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Lack of evidence for an immunosuppressive role for MUC1

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Abstract The in vitro anti-proliferative properties of various supernatants from MUC1-expressing cell lines and of purified preparations of MUC1 were evaluated. We have observed that supernatants from the MUC1- and MUC3-positive cell line T47D, but not from the MUC1- and MUC4-positive cell line MCF7, were able to inhibit proliferation of cells from various haematopoietic cell lines. Although the activity of T47D supernatants could be abrogated by immunodepletion of MUC1, immunopurified MUC1 from T47D was unable to inhibit cell proliferation. Significantly, supernatants from mouse 3T3 cells transfected with a secreted form of MUC1 or from BHK-21 cells infected with a recombinant vaccinia virus coding for the secreted form of MUC1, as well as preparations of purified MUC1 from bile or urine, were likewise unable to inhibit T cell proliferation. Surprisingly, a crude mixture of bile mucins had a suppressive effect on T cell growth. Our results suggest that other molecules, such as amino sugars or other mucins, which can associate with MUC1, are likely to be responsible for the observed anti-proliferative effects of T47D cells.

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Introduction

Immunological surveillance escape is a key element in the progression of solid tumours in vivo, this phenomenon occurring despite the presence of tumour-infiltrating lymphocytes and apparent immune reactivity to the tumour. Several mechanisms by which a tumour may confound the immune response have been described and include the production of various immunosuppressive factors and cytokines [39, 40]. In spite of the large number of reports on tumour-derived products capable of inhibiting lymphocyte proliferation in vitro [25, 33, 34], the exact mechanisms of lymphocyte suppression by tumours and the fate of such anergic cells remain to be elucidated. It has notably been shown that an immunosuppressive factor derived from oesophageal squamous carcinoma cells (OC2 cell line) induces apoptosis in normal and transformed cells of lymphoid lineage [33]. This immunosuppression seems to be "regional" and may inhibit the generation of lymphokine-activated killer cells [34]. Recently, Kolenko et al. [25] have suggested that soluble products from human renal cell carcinomas (RCC-S cell line) can suppress T cell proliferation through a mechanism that involves down-regulation of Jak3 expression and inhibition of interleukin-2 (IL-2)-dependent signalling pathways.

The MUC1 mucin is normally presented on the apical surface of a number of secretory epithelial tissues but is over-expressed and underglycosylated in over 90% of breast, ovarian and pancreatic carcinomas and in 60% of lung cancers [11, 25]. It is well known that glycosylation pathways are altered and often abnormal in tumour cells [8]. Moreover, the inhibition of *N*-acetylgalactosaminyltransferase by antisense oligonucleotide can increase the sensitivity of tumour cells to immune effector cells such as natural killer or lymphokine-activated killer cells [2]. So, while the precise mechanism

underlying the ability of tumours to escape immunological surveillance has not yet been elucidated, these results, taken together, suggest that glycosylation events are important in this process. The alterations involve reappearance, unmasking or altered organization of carbohydrate epitopes as well as altered exposure of the protein core carrying the oligosaccharides. MUC1 is a long transmembrane glycoprotein with a large extracellular domain made up of 25–100 tandem repeats [13] of 20 amino acids (VNTR), each tandem repeat containing potential glycosylation sites. The number of tandem repeats is allelic and appears unrelated to cancer, although cell-surface mucins play a significant role in cell-cell interactions [23]. As MUC1 represents a potential target for antigen-directed immunotherapy of various forms of cancer, several groups have produced vaccine formulations for the MUC1-specific immunotherapy of cancer [1, 6, 11, 19]. These preparations are designed to induce or boost the natural anti-MUC1 immune response developed by some cancer patients, which is usually weak and apparently ineffective. In view of these objectives and since MUC1 has been implicated in the down-regulation of T cell activation [12, 43], it is of crucial importance to elucidate the potential immunoregulatory effects of cancer-associated mucins.

Fung and Longenecker [12] have reported a specific immunosuppressive activity of epiglycanin, a murine mucin-like glycoprotein secreted from breast cancer cells in a mouse model. In addition, it has been suggested that patients with high levels of circulating MUC1 are in a state of MUC1-induced T-cell anergy [29]. Moreover, it has recently been shown that over-expression of MUC1 on the plasma membrane of cultured mammalian cells inhibits their aggregation capacity and their interaction with cytotoxic lymphocytes, probably because of the large, extended and rigid structure of MUC1 [28, 30, 43]. Indeed MUC1 can be a ligand for intercellular adhesion molecule 1 (ICAM-1) and its binding to ICAM-1 on T cells could be involved in immunosuppression by preventing proper costimulation [37]. The inhibition of natural killer cell cytotoxicity has also been suggested as another immunomodulatory property of the MUC1 mucin [32, 46]. More recently it has been shown [14] that culture supernatants from breast cancer cell lines are able to inhibit the in vitro proliferation of human T cells. Further experiments suggest that this inhibition is due to the presence of the tumour-associated glycoprotein MUC1 in the supernatant, and that this cancer-cells-derived MUC1 is able to induce apoptosis in specific lymphocyte populations. Lastly, Agrawal et al. [3] have published data suggesting that purified human MUC1 and synthetic tandem repeats of MUC1 core peptides can cause inhibition of the polyclonal T cell response, which can be reactivated by exogenous IL-2. The potential immune-response-inhibiting, apoptotic activity of MUC1-containing supernatants has been reported to act at the level of immune activation and effector cell function [3, 14]. In contrast to these reports, and in agreement with those of other investigators [7], we have observed that the expression of MUC1 by target cells does not inhibit their interaction with cytotoxic T cells (CTL) or lymphokine-activated lymphocytes. It is therefore important to determine the mechanism of the reported T cell anti-proliferative effect of supernatants from MUC1-expressing cells. To this end, we have attempted to characterize the immunosuppressive activity contained in the supernatant from MUC1-positive cell lines and of purified MUC1 from tumour cells, urine and bile.

Materials and methods

Cells

Breast cancer cell lines MCF7, T47D (human MUC1-positive breast cancer cell lines), Jurkat (human transformed T cell line), HL60 (human promyelocytic leukaemia), K562 (human chronic myelogenous leukaemia), CCRF-CEM (human acute lymphoblastic leukaemia), HeLa (human cervix epitheloid carcinoma), U937 (human histiocytic lymphoma), Balb/C 3T3 (Mouse fibroblast), A549 (human lung carcinoma) and BHK-21 (baby hamster kidney) were obtained from the American Type Culture Collection (Rockville, M.). HF19 (human fetal lung fibroblast) was obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). Epstein-Barr-virus-immortalized B cell lines were derived from human peripheral blood lymphocytes. All cell lines were tested negative for *Mycoplasma* with Hoechst dye, cell culture and the polymerase chain reaction.

Transfectants

P815-MUC1 and 3T3-MUC1 have been described elsewhere [1]. Cells secreting a truncated form of MUC1 were obtained by transfecting 3T3 cells with a plasmid containing a truncated MUC1 cDNA from which the transmembrane and cytoplasmic regions of the MUC1 molecule had been deleted, leading to the production of a secreted form of MUC1 (3T3-MUC1sec). RMA and RMA-MUC1 were generously provided by Dr. J. Taylor-Papadimitriou [16].

Purification of MUC1

Supernatants from confluent breast cancer cell lines were either used unmodified or concentrated in a Centriplus 100 concentrator (Amicon Corp., Beverly, Mass.). The MUC1 antigen was purified from these concentrated fractions by affinity chromatography with anti-MUC1 mAb H23 [41] as described before [20]. Briefly, concentrated supernatants were mixed with CNBr-activated Sepharose 4B (Pharmacia) coupled covalently to anti-MUC1 monoclonal Ab H23 and incubated overnight at 4 °C, and then washed with 0.5 M NaCl/0.1 M TRIS at pH 8. The affinity-bound MUC1 mucin was eluted with 3 M guanidine/HCl at pH 4 and dialysed against complete RPMI culture medium for 2 days (ten changes of buffer) at 4 °C.

The purified biliary form of MUC1 was purified as described before [5].

Samples of Sialyl-Lewis^a or Sialyl-Le^a, carrying mucins from human gall-bladder-associated biles, were purified as detailed elsewhere [10]. Biochemical and immunochemical determinations gave a molar ratio, sialic acid/hexosamine, of 0.17, 0.25 and 0.71 for bile 1, bile 2 and bile 3 respectively, and a sialyl Le^a activity, expressed as a concentration of CA19.9, of 318 U/μg, 823 U/μg and 5434 U/μg protein for bile 1, bile 2 and bile 3 respectively (unpublished results).

The purified urinary form of MUC1, also called B55 Ag, was obtained as outlined before [36].

Supernatants from BHK-21 cells infected with a vaccinia virus encoding the secreted form of MUC1 (VV-MUC1sec [19]) or with a control vaccinia virus were prepared as follows: a culture of 1×10^6 BHK-21 cells was infected for 48 h with 1×10^6 pfu virus. The culture supernatants were then harvested, UV-inactivated and filtered.

Sera from breast cancer patients or from healthy donors were decomplemented for 30 min at 56 °C before use.

The synthetic peptide TAPPA 24 (TAPPAHGVTSAPDTRP-APGSTAPP), corresponding to one tandem repeat of MUC1, was a generous gift from Dr. J. Taylor-Papadimitriou (London, UK).

Detection and quantification of MUC1

A sandwich enzyme-linked immunosorbent assay (ELISA) was used to detect MUC1. ELISA plates were coated overnight at 4 °C with MUC1-specific mAb 1G5 at 4 μg/ml [6] in ELISA coating buffer. Plates were saturated for 1 h with phosphate-buffered saline/3% bovine serum albumin (PBS/BSA). The different sources of MUC1 were then serially diluted in the plates and incubated for 2 h at room temperature. After extensive washing, a polyclonal antiserum obtained from rabbits immunized with a vaccinia virus coding for MUC1 [19] was added. Finally, an enzyme-linked anti(rabbit Ig) Ab was used for the ELISA. The MUC1 content of some supernatants was also quantified by the CA15.3 test (EIA, Roche, Switzerland). The ELISA was standardized against a MUC1-containing solution of known CA15.3 reactivity. By this technique, the following MUC1 concentrations (U/ml) were determined: T47D supernatant (170), MUC1-depleted T47D supernatant (3.5), MCF7 supernatant (155), 3T3-MUC1sec supernatant (145), BHK-21-VV-MUC1sec (115), MUC1 purified from T47D supernatant (1200), MUC1 purified from urine (1500), MUC1 purified from bile (2000), bile mucins sample 1 (1000), bile mucins sample 2 (1400), bile mucins sample 3 (2000), $CA15.3^+$ serum (2000), control serum, A549, 3T3, HF19 and BHK-21-control VV supernatants (0).

Lymphocytes and lymphoid cell proliferation assay

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized whole blood of healthy donors by Ficoll-Hypaque gradient centrifugation. Lymphocyte proliferation was assessed by the uptake of [^{3}H]thymidine. PBMC (5×10^4 cells/well) were stimulated in complete medium with either phytohaemagglutinin (PHA; 1 μg/ml) or 100 ng/ml recombinant human IL-2 (R&D Systems, UK) in the presence of 1 μg/ml OKT3 purified from the OKT3 hybridoma cell line (ATCC, Rockville, Maryland). After 96 h, the cells were pulsed with 1 μCi/well [^{3}H]thymidine. Incorporation of [^{3}H]thymidine into the DNA of proliferating T cells was measured by harvesting cellular DNA onto glass filter-paper (PHD harvester; Cambridge Technology, USA) after 18 h and by counting the radioactivity in a liquid scintillation counter (Beckman, Germany). Jurkat cells were seeded at 5×10^4 cells/well for a 48-h culture. The other cell lines were seeded at 1×10^4 cells/well for a 48-h culture. All experiments were performed in triplicate. Proliferation was also evaluated by using the Cell Titre 96 aqueous non-radioactive cell-proliferation assay (Promega, Madison, USA).

Flow-cytometry analysis of apoptosis

The potential induction of T cell apoptosis by MUC1 was measured after 72 h of co-culturing T47D tumour cells (10^6 cells/test) with Jurkat cells (2×10^5 cells/test). Apoptosis was measured by flow cytometry (FACScan, Becton Dickinson, San Jose, USA) using anti-APO2.7PE-Cy5 mAb (Immunotech, France), which reacts with a 38-kDa mitochondrial membrane protein (7A6 antigen) exposed by cells undergoing apoptosis. As a positive control, apoptotic Jurkat cells induced by anti-CD95-Fas (Immunotech, France) antibody ligation were used. Apoptosis was inhibited by adding inhibitory anti-CD95 Fas monoclonal Ab, which recognizes specifically the human cell-surface antigen Fas (Immunotech, France).

Results

In order to explore the potential inhibition of T cell proliferation by MUC1 [3, 14], we have analysed the consequence of the addition on PHA-activated T cells of culture supernatants from the breast cancer cell lines T47D or MCF7. We have observed that proliferation of PHA-stimulated peripheral blood mononuclear cells (PBMC) was inhibited by T47D (170 MUC1 U/ml) but not by MCF7 (155 MUC1 U/ml) supernatants (Fig. 1). Furthermore, depletion of MUC1 from the T47D supernatant removed the inhibitory effect, as reported previously [14]. Surprisingly, we observed a strong inhibitory effect with the MUC1-negative A549 cell line supernatant. Measurement of the MUC1 content in T47D supernatant and assessment of the activity of diluted samples showed that only 0.28 U/ml was sufficient to induce 70% inhibition. Depletion allowed the removal of 98% MUC1 as measured by ELISA (3.5 U/ml after depletion).

Next, we examined the specificity of the proliferation-inhibitory effect present in the T47D supernatant. T47D supernatant was added to cultures of haematopoietic and non-haematopoietic cells (Table 1). Supernatants of MUC1sec-transfected 3T3 and control 3T3 cells were also tested in the same experiment. We observed an inhibitory effect of the T47D supernatant on a large panel of human and mouse cells of haematopoietic origin (PBMC, Jurkat, HL60, K562, P815, RMA). By contrast, we did not observe any effect on the human non-haematopoietic cells Hela or HF19. Surprisingly, MUC1-transfected cell lines (P815-MUC1, RMA-MUC1) seemed to be slightly less sensitive to this effect than their non-transfected equivalents. Moreover, no significant difference was observed between the effect of the T47D supernatant on PBMC stimulated with either PHA or OKT3 + IL-2, indicating that IL-2 was unable to overcome the inhibitory effect of T47D. Despite the presence of 145 U/ml MUC1 in secretory MUC1-transfected 3T3 supernatants, no inhibitory effect was observed for all cell lines tested.

To determine whether MUC1 was the component responsible for the observed reduction of PBMC proliferation, the inhibitory potential of purified mucins from different origins was tested. In this assay, MUC1 purified from the T47D cell line, bile, urine, MUC1 peptide and bile mucin fractions were added at increasing concentrations. The inhibitory T47D supernatant was included as a positive control. We did not observe any significant inhibition of proliferation with the various purified MUC1 or with the VNTR (TAPPA) 24mer peptide (Fig. 2A). It is of note that both the MUC1-containing serum from a breast cancer patient and the control serum inhibited T cell proliferation, and that the patient's sample seemed slightly more potent in

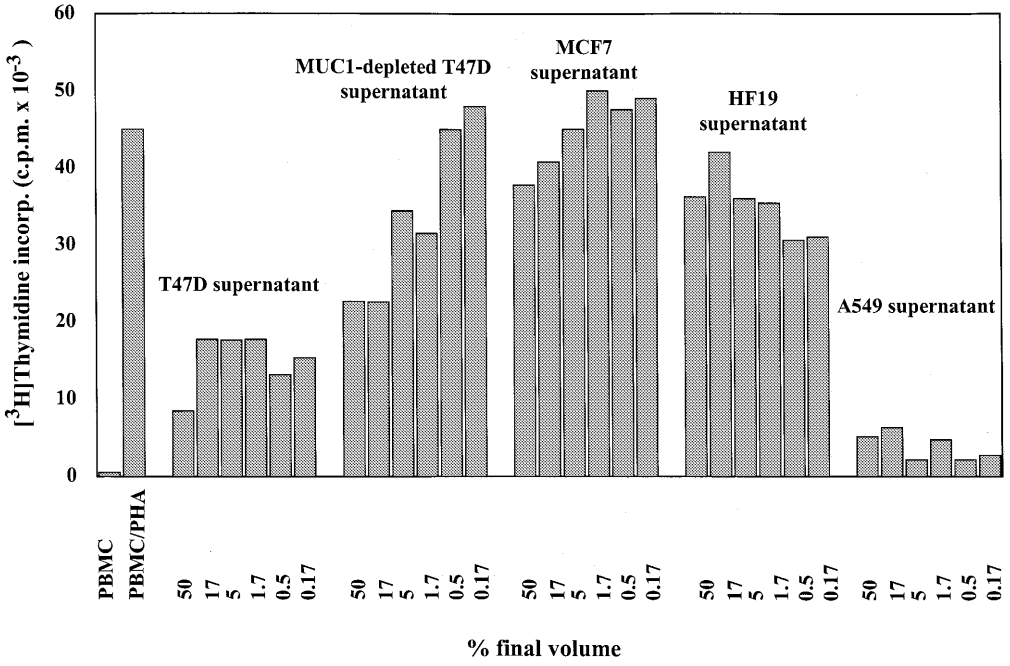

Fig. 1 T cell proliferation in the presence of cancer cell supernatants. Peripheral blood mononuclear cells (*PBMC*; 5×10^4) were stimulated with phytohaemagglutinin (*PHA*; 1 μg/ml). Supernatants taken at confluence from breast cancer cell lines T47D (170 MUC1 U/ml), MCF7 (155 MUC1 U/ml), and two supernatants from MUC1-negative cell lines (A549, HF19; 0 MUC1 U/ml) were added at various concentrations (percentage final volume). T47D supernatant was MUC1-depleted by beads coated with an anti-MUC1 mAb. The depletion efficiency was verified by enzyme-linked immunosorbent (3.5 MUC1 U/ml, e.g. 98% depletion). PHA (1 μg/well) was used as a positive control and showed a stimulation index above 50 (cpm obtained in the presence of PHA/cpm obtained in the presence of medium alone). This experiment is representative of five experiments, all with similar results. Standard deviation, never exceeding 5% of the reported values, not shown

this assay. Interestingly, samples of bile mucins were able to inhibit mitogen-activated PBMC proliferation. Moreover, the inhibitory effect of these samples seems to be related to the degree of sialylation of mucin-associated carbohydrates in the three samples. The less sialylated bile mucins, samples 1 and 2, were more inhibitory than sample 3 for an equivalent MUC1 (U/ml) content. Supernatants from BHK-21 cells infected with a vaccinia virus coding for the secreted form of MUC1 (VV-MUC1sec) or a negative control vaccinia virus were tested on mitogen-activated PBMC in a 5-day proliferation assay (Fig. 2B). We did not observe any anti-proliferative effect of any of these supernatants, despite the fact that the supernatant from VV-MUC1 sec-infected BHK-21 cells contains 115 MUC1 U/ml.

In order to characterize the inhibitory molecules, we have evaluated the activity of boiled (60 min at 100 °C) or filtered T47D supernatant on T cell proliferation and we have observed that boiled and YM10 (cut-off point: 10 kDa) filtered supernatant contained the same

Table 1 Inhibitory effect of MUC1-containing preparations on the proliferation of various hematopoietic and non-haematopoietic cells. Cells were cultured in the presence of 50% of either T47D (170 MUC1 U/ml), 3T3-MUC1sec (145 MUC1 U/ml) or 3T3 supernatants (0 MUC1 U/ml). The proliferation was detected by the uptake of [^{3}H]thymidine or by using the Cell Titer 96 aqueous non-radioactive cell proliferation assay (Promega, Madison, USA). This experiment is representative of three experiments, all with similar results. Standard deviation, never exceeding 10% of the reported values, not shown

Cell type	Cell line	Inhibition (%)		
		T47D supernatant	3T3-MUC1sec supernatant	3T3 supernatant
Haemapoietic	PBMC/PHA	79	2	1
	PBMC/OKT3 + IL-2	85	4	4
	Jurkatt	55	0	0
	HL60	34	17	9
	CEM	4	0,3	0
	U937	27	0	0
	B-EBV	14	13	12
	K562	50	5	0
	P815	59	0	0
	P815-MUC1	26	0	0
	RMA	37	0	6
	RMA-MUC1	0	6	3
Non-haematopoietic	HF19	10	0	0
	HELA	0	0	0
	3T3	14	5	2
	3T3-MUC1sec	0	0	0

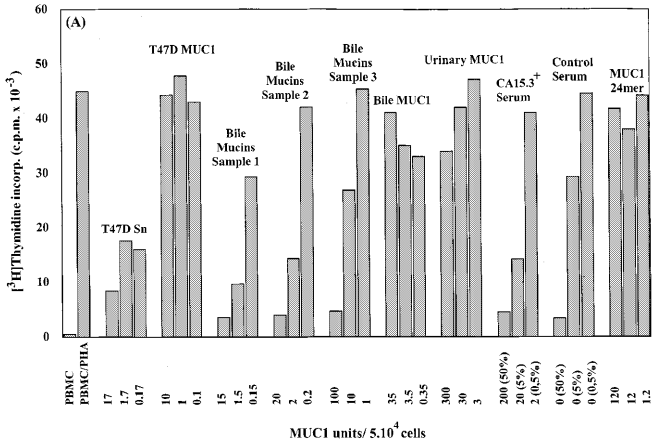

Fig. 2 A In vitro effect of mucin-containing preparations on T cell proliferation. PBMC (5×10^4) were stimulated with 1 μg/ml PHA in a 5-day culture in the presence of increasing concentrations of mucins. MUC1 purified from the T47D tumour cell line (T47D MUC1), purified bile MUC1, purified mixed mucins from bile, urinary MUC1, MUC1-positive serum and 24mer MUC1 peptide preparations have been described in Materials and methods. T47D supernatant (*T47D Sn*) was used as positive control. This experiment is representative of five experiments, all with similar results. Standard deviation, never exceeding 5% of the reported values, not shown.

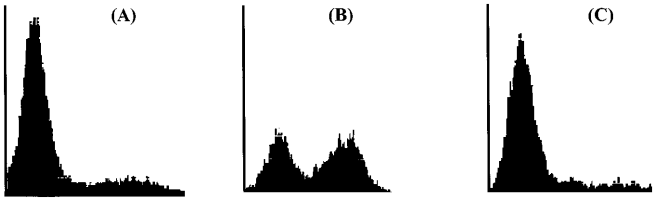

Fig. 3A–C Analysis of apoptotic Jurkat cells in the presence of MUC1. Apoptosis was measured by flow cytometry after 72 h co-culture of T47D tumour cells (1×10^6 cells) and Jurkat cells (2×10^5 cells), using anti-APO2.7PE-Cy5 mAb (**C**). Apoptotic Jurkat cells induced by anti-CD95-Fas antibody ligation were used as a positive control (**B**). As negative control, Jurkat cells were incubated with anti-APO2.7PE-Cy5 mAb (**A**). This experiment is representative of three experiments, all with similar results

anti-proliferative activity as crude supernatant (data not shown), suggesting that the anti-proliferative activity could be a non-proteinaceous agent, since it is resistant to boiling. It is likely to be a small molecule, such as a carbohydrate, since the activity passes through a YM10 filter.

Finally, the induction of T cells apoptosis by MUC1 (Fig. 3) was tested by co-culturing T47D tumour cells (10^6 cells/test) and Jurkat cells (2×10^5 cells/test) for 72 h. Apoptosis was measured by flow cytometry using the anti-APO2.7PE-Cy5 mAb. As a positive control, apoptotic Jurkat cells induced by anti-CD95-Fas antibody ligation were used (Jurkat/anti-CD95 mAb + anti-APO2.7 mAb). We did not observe any significant T cell death or apoptotic bodies in the Jurkat cells from the co-cultures. In agreement with this result, the inhibitory effect on Jurkat cells of T47D supernatant was not modified in the presence of Fas ligand in the culture supernatant (data not shown).

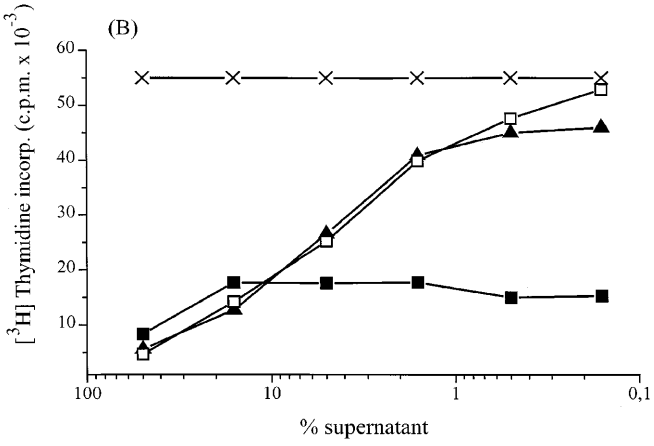

Fig. 2 B In vitro effect of supernatant from BHK-21 cells infected with a vaccinia virus encoding the secreted form of MUC1. PBMC (5×10^4) were stimulated with 1 μg/ml PHA for 5 days in the presence of increasing proportions of culture supernatants. The preparation of supernatants from BHK-21 cells infected with a vaccinia virus encoding the secreted form of MUC1(□; VV-MUC1sec, 115 MUC1 U/ml) or a negative control vaccinia virus (▲; VV-186, 0 MUC1 U/ml) has been described in Materials and methods. The supernatant from T47D cells (■) was used as positive control. Stimulation of the PBMC by PHA (×) gave an incorporation of 55×10^3 cpm. This experiment is representative of three experiments, all with similar results. Standard deviation, never exceeding 5% of the reported values, not shown

Discussion

We have confirmed that T47D cell supernatants contain an anti-proliferative activity and that this activity can be removed by MUC1 immunodepletion as described elsewhere [14]. A control depletion with an irrelevant antibody showed that no MUC1 was removed from T47D supernatant under these conditions (data not shown). However, supernatants from other cell types, such as MCF7 or 3T3-MUC1sec, were unable to induce such an effect despite the fact that they contained similar quantities of MUC1. A strong anti-proliferative activity was observed for the supernatant from the MUC1-negative human tumour cell line A549. Cells from this line do not stain with MUC1-specific antibodies and no MUC1 can be found in the culture supernatants from these cells. However, there are several known anti-proliferative or immunosuppressive factors, such as prostaglandin and nitric oxide, which are reported to be released from these cells [21, 38, 27, 42].

The T cell specificity of the inhibition by T47D supernatant was also examined as described elsewhere [3, 14], and we have observed that the anti-proliferative effect was restricted to cells of haematopoietic lineage, and was less effective on cells already expressing MUC1. To determine whether MUC1 was directly responsible for the observed inhibition of mitogen-induced proliferation, several purified forms of MUC1 were evaluated for their anti-proliferative activity on T cells. We did not observed any effect of purified MUC1 on in vitro T cell proliferation at concentrations at least equal to that

measured in the T47D cell supernatant. Moreover, a 24mer peptide of MUC1 (corresponding to one tandem repeat) was also unable to induce this type of inhibition. Supernatants from cells transfected with MUC1 (3T3-MUC1sec) or infected with a viral MUC1 construct (VV-MUC1sec) were likewise unable to inhibit lymphocyte proliferation. These results are in agreement with the report that MUC1 expression by dendritic cells infected with a recombinant adenovirus is not immunosuppressive in allogenic mixed leukocyte reactions [15, 22]. Interestingly samples of total human bile mucins were able to inhibit strongly the proliferation of PHA-stimulated PBMC, and we hypothesize that this effect is related to the sialic acid content of the bile mucin sample.

Finally, we have tested the ability of the MUC1-expressing tumour cell line T47D to induce the apoptosis in Jurkat cells by co-culture. No apoptosis was measured by flow cytometry in these experiments.

Together these results suggest that MUC1 itself is not anti-proliferative and that the inhibitory activity might be a molecule physically associated with MUC1, as indicated by the experiments of immunodepletion of MUC1 from T47D cell supernatant, which lead to the co-depletion of the anti-proliferative activity. This putative cofactor or MUC1-associated molecule could be another mucin or mucin subfragment since total bile mucins induced inhibition of lymphocyte proliferation and since T47D and MCF7 cells are known to secrete, besides MUC1, other mucins such as MUC3 (T47D) and MUC4 (MCF7) [4, 44]. The procedure for purifying MUC1 from the supernatant of tumour cell lines, urine and bile was very stringent and could thus be responsible for the co-factor elimination, whereas the milder protocol used for the immunodepletion of MUC1 from T47D supernatants could result in co-depletion of the associated cofactor. Alternatively, the cytostatic activity could be lost during the purification of MUC1 as a result of the denaturation of the protein. This hypothesis cannot be ruled out completely but is unlikely as our purification procedure is similar to that used elsewhere [3] and the same as that used by Gimmi et al. [14] and Hayes et al. [20]. Another explanation for our results might be that the MUC1-associated anti-proliferative activity of the T47D supernatant corresponds to a specific glycoform of MUC1 [18] not present in MCF7 supernatants and which does not bind the monoclonal antibody used for the purification. This last point is supported by the observation that anti-MUC1 mAb H23 shows a high dependence on the glycosylation pattern and thus not all forms of MUC1 might be purified from the T47D supernatant. However, the fact that none of the purified MUC1 mucins showed inhibitory activity argues against a direct anti-proliferative effect of the MUC1 glycoprotein.

The observation that the T47D immunosuppressive activity survived boiling and could tentatively be associated with a molecular mass of less than 10 kDa is compatible with the cofactor hypothesis. Low-molecular-mass immunosuppressive factors found in culture supernatants have been reported previously [9, 35] and some are resistant to heat inactivation [9, 17]. Indeed it has been published previously that a putative immunosuppressive protein of 70 kDa [31] was subsequently found to be a small-molecular-mass factor that associates with large proteins in culture and maintains its anti-proliferative activity upon proteolytic degradation, heating and filtration [9]. The MUC1-associated anti-proliferative activity may thus derive from a small molecule like a sugar [24] or a prostaglandin. Indeed, as described elsewhere [26, 45], tumour cells are able to produce free hexosamines or monosaccharides from *N*-acetylated amino sugars, which could interact with mucins such as MUC1 and interfere with immune effector cells. It is interesting that MUC1-transfected cells are less sensitive to the anti-proliferative effects of the T47D supernatant. It is tempting to speculate that this may be due to adsorption of the anti-proliferative co-factor onto the MUC1 outside the cell, thus protecting the cell from its anti-proliferative effects.

The goal of this study was not to characterize small, toxic or anti-proliferative molecules found in some cell culture supernatants. Many such small molecules have been described but very few have been characterized. Rather, our goal was to determine whether any anti-proliferative activity can be attributable to the cancer-associated mucin, MUC1. The results of our experiments indicate that MUC1 is unlikely, in itself, to be immunosuppressive, but rather that small molecules such as amino sugars or other mucins that can associate with MUC1 or anti-MUC1 antibodies are likely to be responsible for the anti-proliferative effects of T47D supernatants observed in vitro.

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