand for intercellular adhesion molecule ICAM-1 on T cells and, by binding, can induce immunosuppression or anergy [34].

A bottleneck for in vitro studies is the limited availability of sufficient material from cancer patients and so far no proliferative studies in PBMC from ovarian cancer patients have been described. This led us to investigate primary cellular immune responses to MUC1 and MUC1 tandem repeat fragments in PBMC of ovarian cancer patients, pregnant women and healthy women and to correlate these cellular responses with circulating levels of MUC1 (CA 15.3 Enzymun-Test) and with the presence of natural antibodies against MUC1, using the recently described PEM.CIg assay [32].

Materials and methods

Patients and controls

Peripheral blood was obtained from 6 healthy women (3 nulligravida, H1–H3, and 3 multipara, H4–H6), from 13 women in the third trimester of pregnancy (P1–P13) and from 21 ovarian carcinoma patients [OvCa1–OvCa21, stage I ($n = 4$), stage II ($n = 3$), stage III ($n = 13$) and stage IV ($n = 1$)]. The tumour stage of the ovarian cancer patients was established at the time of diagnosis according to FIGO criteria. Blood sampling was performed after informed consent had been given by each woman. The clinical status of the ovarian patients at the time of blood sampling is described in Table 2.

Peptides, antibodies and agents

A MUC1 20-mer tandem-repeat peptide (NH_2-HGVTSAPDTRPAPGSTAPPA-COOH), a MUC1 60-mer tandem repeat (3×20-mer) and a control 60-mer peptide corresponding to three reverse sequences of the 20-mer tandem-repeat peptide ($3 \times NH_2$-GHAPPATSGPAPRTDPASTV-COOH) were synthesized and purified by Dr. L. Vernie (The Netherlands Cancer Institute, Amsterdam, The Netherlands) using a solid-phase procedure on a MilleGen 9050 synthesizer (B&L Systems, Maarssen, The Netherlands). All peptides were dissolved in phosphate-buffered saline (PBS) at a stock concentration of 10 mg/ml and stored at −20 °C. Bovine serum albumin conjugation of the 60-mer tandem repeat to bovine serum albumin (BSA) was performed with an Imject Immunogen EDC conjugation kit (Pierce, Rockford, Ill.). Three different MUC1 mucin preparations were used. The first, a kind gift from Dr. M. Price (Cancer Research Laboratory, University of Nottingham, UK), was isolated from the urine of a healthy donor. The second preparation, isolated from the breast cancer cell line ZR-75-1 [12], was donated by Dr. P. Maimonis (Chiron Diagnostics, Walpole, Mass). The third preparation was isolated in our laboratory from supernatant of the same breast cancer cell line, cultured for 5 days in serum-free medium. Isolation and purification were done on the basis of molecular size (Ultrafree 15 centrifugal filter device, Biomax-100k NMWL Membrane, Millipore) and affinity binding (CNBr-activated Sepharose 4 fast flow, Pharmacia). The purity of the large glycoprotein was tested by sodium dodecyl sulfate gel electrophoresis and Western blotting. Affinity binding and Western blotting was performed with MUC1 mAb 139H2 [41] kindly provided by Centocor B.V, Leiden, The Netherlands. The concentrations of the MUC1 mucin preparations and the MUC1 plasma levels in the patients and controls were measured with the Enzymun-Test CA 15-3 (Boehringer Mannheim, Tutzing, Germany) on the fully automated Enzymun-Test system ES 300 [22, 26]. Phytohaemagglutinin (Murex Diagnostic Ltd., Dartford, England), tetanus toxoid (RIVM, Bilthoven, The Netherlands) and *Candida albicans* (HAL Allergen Laboratory, Haarlem, The Netherlands) were used as control stimulating agents. Human recombinant interleukin-2 (rIL-2) was purchased from Cetus Corp., Emeryville, Calif.

Plasma collection and PBMC isolation

A 30-ml sample of heparinized blood was collected from each patient and control. After centrifugation, plasma was collected, divided into aliquots and stored at −80 °C. PBMC were isolated by density centrifugation with Ficoll-Hypaque (1.077 g/ml, Pharmacia). Cells were washed three times with Hanks balanced salt solution (Gibco, Paisley, Scotland) supplemented with 2% fetal calf serum (HyClone Laboratories Inc., Logan, Utah) and diluted to a final concentration of 10^6 cells/ml in Iscova's modified Dulbecco medium (Gibco) supplemented with 10% autologous plasma and gentamicin (80 μg/ml).

PBMC stimulation and proliferation measurement

PBMC were seeded in flat-bottom 96-well culture plates (Costar, Cambridge, Mass., USA) at a concentration of 2×10^5 cells/well and stimulated with 10 μg/ml or 100 μg/ml MUC1 tandem-repeat peptides or with 10 U/ml or 100 U/ml MUC1 protein in the presence or absence of 1–10 U/ml rIL-2. Each experiment was carried out in triplicate. Phytohaemagglutinin (10 μg/ml), tetanus toxoid (10 μg/ml) and *C. albicans* (1 μg/ml) were used as positive controls, and as negative control we used the reverse 60-mer peptide. In additional experiments, PBMC were simultaneously incubated with *C. albicans* (1 μg/ml) and different concentrations of MUC1 (0.5–100 U), again with or without IL-2 (up to 2 U/ml). Proliferation was determined 7 days after stimulation by measurement of the incorporation of [^{3}H]thymidine (13 kBq/well; Radiochemical Centre, Amersham, UK) during the last 16 h of incubation.

MUC1 Ab detection in plasma of donors

MUC1 Ab in the plasma were measured by the PEM.CIg assay [32]. In short: a BSA-conjugated MUC1 60-mer tandem-repeat peptide (250 ng/well in PBS) was used to coat 96-well ELISA plates (Costar) overnight at room temperature. After washing with PBS/0.1% Tween-20, the plates were incubated with 1% BSA (grade V; Sigma) for 3 h at 37 °C, washed and incubated overnight with the plasma samples in PBS + 1% BSA + 0.02% azide. Determinations were performed in duplicate with plasma dilutions of 1:100 for IgG and 1:500 for IgM. After six washes the plates were incubated with anti-(human IgG) or anti-(human IgM) (Dako; dilution 1:10 000) coupled to horseradish peroxidase for 1 h at room temperature. After six washes, tetramethylbenzidine (0.06 mg/ml) plus H_2O_2 (0.1 µl/ml) in 0.1 M citrate/acetate buffer (pH = 4.0) was added as substrate. After 1 h the reaction was stopped with 1.6 M H_2SO_4. The absorbance was measured in an enzyme-linked immunosorbent assay reader at 450 nm. A MUC1- IgG- or IgM-positive serum sample was used to construct a four-point reference curve in each plate and an arbitrary value of 1 was ascribed to the 1:2000 dilution of this positive serum sample.

Statistical analysis

Data are expressed as means ± SEM. Statistical analysis was performed with SPSS-PC software (SPSS, Chicago, Ill.) and Graphpad InStat V2.02. Significance was determined with the unpaired Student *t*-test (taking two-tail probability below 0.05 as significant) or, in the case of a non-Gaussian distribution, with the Mann-Whitney *U*-test. The normality of distribution of the data was evaluated with the Kolmogorov-Smirnov test. Linear regression analyses were used to correlate cellular and humoral responses.

Results

Effect of the 20- and 60-mer MUC1 tandem-repeat peptides on PBMC proliferation

The effect of the addition of 10 µg/ml and 100 µg/ml 20-mer and 60-mer tandem-repeat peptides on the proliferation of PBMC of all women (except for OvCa16–OvCa21) was determined. In all experiments the addition of 10 µg/ml peptides had a much less stimulating effect than the addition of 100 µg/ml. Only in 2 ovarian cancer patients could 10 µg/ml peptide induce a significant stimulating effect (data not shown). Addition of 1–10 U/ml IL-2 during incubation strongly enhanced the initial proliferation in all subjects and improved the stimulation index (SI) in part of the population.

Only in one multiparous woman of the 6 healthy women could a very weak stimulating effect of 100 µg/ml 20-mer peptide be detected in the presence of IL-2 (SI = 1.434). In 5 out of the 13 pregnant women (38%) weak but significant stimulations were induced by 100 µg/ml 20-mer and/or 60-mer peptides (SI ≤ 3; Figs. 1, 2). In 8 out of 15 (OvCa1–OvCa15) ovarian cancer patients (53%) a significant primary stimulation could be induced in freshly isolated PBMC by 100 µg/ml 20-mer and/or 60-mer peptides (SI ≤ 5.0; Table 1; Figs. 1, 2). Statistical analysis of the data obtained in the different experimental groups demonstrates an increase of proliferation in the ovarian cancer patients by the 20-mer MUC1 peptide in the absence and presence of IL-2 (SI = 1.79 ± 0.37, $P = 0.0542$, and SI = 1.62 ± 0.25, $P = 0.0287$) and by the 60-mer MUC1 peptide in the absence and presence of IL-2 (SI = 1.49 ± 0.18, $P = 0.0173$, and 1.21 ± 0.088, $P = 0.0335$).

Effect of MUC1 on PBMC proliferation

The effect of MUC1 from different sources on the proliferation of PBMC was determined in a total of 3 healthy women (H4–H6) and 16 ovarian cancer patients (OvCa8–OvCa21). Increasing doses of MUC1 mucin isolated from urine of a healthy donor were tested on PBMC of 2 healthy women and 4 ovarian cancer patients. In the 2 healthy women MUC1 had no stimulating effect and in 1 of them MUC1 had an inhibiting effect on PBMC proliferation ($P = 0.0298$). In one of the 4 ovarian cancer patients tested with this MUC1 mucin preparation a significant stimulating effect was found (SI = 4.47; $P < 0.0001$). The two preparations of MUC1 mucin isolated from the supernatant of the breast cancer cell line ZR-75-1 were tested on PBMC of 3 healthy women and 12 ovarian cancer patients. No significant effects were seen in the 3 healthy women. In 3 of the ovarian cancer patients a significant stimulating effect was seen (SI ≤ 20; $P = 0.0243$, $P = 0.0018$ and $P < 0.0001$; Fig. 3) whereas, in 1 patient, an inhibiting effect with high doses of MUC1 was found ($P = 0.0191$).

Effect of MUC1 on proliferation of *C. Albicans*-stimulated PBMC

We incubated PBMC of 9 ovarian cancer patients (OvCa13–OvCa21) with MUC1 together with *C. albicans* extract to investigate whether MUC1 not only has stimulating effects on PBMC proliferation but also may exert immunosupressive actions in activated PBMC of these patients. *C. albicans* extract alone gave, in almost all donors, a strong induction of PBMC proliferation. Addition of each of the MUC1 mucin preparations, in the range 0–100 U/ml, to *C. albicans*-stimulated PBMC gave a dose-dependent and significant inhibition (up to $P < 0.0001$; Fig. 4A–C). In the presence of 2 U/ml IL-2, the inhibition induced by MUC1 was partially abrogated and only significant at higher concentrations (Fig. 4B, C).

MUC1 antigen (CA 15.3) and free circulating MUC1 IgG and IgM (PEM.CIg) measurements

In all subjects the plasma concentrations of MUC1 antigen (CA 15.3) and the free circulating MUC1 IgG and IgM Ab (PEM.CIg) were determined. The individual data of the 21 ovarian patients are displayed in Table 2. At the moment that the blood was sampled for the experiments, 10 of the 21 ovarian cancer patients had

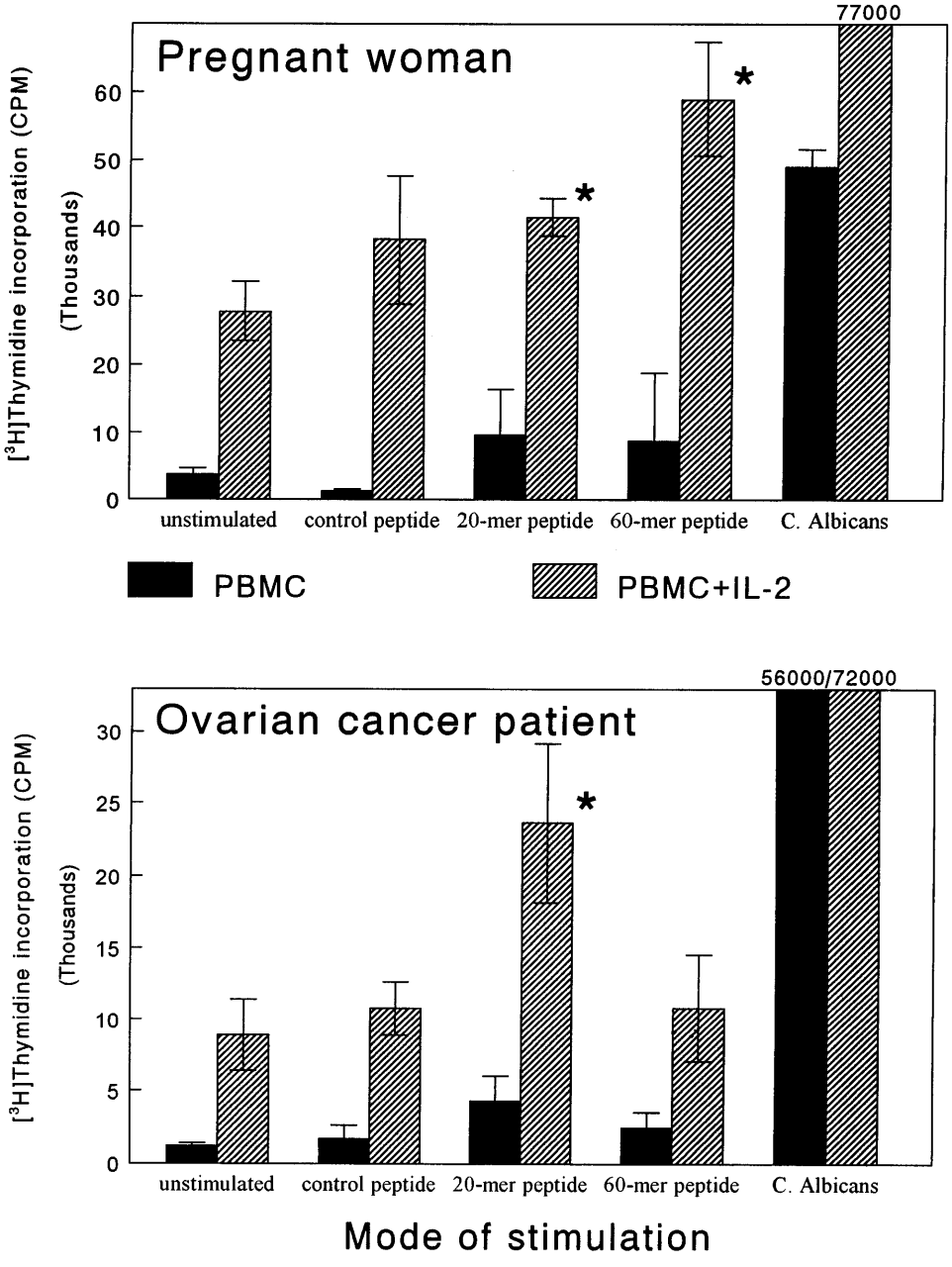

Fig. 1 Proliferative effect of 100 μg/ml 20-mer or 60-mer MUC1 tandem-repeat peptide on peripheral blood mononuclear cells (PBMC) from a pregnant woman (P13) and an ovarian carcinoma patient (OvCa4) as determined by [^{3}H]thymidine incorporation. The 60-mer peptide, corresponding to three reverse sequences of the 20-mer tandem repeat, was used as a control peptide and an extract of *Candida albicans* was used as a control antigen-specific stimulating agent. * $P < 0.05$: statistically significantly different from results with unstimulated PBMC + interleukin-2

progression of disease and/or were under treatment while 10 patients showed no evidence of disease. The MUC1 antigen plasma concentrations in the 10 patients with no evidence of disease (13.6 ± 2.5 U/ml) were significantly lower than in the other 10 ovarian cancer patients (25.1 ± 2.8 U/ml, $P = 0.0007$). There was no correlation between free circulating MUC1 antibody plasma levels and the disease status.

The MUC1 antigen plasma concentrations were significantly higher in the pregnant women (22.5 ± 3.3 U/ml, $P = 0.0221$) and in the ovarian cancer patients (19.4 ± 1.8 U/ml, $P = 0.0134$) than in the healthy women (9.8 ± 1.5 U/ml). None of the 6 healthy women had elevated MUC1 levels. MUC1 values were above the accepted cut-off of 30 U/ml [40] in 3 of the 13 pregnant women and in 4 of the 21 ovarian cancer patients.

MUC1 IgG arbitrary values were significantly higher in the plasma samples obtained from the ovarian cancer patients (1.94 ± 0.18, $P = 0.0488$) compared to those of the healthy women (1.26 ± 0.10), while MUC1 IgG arbitrary values in the pregnant women (1.63 ± 0.55) were not significantly different from those of the healthy women. MUC1 IgM arbitrary values in the plasma of the pregnant women and of the ovarian cancer patients, respectively 1.54 ± 0.57 and 0.64 ± 0.25, were not significantly different from each other or from the values of the healthy women (1.56 ± 0.75).

Correlations between cellular and humoral responses

In 2 pregnant women and 2 ovarian cancer patients cellular immune responses and MUC1 or MUC1 Ab levels were concordantly elevated. In the ovarian cancer patients as a group, the MUC1 plasma levels, the MUC1 IgG Ab plasma levels and the cellular immune responses were significantly higher than in the healthy women. No significant correlations were found between the MUC1 antigen, MUC1 IgG or IgM levels (OvCa1–OvCa15 in Table 2) and the cellular response to the 20-mer or 60-mer tandem repeats (Table 1).

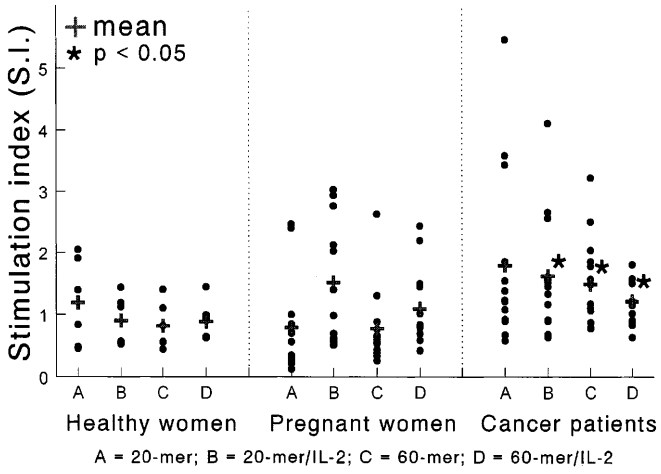

Fig. 2 Stimulation indices (*SI*) of 6 healthy women, 13 pregnant women and 15 (OvCa1–15) ovarian cancer patients. PBMC were stimulated with 100 μg/ml 20-mer and 60-mer MUC1 tandem-repeat peptides in the absence or presence of 1–10 U/ml interleukin-2 (IL–2). *A* 20-mer, *B* 20-mer + IL-2, *C* 60-mer, *D* 60-mer + IL-2. The stimulation index is the ratio of stimulated to unstimulated PBMC. * $P < 0.05$: the incubation of PBMC from ovarian cancer patients with a MUC1 20-mer peptide + IL-2 or MUC1 60-mer peptide with or without IL-2 induced a significantly different stimulation index from SI = 1

Discussion

In this study we found that significant PBMC proliferation can be induced in more than 50% of the ovarian cancer patients by the 20-mer and/or 60-mer MUC1 tandem-repeat peptide. This confirms the presence of MUC1-antigen-specific T cells in the blood of ovarian cancer patients, and it demonstrates that the measurement of an in vitro cellular response is an immunological tool applicable to monitoring ovarian carcinoma patients during a MUC1-based immunotherapy trial. In most cases the SI values were very low, leading one to question the possible efficacy of these cellular responses in the attack of an ovarian tumour, although it does not rule out the posibility that the presence of antigen-specific T cells can be much higher at the tumour site. In a limited number of breast, colon, stomach and rectum cancer patients Karanikas et al. [28] found even fewer proliferative responses. After repeated injection of a mannan-MUC1 fusion protein they could induce T cell proliferation in 4 of the 15 tumour patients.

To circumvent the possibility that part of the MUC1-antigen-specific T cells in the PBMC of our subjects has become anergic [10] we also performed our proliferation assays in the presence of different concentrations of IL-2 (1–10 U/ml). In general, IL-2 induced higher proliferation and reduced the standard deviation of the experiments, but the SI were not higher, indicating that no putative anergic antigen-specific T cells were induced to proliferate.

Table 1 Stimulation indices (SI) determined after antigen-specific peripheral blood mononuclear cell stimulation with MUC1 20-mer and 60-mer tandem-repeat peptides in ovarian cancer patients. Bold figures indicate significantly different stimulation indices ($P < 0.05$). In 8 out of 15 ovarian cancer patients (53%) a significant proliferation could be induced by the MUC1 20-mer and/or 60-mer tandem-repeat peptides. The tumour stage shown is that at the time of diagnosis. *IL-2* interleukin-2, *ND* not determined

Subject	Tumour stage	Stimulation index					
		60-mer (reverse)	60-mer/IL-2 (reverse)	60-mer	60-mer + IL-2	20-mer	20-mer + IL-2
OvCa1	IIIC	ND	ND	1.88	1.50	**5.46**	**2.56**
OvCa2	IIIC	1.43	1.25	0.79	1.21	1.85	4.11
OvCa3	IIIC	1.04	1.55	1.06	1.02	0.58	1.51
OvCa4	IIIB	1.41	1.21	2.04	1.22	3.58	**2.66**
OvCa5	IC	1.30	0.63	1.78	0.63	**3.43**	0.91
OvCa6	IIIC	1.46	1.21	1.20	**1.62**	ND	ND
OvCa7	IIIB	1.06	0.81	0.87	1.58	1.21	1.16
OvCa8	IIIC	1.74	1.45	**3.22**	1.17	1.23	0.89
OvCa9	IIIC	0.75	0.87	0.91	1.10	1.23	0.63
OvCa10	IIIC	1.18	1.34	1.49	1.15	0.92	**1.56**
OvCa11	IV	3.34	1.65	1.07	0.83	1.38	1.45
OvCa12	IIA	1.01	0.87	0.77	0.89	0.89	0.67
OvCa13	IIIC	0.93	1.77	1.17	**1.81**	0.67	1.61
OvCa14	IA	0.71	1.23	**2.50**	**1.57**	1.08	**1.56**
OvCa15	IIB	2.01	0.92	1.57	0.84	1.54	1.33

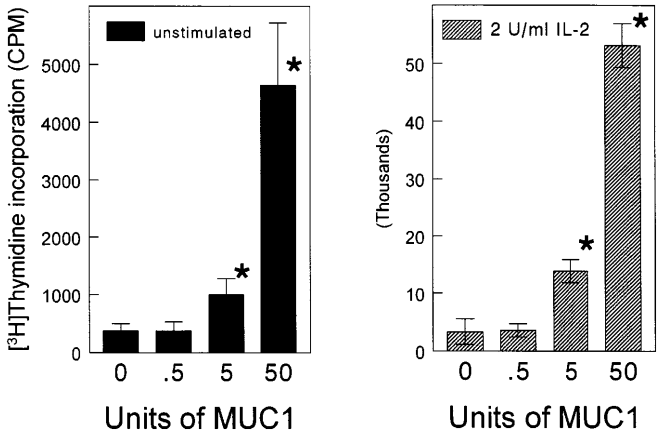

Fig. 3 Stimulating effect of MUC1(U/ml) on proliferation of PBMC (10^6 cells/ml) from an ovarian cancer patient. * $P < 0.05$: significantly different from PBMC in the absence of MUC1. MUC1 was isolated in our laboratory from breast tumour cell line ZR-75-1

Flow cytometry (data not shown) for monitoring T cell expression and activation (CD3, CD4, CD8 and CD25) before and after 1 week of stimulation with the MUC1 peptides was performed in PBMC from a number of ovarian cancer patients. Large variations in the CD4/CD8 ratios were found and no shift was seen after 1 week of stimulation towards either CD4 or CD8. Even in those cases with significant stimulations, no steering towards cytotoxic ($CD8^+$) T cells could be demonstrated.

When the MUC1 molecule was used as a stimulating agent, a stimulation could be induced in freshly isolated PBMC from 4 of 16 ovarian cancer patients. This proliferative response was stronger with the whole mucin molecule than with the 20-mer and 60-mer peptides. Essentially the processing of the whole molecule by antigen-presenting cells (monocytes and B cells present in the PBMC) is not different from that of the smaller tandem-repeat peptides: in both cases the molecules are broken down to small peptides and the dominant epitopes presented on the cell surface. One difference may be the presence of antigenic carbohydrate groups on the glycosylated MUC1 molecule. Possibly in the PBMC of the ovarian cancer patients there are not only antigen-specific T cells against naked (non-glycosylated) MUC1 antigens present, but also T cells with specificity for cryptic carbohydrate epitopes expressed on the MUC1 molecule (e.g. STn, Tn, TF, Lewis, etc). In fact, T cells specific to the TF carbohydrate epitope (Galβ1-3GalNAcα 1-3-bound to Ser/Thr) have recently been described in colorectal cancer patients [6].

MUC1 forms very large extended rods, which protrude from the cell surface [18] and may in this way shield other cell-surface molecules from their ligands and protect the cell from attack by other cells [39]. Of course, this phenomenon is also valid for adenocarcinomas and can partly explain why our immune system fails to destroy these tumours. On the other hand, MUC1 may play an adhesive role by presenting carbohydrates as

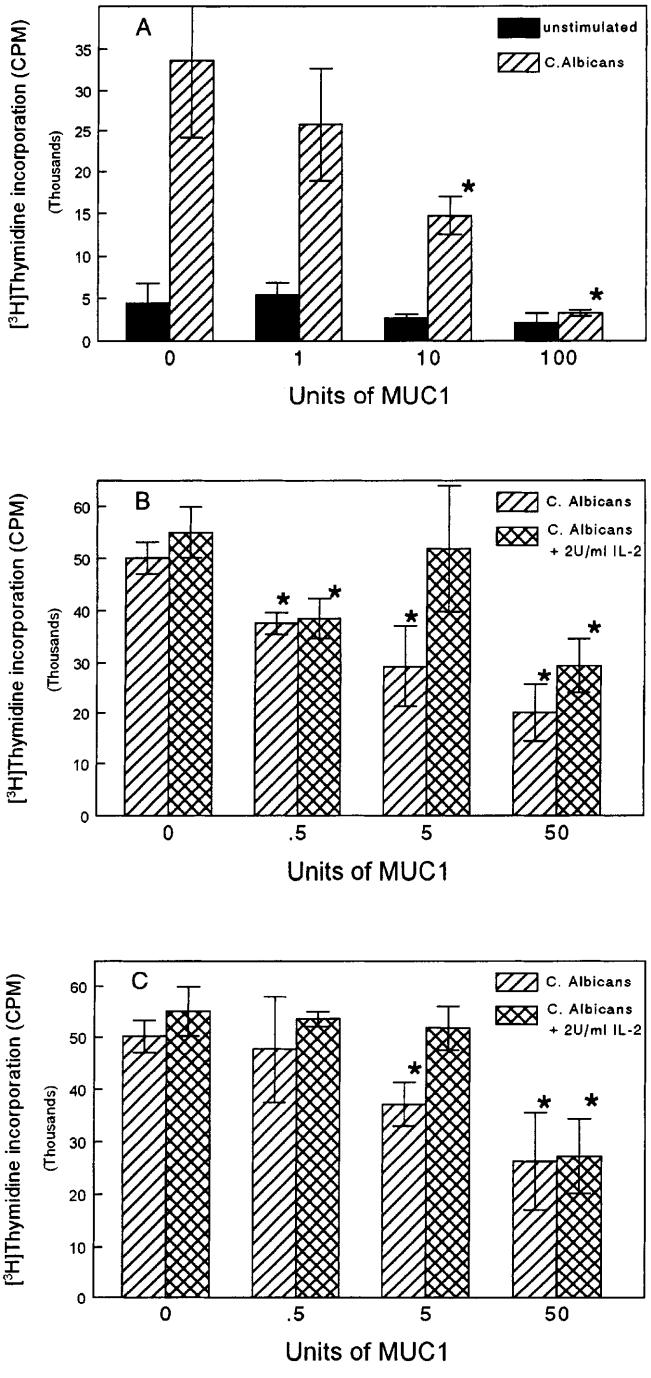

Fig. 4A–C Strong inhibiting effects of three different batches of MUC1 mucin on *C. albicans*-stimulated PBMC from three ovarian cancer patients (**A** OvCa13; **B**, **C** OvCa16). * $P < 0.05$: significantly different from *C. albicans*-stimulated PBMC in the absence of MUC1. Addition of IL-2 resulted in no or less pronounced inhibition. MUC1 was isolated from urine of a healthy donor (**A**) or from the breast tumour cell line ZR-75-1, (from own laboratory (**B**) and from Chiron Diagnostics (**C**)

ligands for selectin-like molecules [16]. Compared to normal glandular epithelial cells, MUC1 on adenocarcinoma cells is over-expressed, aberrantly glycosylated and relocated over the entire cell surface [19]. These differences can explain why we were able to find antigen-

Table 2 MUC1 (CA 15.3) and free circulating MUC1 antibody (PEM.CIg) plasma values in ovarian cancer patients. The tumour stage shown is that at the time of diagnosis, and the status is that of the patient at the time of blood sampling. *NED* no evidence of disease

Subject	Tumour stage	Status	MUC1 (U/ml)	MUC1 IgG (arbitrary U)	MUC1 IgM (arbitrary U)
OvCa1	IIIC	Pretreatment	20	0.76	0.19
OvCa2	IIIC	10 days after debulking	12	2.11	0.26
OvCa3	IIIC	Pretreatment	34	1.34	0.25
OvCa4	IIIB	NED	20	1.13	0.17
OvCa5	IC	NED	9	2.88	0.33
OvCa6	IIIC	Pretreatment	19	3.24	0.79
OvCa7	IIIB	NED	19	1.18	1.50
OvCa8	IIIC	NED	17	3.10	0.16
OvCa9	IIIC	During chemotherapy	20	1.61	0.65
OvCa10	IIIC	NED	8	1.99	0.24
OvCa11	IV	5 days after chemotherapy	31	0.71	0.35
OvCa12	IIA	Progression	21	3.38	0.30
OvCa13	IIIC	NED	16	1.78	0.28
OvCa14	IA	NED	7	1.95	0.33
OvCa15	IIB	NED	11	2.93	0.49
OvCa16	IIIC	NED	19	1.18	5.41
OvCa17	IIC	NED	10	2.18	0.24
OvCa18	IB/IC	Progression	36	1.47	0.24
OvCa19	IIIC	Progression	31	1.75	0.73
OvCa20	IIIA	Unknown	21	1.44	0.21
OvCa21	IC	During chemotherapy	26	2.53	0.49

specific T cells to the MUC1 20-mer and/or 60-mer tandem repeat peptide in more than 50% of the ovarian cancer patients as compared to 17% (1 subject) in the healthy women. A very weak but significant PBMC proliferation against the peptides was also found in 38% of the third-trimester pregnant women. Taking into account that the MUC1 levels in the plasma from the pregnant women were elevated as well, it may be possible that, in these women, MUC1 is over-expressed and can already evoke a cellular response.

Although an apoptotic effect of MUC1 on activated T cells [14] could not be confirmed [1], there are strong indications that MUC1 has inhibiting effects on allogenic, phytohaemagglutinin- or CD3-mAb-stimulated T cells of healthy donors. In a pilot study we could confirm this inhibiting action of MUC1 (isolated from the urine of a healthy donor) on phytohaemagglutinin-stimulated PBMC of ovarian cancer patients. In the present study we clearly demonstrate the immunosuppressive effect of different sources of MUC1 on *C. albicans*-stimulated PBMC. Agrawal et al. [1] could abrogate inhibition by addition of CD28 mAb or high concentrations of IL-2 (50 U/ml). In the presence of 2 U/ml IL-2 we found that MUC1 at high concentrations induced inhibition. At lower MUC1 concentrations even this relatively low amount of IL-2 could partially abrogate the inhibiting effect of MUC1.

A major object of this study was to correlate the humoral responses to MUC1 with the cellular responses to MUC1 and its tandem repeats. The weak proliferative responses we found made it impossible to find positive or negative correlations. Even in the ovarian cancer patients in which MUC1 levels are elevated (above 30 U/ml) or in which MUC1 IgG or MUC1 IgM values were relatively high (above 3 arbitrary U/ml), the SI induced by MUC1 tandem repeats or MUC1 were not significantly higher. A correlation was present between the clinical status and the MUC1 antigen plasma levels of the patients. This is in agreement with the previously described positive correlation between disease status and serum MUC1 antigen levels in ovarian cancer patients [6].

Tolerance is a major hindrance in the development of effective immune responses to tumours. It protects against autoimmune reactions in healthy subjects but may prevent effective immunotherapy for tumours. Although MUC1 is a genuine self-protein, the fact that it is over-expressed and aberrantly glycosylated on tumour cells, and can induce cellular responses [6, 11, 21, 24], leads us to think that modified MUC1 derivatives can be putative candidates for a cancer vaccine. In this respect, treatments of MUC1 transgenic mice with tumour load with different glycosylated MUC1 derivatives may help us to find the best vaccine [36]. In conclusion, the possibility of demonstrating not only MUC1 and MUC1 mAb but MUC1-antigen-specific T cells in the blood of ovarian cancer patients will provide an additional tool to monitor patients in MUC1-based immunotherapy studies.

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