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University of Alberta

MUC1 Synthetic Peptide Inhibition of ICAM-1 and MUC1 Binding is Dependent on the Number of Tandem Repeats

by

Janice Lik Ming Kam

A thesis submitted to the Faculty of Graduate Studies and research in partial fulfillment of the requirement for the degree of Master of Science

Department of Laboratory Medicine and Pathology

Edmonton, Alberta

Fall, 1998

University of Alberta

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Dr. Kim Solez, M.D. (Chair)

Dr. Judith Hugh, M.D. (Supervisor)

Dr. Linda Pilarski, Ph.D. (Advisor)

29th June, 98

Dr. David Rayner, M.D. (Advisor)

Abstract :

MUC1 is a heavily glycosylated transmembrane glycoprotein. In normal breast, MUC1 is located on the apical surface of epithelial cells. In breast cancer cells, MUC1 is underglycosylated and over-expressed in a non-polarized fashion. It was recently reported that the breast tumor-associated MUC1 is a ligand for intercellular adhesion molecule 1 (ICAM-1) (1), and it was suggested that the adhesion of the molecules is important in the process of blood borne metastases. This study used a competitive indirect binding assay to detect the molecular requirements for binding between ICAM-1 and MUC1. The addition of anti MUC1 antibody or affinity purified tumor MUC1 blocked the binding of rhICAM-1 to a murine breast adenocarcinoma cell line transfected with human MUC1. The addition of a library of human MUC1 synthetic peptides ranging from 9 mer to 24 mer showed little or no inhibition of binding. However, a 120 mer peptide which corresponds to 6 tandem repeats of the human mucin MUC1 was as an effective an inhibitor as purified tumor MUC1 and MUC1 epitope (APDTRP) specific antibody (B27.29). Hence, the number of MUC1 tandem repeats necessary for an ordered tertiary structure is also important for ICAM-1 recognition. These findings are identical to those recently described for MUC1 induction of T cell anergy (2). As such, MUC1 synthetic peptides may be important for anti-metastatic therapy as well as immunotherapy.

Dedication

Mom, Dad and Marten

Acknowledgements

First, I would like to express my heartfelt thanks to my supervisor, Dr. Judith Hugh, for her guidance and support throughout my graduate program. I am also thankful to the members of my committee: Drs. Linda Pilarski, David Rayner and Kim Solez for their valuable advice. Last, but not least, I thank the colleagues in the labs of Drs. Linda Pilarski, Robert Coupland, Carol Cass and Judith Hugh for excellent technical assistance.

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List of Abbreviations

1. ICAM-1	Intercellular adhesion molecule - 1
2. rhICAM-1	Recombinant human intercellular adhesion molecule - 1 Immunoglobulin G fusion protein
3. ECM	Extracellular matrix
4. E selectin	Endothelial selectin
5. APC	Antigen presenting cell
6. $sLe^{x/a}$	Sialylated Lewis x and a antigens
7. Ig	Immunoglobulin
8. IL-1	Interleukin-1
9. LFA-1	Lymphocyte function-associated antigen-1
10. Mac-1	CD11b/CD18 β2 integrin
11. Ab	Antibody
12. mAb	Monoclonal antibody
13. kD	Kilo-dalton
14. TNFα	Tumor necrosis factor α
15. VNTR	Variable number of tandem repeats
16. sTn	Sialylated Tn antigen
17. TF	Thomsen-Friedenreich antigen
18. RPMI	RPMI 1640 growth medium
19. FCS	Fetal calf serum

20. PBS	Phosphate buffered saline
21. DMEM	Dulbecco's modified Eagle's medium
23. rpm	Revolution per minute
24. BSA	Bovine serum albumin
25. CTL	Cytotoxic T lymphocyte
26. HMFG	Human milk fat globule
27. INFγ	Interferon γ
28. TCR	T cell receptor
29. MHC	Major histocompability complex
30. ICAM-3	Intercelluar adhesion molecule - 3
31. P-selectin	Platelet selectin

Chapter 1

INTRODUCTION

1.1. Background

Cancer is a collective term for abnormal cells which have uncontrolled cell growth and metastatic spread.

Breast cancer is now the most common malignancy among women in North America and Western Europe (3). Between 10 to 11 percent of women in these populations who live to age 80 will develop breast cancer. In the decade of the 1990s, it is predicted that more than 1.7 million women in U.S and Canada will be diagnosed with breast carcinoma (3).

Metastases are the major cause of treatment failure in cancer patients. A metastasis is defined as a growth, separate from the primary tumor, that has arisen from detached, transported fragments of the primary tumor. Metastases do not occur randomly, but are the end result of a complicated series of tumor-host cell adhesion interactions (4). Hence, it may be possible to selectively interfere with the tumor cell adhesive processes without blocking normal adhesion interactions in the cancer patients.

1.2. The Metastatic Cascade

A successful metastatic process includes invasion of the extracellular matrix, intravasation of peritumoral vessels, arrest at the target organ endothelium, extravasation to the secondary growth site and angiogensis (Figure 1) (4).

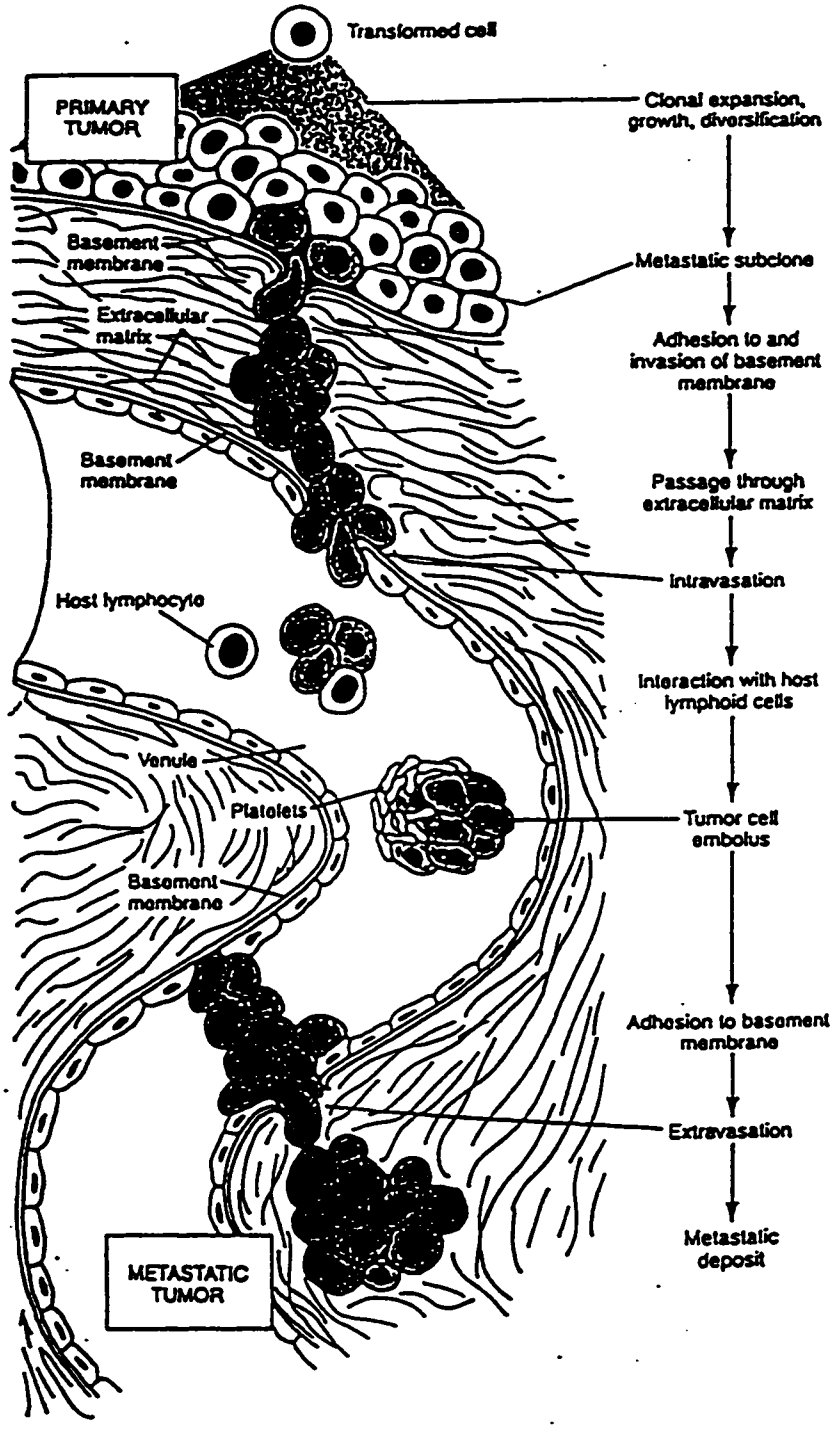


Figure 1. The cascade of blood-borne metastasis. (taken from ref. 5)

Our laboratory recently reported that a breast tumor associated molecule, MUC1, could bind to a common adhesion molecule, intercellular adhesion molecule, ICAM-1 (CD54). There are two areas within the metastatic cascade where the MUC1-ICAM-1 interaction may be important.

During the transition from in-situ to invasive disease, malignant cells attach to the basement membrane via tumor cell surface receptors such as the laminin receptor, then tumor-associated proteolytic enzymes like Type IV collagenase are released to degrade the ECM components. Subsequent movement through the extracellular matrix requires a repetitive sequence of attachment and detachment. Adhesion molecules on the surface of the tumor cells bind and allow tumor cells to pull themselves along the adhesion ligands expressed on the cells and matrix of the ECM. In breast cancer, the increased expression of ICAM-1 on stromal fibroblasts surrounding the tumor (6) provides a possible mechanism for the MUC1 positive tumor cells to move through the ECM. Degradation of the perivascular basement membrane is followed by intravasation of the tumor cells into the bloodstream (7). The circulating malignant cells tend to aggregate with platelets and are protected from attack by lymphoid cells (6). This aggregate will arrest at the target organ endothelium via specific tumor-associated adhesion molecules such as $sLe^{x/a}$ which bind to the P and E selectins on endothelium (8) (Figure 2). This process is thought to be similar to leukocyte extravasation. The MUC1 mucin on breast cancer cells may also mediate

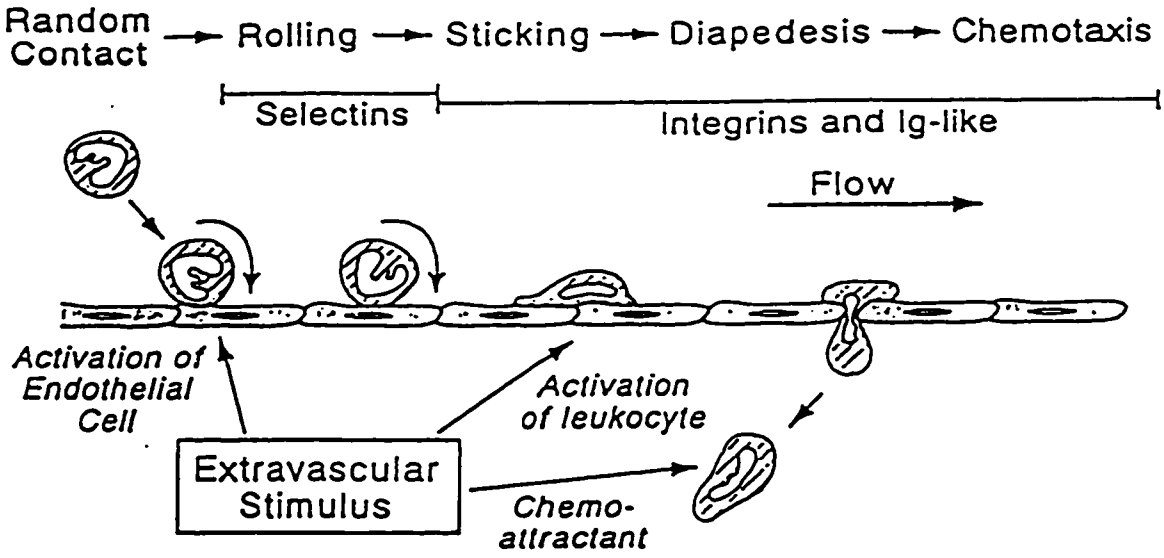


Figure 2. Process of leukocyte extravasation. (taken from ref. 8)

arrest and extravasation through binding to endothelial ICAM-1. After adherence of the tumor cells, the endothelial cells retract exposing the basement membrane for degradation, and tumor cells extravasate to a secondary growth site accompanied by angiogenesis to sustain the growth of tumor (9).

The possible role of MUC1-ICAM-1 in facilitating breast cancer metastases is an area of active research in our lab. The molecular requirement for MUC1-ICAM-1 binding is unknown, and is the subject of this thesis. If the binding characteristics are known, it is possible that this may lead to the development of treatment strategies which selectively interfere with the tumor cell adhesive processes without blocking physiological adhesion interactions in cancer patients.

1.3. The Human MUC1 Mucin

1.3.1. MUC1 Structure

MUC1 is a heavily glycosylated transmembrane glycoprotein membrane of the mucin family which occurs in all mammals (10). The human MUC1 gene is located on chromosome 1q21, and both alleles are expressed codominantly (10). The gene contains seven exons, where the second exon encodes a tandem repeat region. The MUC1 protein consists of three distinct domains (Figure 3).

The longest domain is composed of a variable number of tandem repeats (VNTR) of a twenty amino acid sequence. The number of tandem repeats varies from thirty to ninety

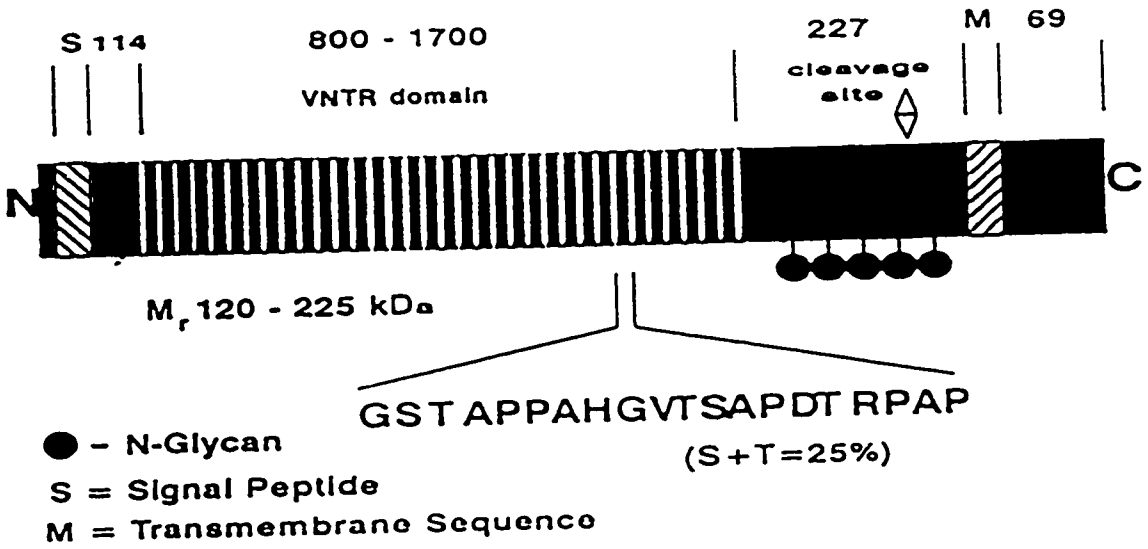


Figure 3. The structure of the mucin MUC1 shows a transmembrane protein with short cytoplasmic and transmembrane domains, followed by a VNTR region flanked by degenerate tandem repeats. (taken from ref. 11)

due to genetic polymorphism in the population (12). This variation coupled with differences in MUC1 glycosylation results in MUC1 species ranging in size from 200-500 kD (13). The protein's amino terminus is composed of a hydrophobic signal peptide and degenerate tandem repeats preceding and following the hydrophilic VNTR domain. Its carboxy terminus consists of a 31 amino acid transmembrane region followed by a 69 aa cytoplasmic tail, with tyrosine phosphorylation sites, linked to the actin filament of the cytoskeleton (14) (Figure 4). The transmembrane and cytoplasmic domains share 87% homology between species suggesting that these regions are functionally important. However, interspecies homology of the VNTR domain is low, with only 34% homology between human and mouse.

Threonines, serines, prolines, alanines and glycines account for 80% of the amino acids in the tandem repeats, with each repeat containing two serines and three threonines. Four of these five amino acids occur as doublets for O-linked glycosylation. Prolines, induce a β-turn helix, and space the serines and threonines thus giving MUC1 a rigid structure extending well above (200-500 nm) the glycocalyx (15) (Figure 4). The glycines and alanines facilitate the close approach of glycosyltransferase to the MUC1 (13).

The native MUC1 is thought to assume a stable and ordered rod shape conformation but with less than three tandem repeats, MUC1 has a random coil structure. Physical modeling has shown that the VNTR*s* have a knob-like structure with adjacent spacer

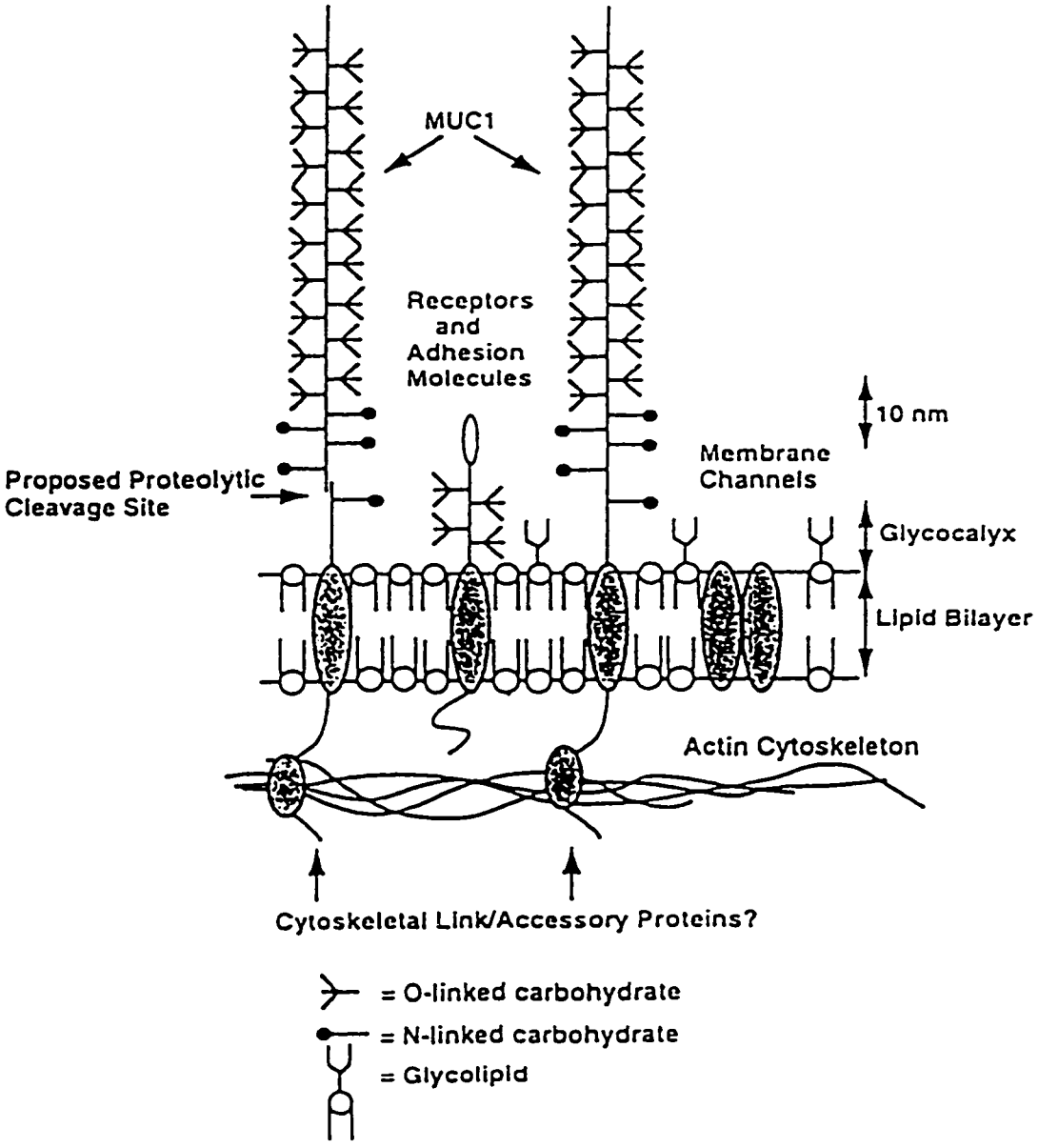


Figure 4. Expression and structure of MUC1 glycoprotein on cell surface. (taken from ref. 13)

regions. The tip of the knob contains a highly immunogenic epitope (APDTR), and this is flanked by O-linked glycosylation sites (Figure 5).

Physiological MUC1 has a molecular composition which is 50% carbohydrate, 30% of which is sialic acid. Galactose and N-acetylglucosamine are the dominant O-linked sugars both of which confer a negative charge to the glycoprotein (16).

1.3.2. MUC1 Expression

MUC1 is expressed in epithelial cells of various organs such as mammary and salivary glands, the pancreas and lung. Within the breast, MUC1 is expressed on the epithelial cells lining the milk ducts (13). In normal breast epithelial cells, MUC1 is glycosylated and is located along the apical surface of the membrane (17), and consequently is present in large amounts in the human milk fat globule. In malignant breast epithelium, MUC1 undergoes several changes: (a) MUC1 is underglycosylated exposing the immunogenic repeated epitope (APDTRP) recognized by most mAbs (18, 72), (b) it is over-expressed possibly due to hypomethylation of the MUC1 gene (19) (Figure 6), (c) the membrane polarity in cancer cells is lost, and (d) MUC1 is shed into the circulation. This circulating MUC1 serves as a marker of tumor load, and it may also form complexes with autoantibodies. These complexes have been associated with a variable prognosis (20).

MUC1 matures with respect to its full sialic acid content as a result of membrane cycling to and from the cell surface (21). Glycosylation of MUC1 is tissue specific. In malignant

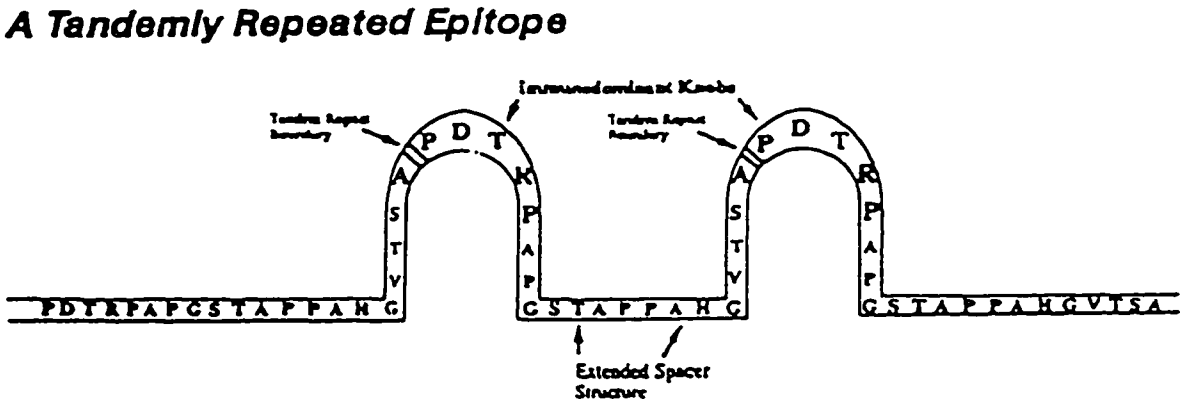


Figure 5. The repeating and protruding knob-like structure of MUC1 tandem repeats. (taken from ref. 76)

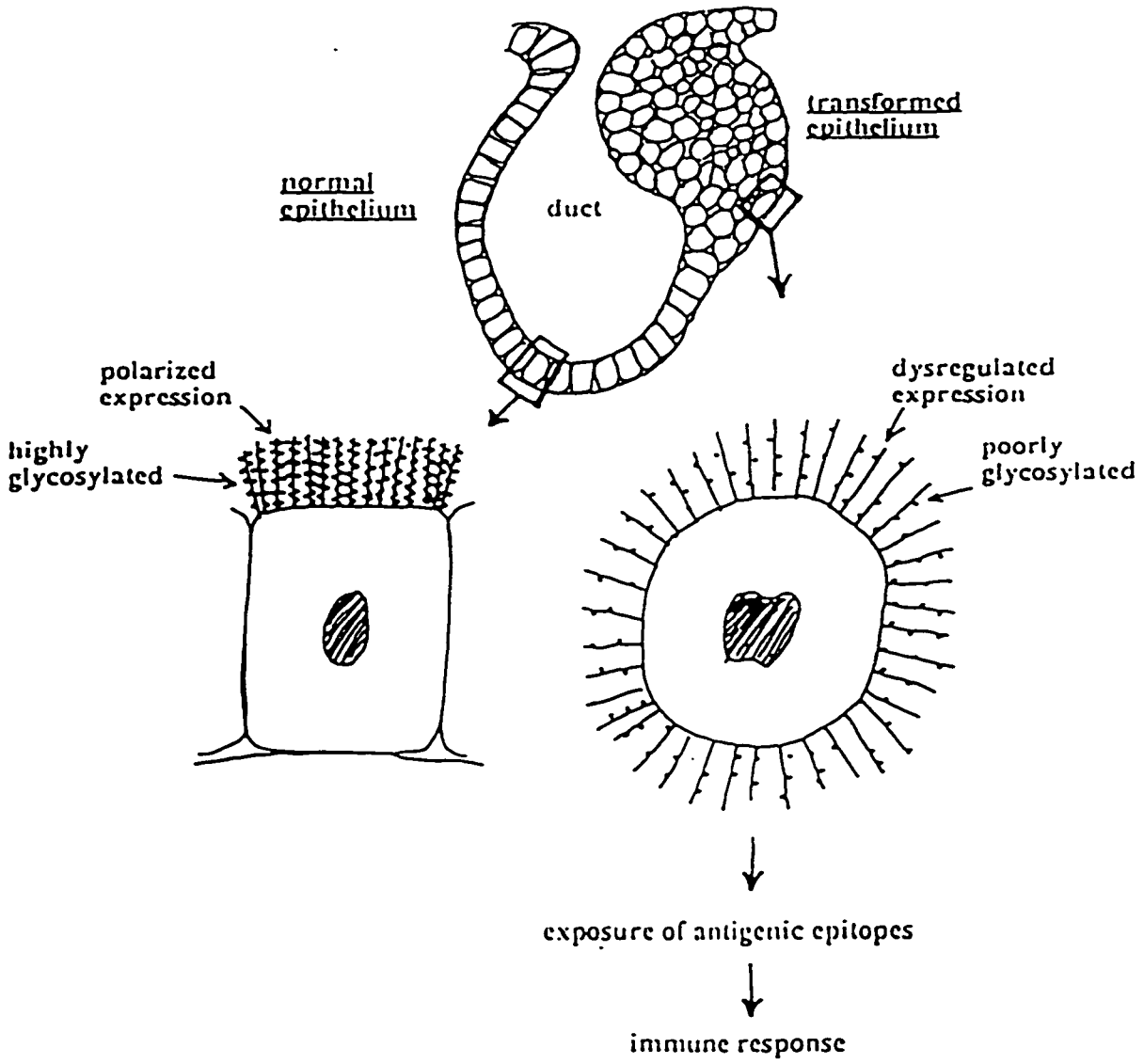


Figure 6. Difference between normal and cancer MUC1 mucin expression. (taken from ref. 23)

cells, the aberrant glycosylation is due to alteration in glycosyltransferase activity resulting in the expression of cancer-associated antigens such as TF, Tn and sTn, which are the targets for immunotherapy (22).

1.3.3 MUC1 Functions

The function(s) of the MUC1 glycoprotein is unknown but several possible functions have been suggested. MUC1 mucins secreted by epithelial cells form mucus, which acts as a physical barrier against microorganisms and degradative enzymes (24), and it also helps maintain the osmolarity and pH in bladder epithelium (25). MUC1 also occurs in the human milk fat globule where it provides protection against bacterial colonization in neonatal gut. In normal breast epithelium, the high sialic acid content of MUC1 makes the extracellular domain extremely hydrophilic. This is thought to shield the shorter glycocalyx cell membrane proteins, thereby suppressing cell adhesion and maintaining patency of the milk ducts.

Contrary to these anti-adhesive effects, in breast cancer cells, the exposed MUC1 peptide core and Lewis carbohydrate antigens bind to ICAM-1 and L-selectin respectively. These pro-adhesive effects have been proposed as mechanisms to facilitate metastasis (1). MUC1 also has diverse immunomodulatory functions.

MUC1 has been proposed as a potential cancer vaccine and has demonstrated promising results in *vivo*. A brief summary of the animal trial data and different formulations attempted include: a) MUC1 peptides packaged within liposomes have been shown to

stimulate an efficient cellular response against tumor cells in mice (26), b) MUC1 peptides composed of five tandem repeats conjugated with adjuvants or MUC1 fusion protein can induce MUC1 specific immune response (27-31, 73), c) intra-muscular immunization with MUC1 cDNA results in both humoral and cellular responses (32), d) Epstein-Barr virus-immortalized B cells transfected with the human MUC1 gene can activate MUC1 specific CTL in chimpanzees (33) and e) after inoculation of mice with 410.4 cells transfected with human MUC1, subsequent tumor challenge showed no tumor growth (34). These experiments show the marked immunogenicity of this tumor-associated molecule.

Cellular immunity, leading to tumor rejection, could involve either a non-specific (natural killer cell) or specific cytotoxic (T lymphocyte) response (35). In breast cancer, secreted MUC1 is able to inhibit both mechanisms showing a dose-dependent inhibition of target cell lysis by NK cells (36), and inhibition of T cell proliferation (2). Interestingly, the tumor reactive CTL*s* from draining lymph nodes have been shown to recognize cancer MUC1 as their target in a non-major histocompatibility complex restricted fashion (37-40). Cytotoxicity of these CTL*s* is inhibited by anti-CD3, anti-αβ TCR and anti-MUC1 mAb (39, 40). This has been taken as evidence that MUC1 may act as a superantigen, with the tandem repeat region interacting directly with the TCR rather than the MHC/TCR complex. In support of this concept, it has been determined that most human HLA alleles do not bind mucin peptides which contain the epitope recognized by CTL (41).

Most pertinent to this thesis is a recent demonstration that the cancer-associated MUC1 as well as some but not all MUC1 synthetic peptides can inhibit T cell proliferation. The anergy was dose-dependent and refractory to stimulation by antibodies to CD3 and CD28.

However, the anergy was reversible through the addition of IL-2 (2). This pattern of immune suppression is identical to that described in the lymphocytes of many cancer patients. Since ICAM-1 is also expressed on activated T lymphocytes, MUC1 interacting with ICAM-1 on the surface of NK cells and T lymphocytes may account for some of the unusual immunomodulatory properties of MUC1. Thus, the parameters of the MUC1-ICAM-1 binding examined in this thesis may be applicable to the immune effects of MUC1.

1.4. The Human ICAM-1

1.4.1. ICAM-1 Structure

ICAM-1 (CD54) is a type I transmembrane glycoprotein which belongs to the Ig superfamily (42) (Figure 7). It has a rod shaped structure with a length of 19 nm and width of 2 nm (43). The five Ig-like unpaired extracellular domains share little sequence homology between species (44). The tandem duplication is thought to represent an evolutionary mechanism creating multiple, divergent and independent binding sites along the ICAM-1 molecule (45). All Ig-like domains, except domain 4 where cysteine is replaced by leucine, are stabilized by disulfide bridges between conserved cysteine residues (46). The human ICAM-1 gene is located on chromosome 19, and it has seven exons separated by six introns, and each Ig-like domain is encoded by an individual exon (47).

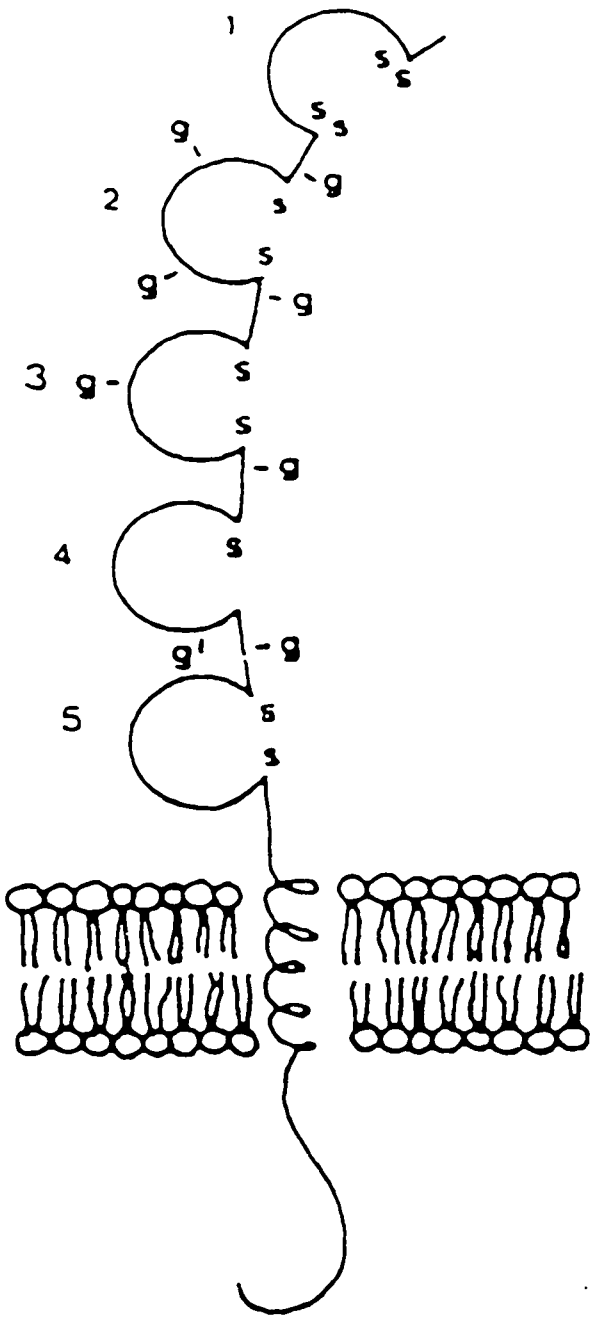


Figure 7. The structure of membrane-bound ICAM-1. (taken from ref. 44)

The extracellular domain consists of 450 amino acids, followed by a 24 hydrophobic amino acid transmembrane region and a charged cytoplasmic domain with 28 residues (46). There are eight N-linked glycosylation sites on the extracellular region of ICAM-1. There are also sites for O-linked glycosylation but the exact location of these is unknown. Glycosylation is cell type specific and the resulting physiological size of ICAM-1 is between 60-114 kD (48). The homology of the cytoplasmic domain is well conserved between species(49), suggesting that it serves an important function. One possibility is that the cytoplasmic domain mediates the internalization of ICAM-1 depending on the membrane ICAM-1 density, in order to selectively modulate adhesive interactions between cells (50).

1.4.2. ICAM-1 Expression

ICAM-1 is expressed on both haematopoietic and nonhaematopoietic cells in a non-polarized fashion (48). On activated endothelium, ICAM-1 is located exclusively along the luminal membrane (50), and its cytoplasmic tail has been shown to associate with the cytoskeleton-binding protein α-actinin regulating the distribution of membrane bound ICAM-1 (51).

Many agents can up- or downregulate ICAM-1 expression, including inflammatory cytokines and steroid hormones. TNFα, interferon γ and IL-1 are the most common inducers of ICAM-1 (52), whereas glucocorticoids are the most important inhibiting agent (53). Interestingly, ICAM-1 can also be downregulated by angiogenic factors. These

factors decrease the sensitivity of endothelial cells to inflammatory cytokines and may provide a mechanism for inhibiting the infiltration of leukocytes into tumors (54).

ICAM-1 expression is regulated by a variety of methods including signal transduction, transcriptional and posttranscriptional pathways. Signal transduction induced by cytokines is cell type specific, and is up-regulated by intracellular second messengers including protein kinase C, cAMP, Ca2+ and phospholipase A2 (53). Other second messenger proteins including cGMP (55) and tyrosine kinase downregulate ICAM-1 expression (56). At the transcriptional level, TATA boxes and enhancers such as AP1 and κB in the promoter region are responsive to TNFα and IL-1 upregulating the mRNA of ICAM-1 (53). After transcription, ICAM-1 expression can be regulated by IFNγ which stablizes the mRNA. This mechanism is especially relevant in endothelial cells which constitutively express a low level of ICAM-1 due to the short half-life of the mRNA. (57).

1.4.3 ICAM-1 Function

The basic function of ICAM-1 is the induction of cell to cell adhesion. This is essential for leukocyte recruitment to sites of inflammation and mediation of humoral and cellular immune responses. During inflammation, activated endothelial cells expressing ICAM-1 bind to leukocyte integrins such as LFA-1 and MAC-1 (58, 71), as well as CD43 (59). LFA-1and Mac-1 recognize the NH_2-terminal first and third Ig-like domains of CD54 respectively (45, 46).

Membrane-bound ICAM-1 exists physiologically as a dimer, through interaction between adjacent transmembrane domains and NH_2-terminal domain 3 (60). Homodimers exhibit a higher avidity for the LFA-1 ligand than the monomer (61). Moreover, ICAM-1 can function as a costimulatory molecule together with anti CD3 to activate MHC class I CTL (62) or MHC class II cells thereby generating an appropriate immune response.

In breast cancer, it is possible that the mechanism by which tumor cells migrate through the extracellular matrix endothelium as well as modulate the immune response can be mediated by underglycosylated MUC1 adhesion to ICAM-1 (1). As such, the ability of the MUC1 synthetic peptide to inhibit the ICAM-1 and MUC1 binding may have significant therapeutic implications.

1.5. The Human ICAM-3

ICAM-3 (CD50) is a 120 kDa transmembrane glycoprotein, which contains an extracellular domain with five Ig-like regions similar to ICAM-1. ICAM-3 is expressed constitutively only on hematopoietic cells such as resting lymphocytes (63, 64). Though CD50 is absent on endothelial cells in normal tissue, its expression on tumor endothelia is high (65). ICAM-3 has a 456 aa residue in the extracellular segment, as well as a 25 aa transmembrane and a 37 aa cytoplasmic region. Human ICAM-3 shows virtually no homology to ICAM-1 in the transmembrane and cytoplasmic regions but in the extracellular NH_2-terminus domain five and two, they share 31% and 77 % homology respectively (66).

β_2 integrins such as LFA-1 and $\alpha d\beta 2$ are the counter-receptors for ICAM-3, and the abundance of ICAM-3 on resting T cells has led to speculation that this molecule has a function in T cell and APC interaction (67,68). Knowing the structural and ligand similarity between ICAM-3 and ICAM-1, as well as the increased level of ICAM-3 expression in tumor endothelium, this thesis will also look at the possibility of ICAM-3 and MUC1 interaction.

1.6 Objective

This study aims to demonstrate the molecular requirements for ICAM-1 and MUC1 adhesion through the following sequence of experiments:

1) Development of a reproducible MUC1 - ICAM-1 binding inhibition assay
2) Determination of the protein sequence of MUC1 essential to MUC1- ICAM-1 binding using a library of synthetic peptides in the assay developed in (1).
3) Determination of the adhesion between MUC1 and ICAM-3, which has a moderate level of homology to ICAM-1 and shares the integrin ligand LFA-1.

1.7 Hypothesis

Binding of ICAM-1 to MUC1 involves specific protein sequence recognition and therefore can be competitively inhibited by peptide fragments of MUC1.

The work of Regimbald et al. (1) showed that the MUC1 - ICAM-1 interaction was inhibited by the B27.29 Ab, which is against the MUC1 protein core, and similarly the CTL - MUC1 recognition (39) was inhibited by SM3 Ab (anti-MUC1 protein core).

Subsequently, Agrawal et al. (2) demonstrated that both native MUC1 and synthetic MUC1 peptides with six repeats could induce T cell anergy. These reports suggest that the ICAM-1 binding site on MUC1 lies within the underglycosylated peptide core in breast cancer, and that synthetic peptides may block the MUC1-ICAM-1 interaction.

The two cell lines used in the following *in vitro* experiments are 410.4 and GZ.Hi. Both are murine breast adenocarcinoma cell lines but the daughter cell line GZ.Hi, has been transfected with the human MUC1 gene. In chapter two of this thesis, the following experiments will be addressed:

1. Analysis of 410.4 and GZ.Hi for expression of possible counter-receptors for ICAM-1.
2. Adhesion of human dimeric rhICAM-1 and human MUC1 on 410.4 and GZ.Hi demonstrated by an indirect binding assay.
3. Confirmation of the specificity of rhICAM-1 and membrane bound MUC1 binding by B27.29 Ab (anti-MUC1) and tumor-associated affinity purified MUC1 inhibition.
4. Inhibition of rhICAM-1 and MUC1 binding by a library of MUC1 synthetic peptides.
5. Verification of the antigenicity of the MUC1 synthetic peptides.
6. Adhesion of human dimeric ICAM-3 to GZ.Hi cells using the indirect binding assay.

Chapter 2

EXPERIMENTAL METHODS AND RESULTS

2.1 Phenotypical analysis of 410.4 and GZ.Hi cell lines

Objective:

To phenotype the murine breast adenocarcinoma parental cell line, 410.4 and the human MUC1 transfectant, GZ.Hi for the expression of counter-receptors for human ICAM-1, which are LFA-1, Mac-1, CD43 and MUC1.

Materials and Methods:

Abs

Monoclonal anti-E selectin as a control Ab (1.2B6) was obtained from Serotec Canada. Monoclonal anti-CD43 (MT1) was donated by Laith Dabbagh, Department of Laboratory Medicine, Cross Cancer Institute, Edmonton, Alberta. Monoclonal anti-LFA-1 (Clone no. 0157) was obtained from AMAC, Inc., Westbrook, ME. Mac-1 antibody was kindly provided by Dr. Linda Pilarski, Department of Oncology, Cross Cancer Institute. RPE-labeled goat anti mouse Ab (light and heavy chains specific) as a secondary Ab was purchased from Southern Biotechnology Associates Inc.. Anti human ICAM-1 (18E3D) was donated by ICOS Co., Bothell, WA., and monoclonal anti MUC1 (B27.29) was a gift from Biomira, Co., Edmonton, Alberta.

Cells and Reagents

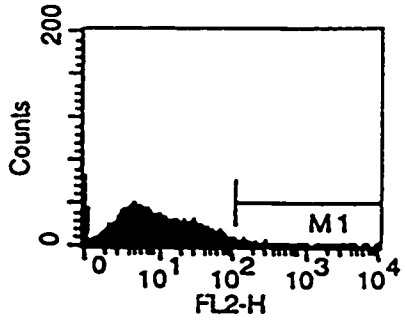
410.4 and GZ.Hi cells were gifts from Biomira Inc., Edmonton, Alberta, and were maintained in RPMI with 10% FCS and supplemented with 2mM L-glutamine, 100U/ml penicillin (Gibco) at 37°C in a humidified incubator containing 5% CO_2.

Flow Cytometric Phenotyping

Cultured cells were harvested using 0.05% (w/v) trypsin with 0.53 mM EDTA. 0.5×10^5 cells were washed in cold PBS. Cells were resuspended in 100ul, 1/20 diluted primary monoclonal Ab, and incubated for 40 min on ice. Subsequently, cells were washed in cold PBS and incubated for another 40 min on ice with 50ul at 1/25 dilution of 0.5 mg/ml RPE-labeled goat anti mouse Ab. This was followed by washing and fixation with 250 ul of 1 % paraformaldehyde and immediate analysis by flow cytometry using FACScan (Becton Dickinson). For each analysis, 10000 events were collected.

Results:

Both 410.4 and GZ.Hi cells showed no expression of CD43, LFA-1 and Mac-1 compared to the control antibody, anti E-selectin. Human ICAM-1 expression was also absent on the cells. The human MUC1 transfectant, GZ.Hi when stained for MUC1 expression, showed 45% of the population to be positive when the GZ.Hi cells were grown in non-selective medium (Figure 9). Sample histograms are presented in Figure 8. The mean and standard deviation are summarized in Figure 9. Each value in all the figures of this thesis represents the mean of three replicates; *bar*, SD. The experiments shown are representative of three independent experiments of each type. The histograms shown are representative of each experiment.



a) anti E-selectin control

Total Events: 10000

Events	% Total	Mean	Peak Ch
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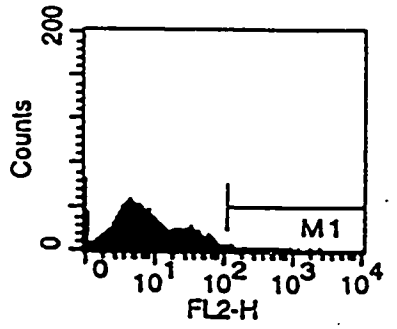
545	5.45	1137.14	127



b) anti CD 43

Total Events: 10000

Events	% Total	Mean	Peak Ch
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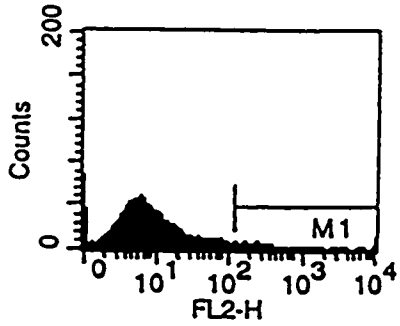
240	2.40	665.82	136

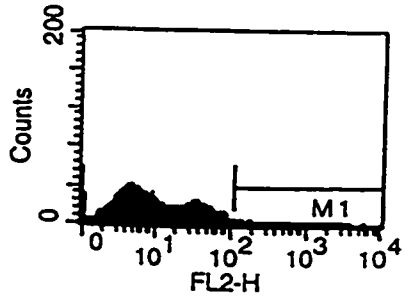


c) anti LFA-1

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	123.49	1
860	8.60	1288.28	9910

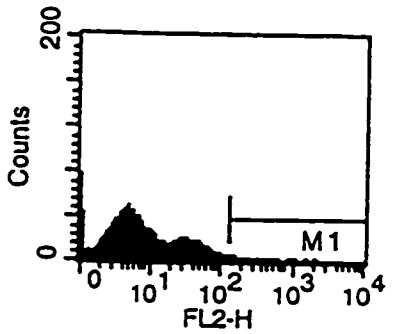
Figure 8. Flow cytometric analysis for the expression of counter-receptors of ICAM-1 on GZ.Hi. Histogram (a) is a negative allowing establishment of gates used for the series of experiments. (b), (c) and (d) show no expression of CD43, LFA-1 and Mac-1 respectively; (e) ICAM-1 expression on GZ.Hi was absent; (f) GZ.Hi are positive for MUC1 expression.



d) anti Mac-1

Total Events: 10000

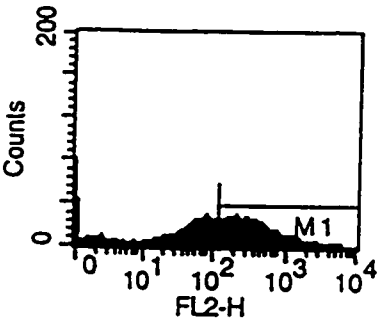
Events	% Total	Mean	Peak Ch
10000	100.00	55.13	1
368	3.68	1051.25	120



e) anti ICAM-1

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	36.76	1
276	2.76	812.97	132



f) anti MUC1 on GZ.Hi cells

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	271.17	1
5099	50.99	487.80	163

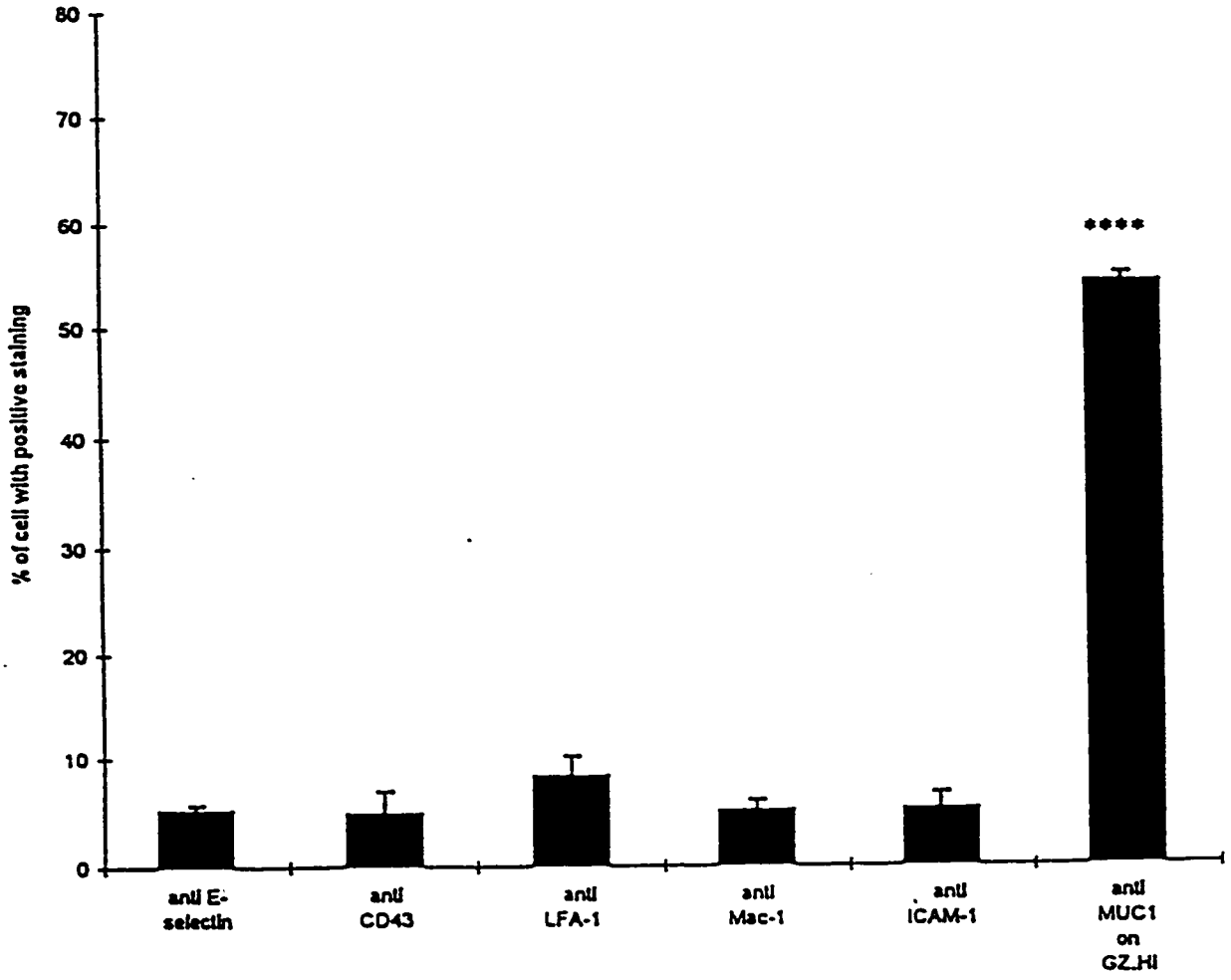


Figure 9. Phenotype of counter-receptors for ICAM-1 on 410.4 / GZ.Hi cells. The graph shows the absence of any significant expression of the lymphocyte counter-receptors for ICAM-1. These are compared to the presence of human MUC1 expression on GZ.Hi. MUC1 expression on 410.4 cells was comparable to the negative controls (see Fig. 13). The data were subjected to a two tailed t-test (Dunnett's test is from Release 6.12, 1989-1996 by SAS Institute Inc.). Dunnett's test tests if any treatments are significantly different from a single control. * , α = 0.05; ** , $\alpha = 0.01$; ***, $\alpha = 0.001$; ****, $\alpha = 0.0001$.

2.2 Binding of rhICAM-1 to 410.4 and GZ.Hi

Objective:

To develop an indirect binding assay (Figure 10) to determine the in *vitro* adhesion between rhICAM-1 and the parental cell line, 410.4, as well as the human MUC1 transfectant, GZ.Hi.

Materials and Methods:

mAb*s* and protein

Monoclonal anti-ICAM-1 (164B) was kindly provided by ICOS Corp.. This Ab blocks LFA-1/ICAM-1 binding and has little effect on MUC1/ICAM-1 adhesion (1). 164B Ab was conjugated with FITC before use (see below). Goat anti mouse conjugated with FITC was used as a secondary Ab, and was obtained from Southern Biotechnology Associates Inc.. Anti MT1 Ab was an isotype control. Human IgG conjugated with FITC was purchased from Sigma. rhICAM-1 and rhICAM-3 are dimeric recombinant human immunoglobulin fusion proteins. Both were gifts from ICOS Corp..

Cells and Reagents

Both 410.4 and GZ.Hi were maintained in the same growth medium as in section 2.1 but 0.6 ug/ml geneticin (Gibco) was added to the medium for GZ.Hi as a selective agent to upregulate the human MUC1 expression.

Conjugation of 164B (anti-ICAM-1) Ab with FITC

1 ml of 1.0 mg/ml 164B Ab in PBS was adjusted to pH 9.0 with 5% sodium carbonate. 0.5mg FITC was added to the Ab and vortexed briefly. The solution was kept in the dark for 45 min at room temperature, and subsequently centrifuged at 200x g, 1000 rpm for 5 min to separate the labeled Ab (in supernant) from unconjugated FITC (in pellet). The labeled Ab was purified by a 10 ml Sephadex G-25 column and eluted with PBS in consecutive 1 ml fractions. The FITC-labeled Ab was pooled and titred out.

Indirect Binding Assay (Figure 10):

After reaching confluence in the second passage, cells were harvested using cell dissociation solution (Sigma), and 2 x 10^5 cells/well were grown on flat-bottom 24 well tissue culture plates (Costar) overnight at 37 °C at 5% CO_2. When cells became confluent after overnight growth, they were washed with PBS prior to use. 200ul rhICAM-1 at 70 ug/ml in PBS was added to each well and incubated for 90 min at 37 °C. The amount of rhICAM-1 used in these experiments was above the determined saturating concentration for the binding assay (Figure 11). Subsequently, cells were washed with PBS to remove unbound rhICAM-1 and transferred to tubes using cell dissociation solution, then washed in PBS again. Cells were centrifuged at 1200 rpm, 4 °C for 5 min. FITC-labeled anti-ICAM-1 Ab (164B) was added to the cells and incubated for 40 min on ice. After washing with PBS, cells were fixed with 250 ul of 1% paraformaldehyde and analyzed by flow cytometry immediately. Positive staining indicated the presence of bound rhICAM-1

detected by 164B-FITC Ab (Figure 12). Each value represents the mean of three replicates; *bar*, SD. The experiments shown are representative of three independent experiments of each type.

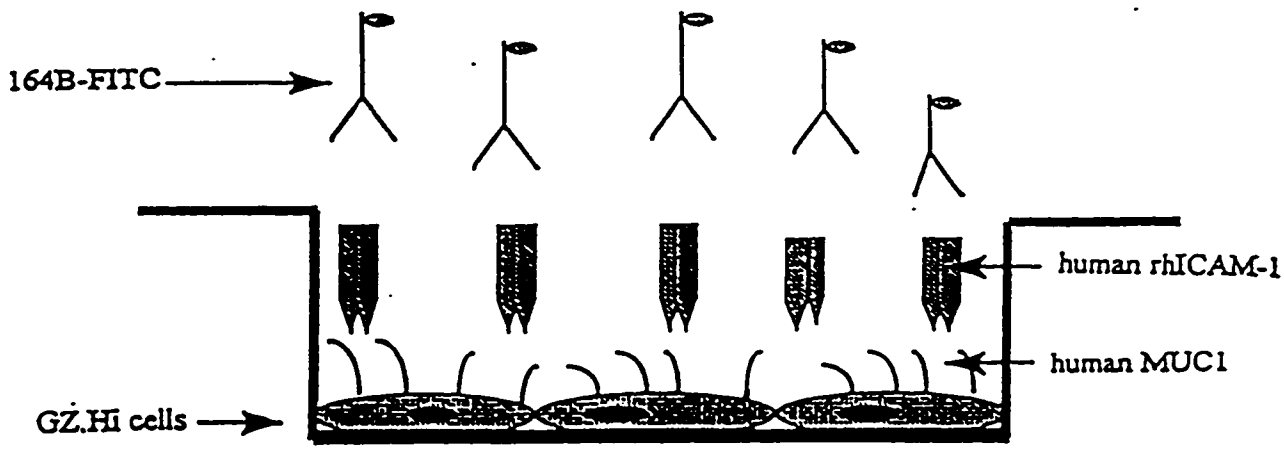


Figure 10. The indirect binding assay detects the adhesion between rhICAM-1 and membrane-bound MUC1 on GZ.Hi cells. Cells were grown to confluence. rhICAM-1 proteins were incubated with the cells at 37°C. Anti ICAM-1 Ab (164-B) conjugated with FITC was added to detect the bound rhICAM-1 on cells. The cells were then analyzed by flow cytometry.

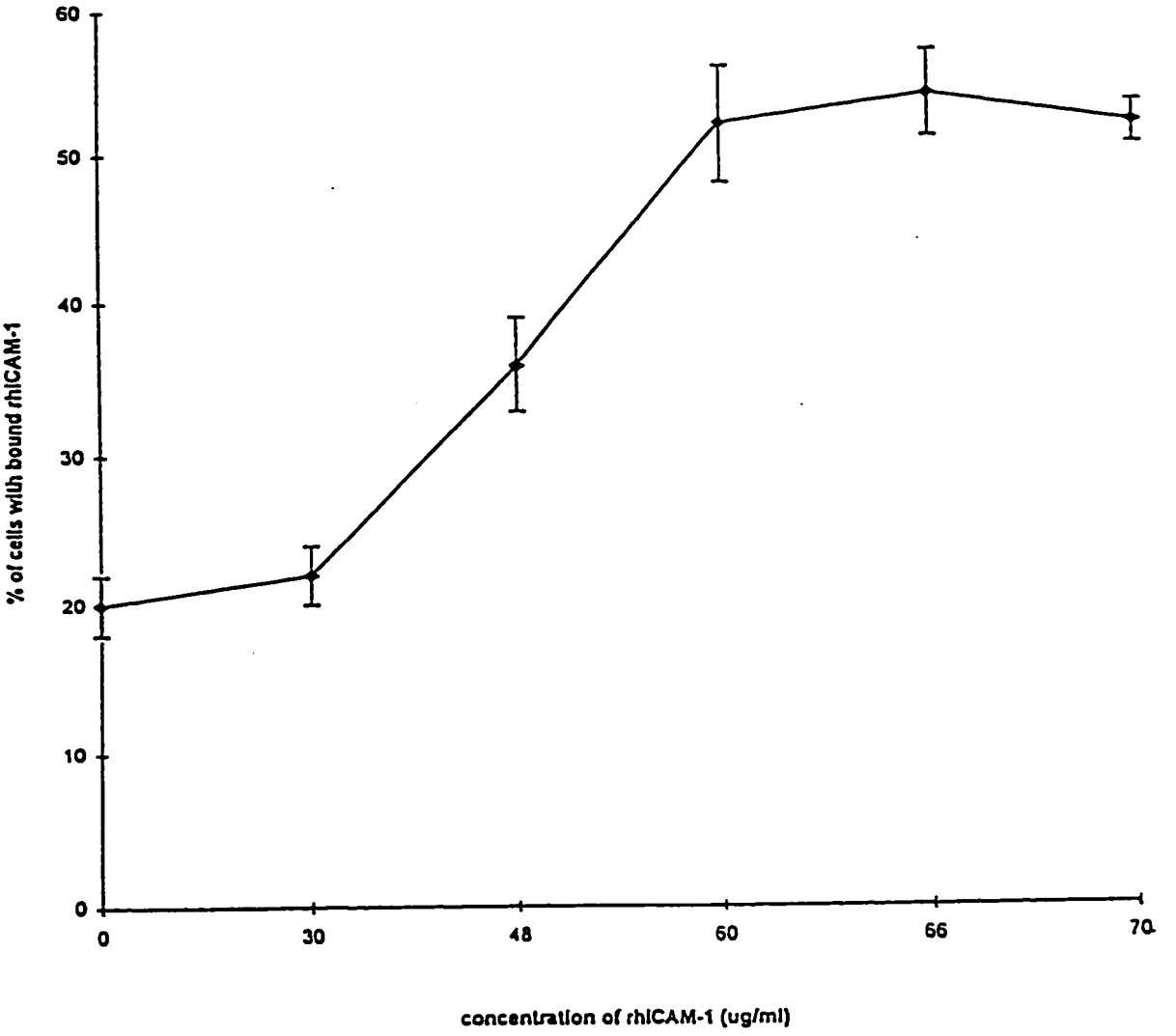
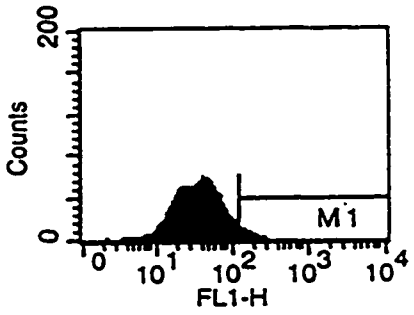


Figure 11. Titration of rhICAM-1 binding to GZ.Hi cells. Cells bound with rhICAM-1 as detected by 164B-FITC Ab increases with increasing concentration of rhICAM-1.

Results:

Human MUC1 expression was again confirmed to be absent on 410.4. No bound rhICAM-1 could be detected on 410.4 cells stained with anti ICAM-1, 164B-FITC Ab. Representative histograms are shown in Figure 12, the mean and standard deviation of three independent experiments performed in triplicate is presented in Figure 13.

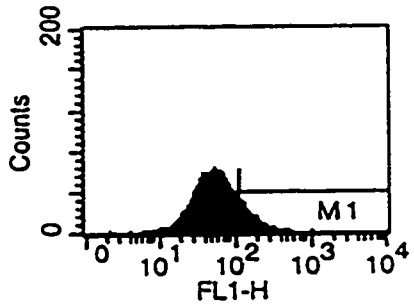
75% of GZ.Hi cells cultured in the selective antibiotic media expressed human MUC1. 53% of the total GZ.Hi population had detectable rhICAM-1 bound to their surface (Figure 14f). The absence of bound human IgG conjugated with FITC bound to either 410.4 or GZ.Hi cells (Figure 12b & 14b) excluded the possibility that the immunoglobulin portion of rhICAM-1 was binding to any cell surface receptors.



a) 410.4 cells only

Total Events: 10000

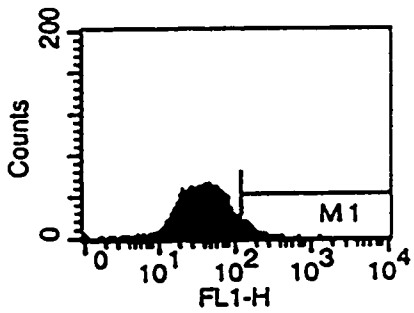
Events	% Total	Mean	Peak Ch
10000	100.00	42.09	40
344	3.44	158.99	127



b) hIgG-FITC binding

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	71.52	52
1205	12.05	213.95	118

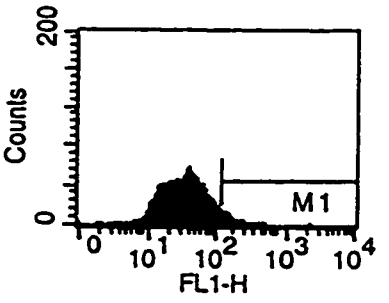


c) anti MT1 control

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	53.63	38
661	6.61	234.95	122

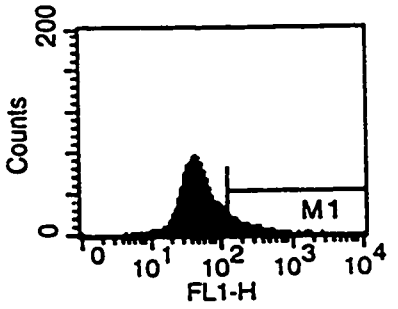
Figure 12. Flow cytometric analysis for human MUC1 expression and rhICAM-1 binding to 410.4 cells. Histogram (d) and (f) shows that both MUC1 expression and bound rhICAM-1 were absent on 410.4, compared to the controls, (c) and (e). (a) and (b) have a similar peak indicating hIgG-FITC did not bind to 410.4.



d) human MUC1 expression

Total Events: 10000

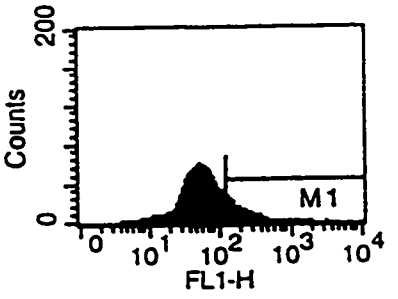
Events	% Total	Mean	Peak Ch
10000	100.00	50.38	38
596	5.96	252.10	130



e) 164B-FITC control

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	81.42	34
1247	12.47	328.50	162



f) rhICAM-1 binding

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	95.32	46
1759	17.59	300.07	119

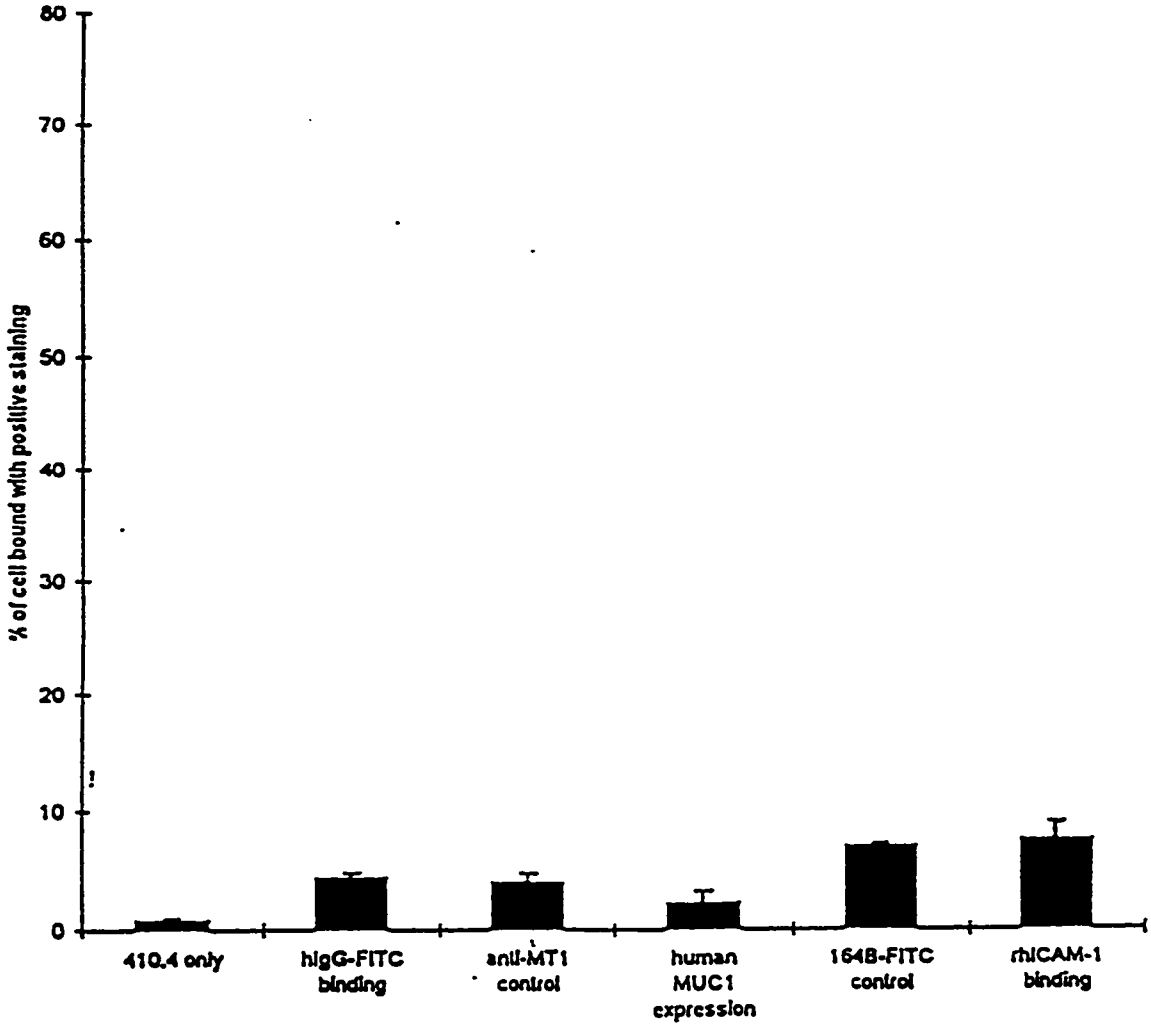
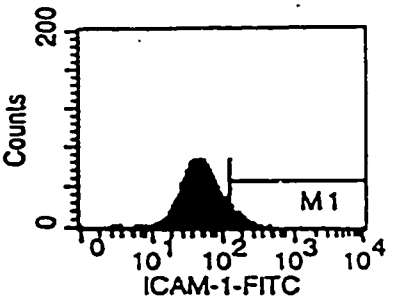


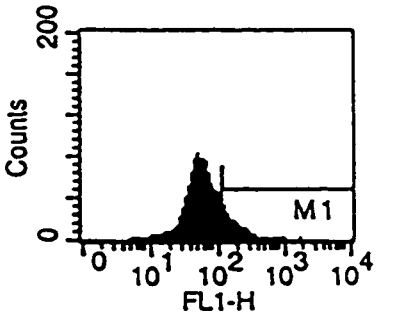
Figure 13. Human MUC1 expression and rhICAM-1 binding to 410.4 cells. Both human MUC1 expression and rhICAM-1 adhesion on 410.4 were absent, when compared to the anti-MT1 and 164B-FITC controls. hIgG conjugated with FITC shows that the Ig portion of rhICAM-1 did not bind to the surface of 410.4.



a) GZ.Hi cells only

Total Events: 10000

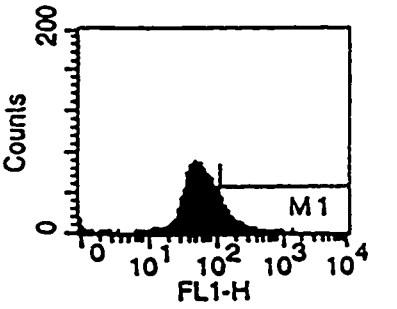
Events	% Total	Mean	Peak Ch
10000	100.00	54.06	49
617	6.17	182.15	134



b) hIgG-FITC binding

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	75.80	50
1194	11.94	237.38	115

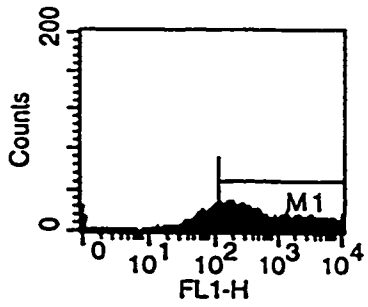


c) anti MT1 control

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	84.12	50
1330	13.30	269.84	117

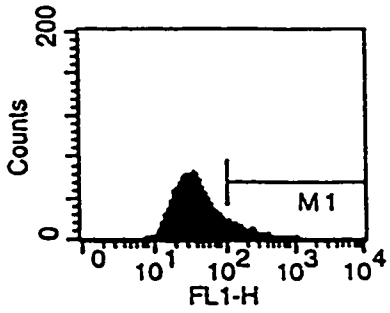
Figure 14. Flow cytometric analysis for human MUC1 expression and rhICAM-1 binding to GZ.Hi. The histogram (d) shows the antibiotic selected GZ.Hi to express high levels of MUC1 when compared to the 410.4, which had no MUC1 expression (Figure 12d). In (f), the GZ.Hi bound with rhICAM-1 displays a dominant positive peak at high intensity, whereas the control 164B-FITC binding to GZ.Hi, (e) shows a single negative peak.



d) human MUC1 expression

Total Events: 10000

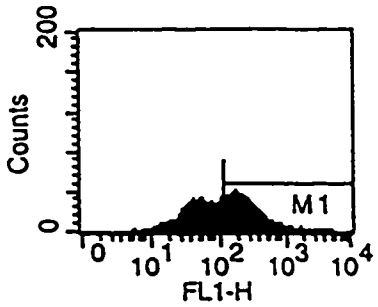
Events	% Total	Mean	Peak Ch
10000	100.00	1234.65	1
7215	72.15	1687.55	239



c) 164B-FITC control

Total Events: 10000

Events	% Total	Median	Peak Ch
10000	100.00	34.60	24
1187	11.87	171.54	116



f) rhICAM-1 binding

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	199.94	153
4821	48.21	356.32	153

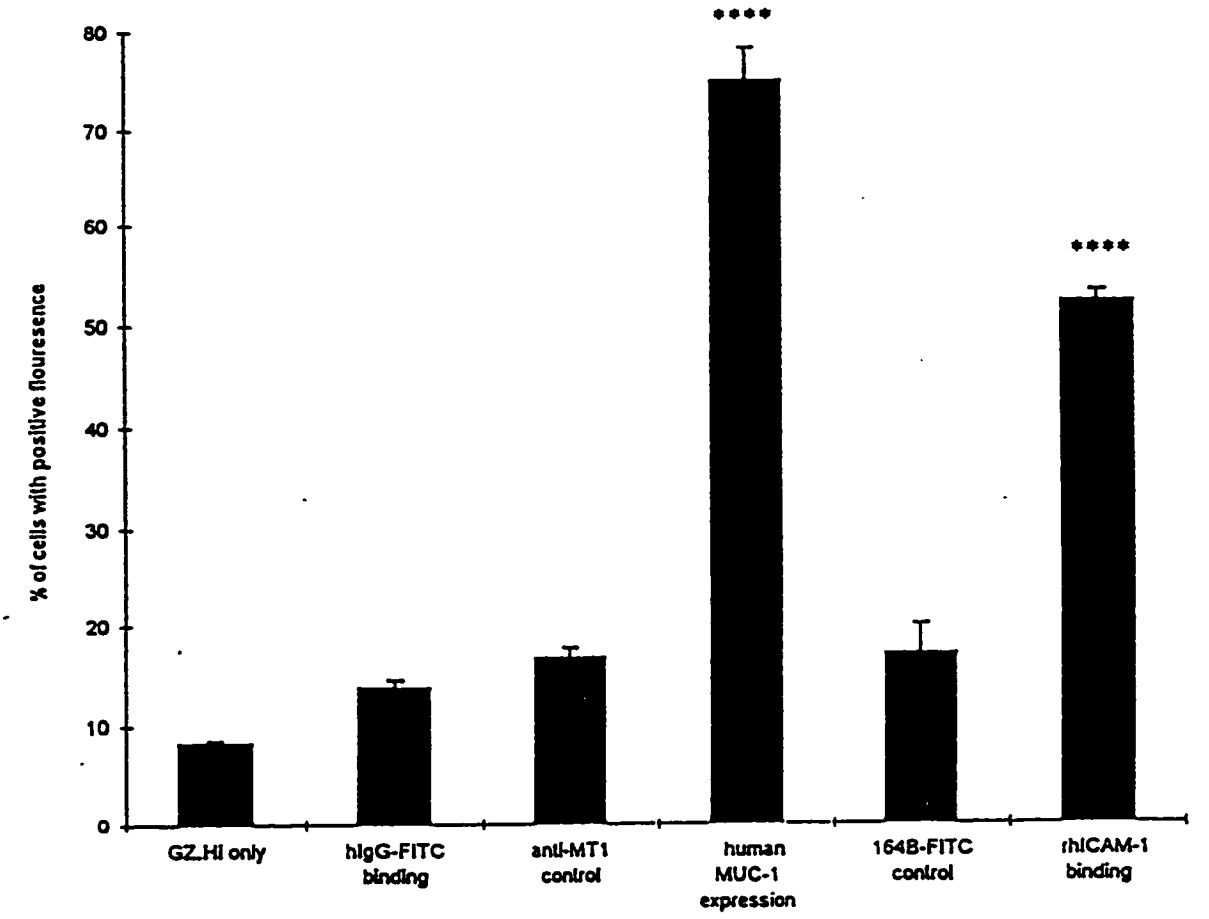


Figure 15. Human MUC1 expression and rhICAM-1 binding to GZ.Hi cells. GZ.Hi cells express high level of MUC1 protein and show a significant amount of bound rhICAM-1. The absence of hIgG-FITC on GZ.Hi indicates the Ig portion of rhICAM-1 is not responsible for cell binding. The data were subjected to a two tailed t-test (Dunnett's test is from Release 6.12, 1989-1996 by SAS Institute Inc.). Dunnett's test tests if any treatments are significantly different from a single control. * , $\alpha = 0.05$; ** , $\alpha = 0.01$; ***, $\alpha = 0.001$; ****, $\alpha = 0.0001$.

2.3 Summary of 2.1 and 2.2

1) Both the parental cell line, 410.4 and the human MUC1 transfectant, GZ.Hi did not express any counter-receptors for human ICAM-1, except the membrane bound human MUC1 on GZ.Hi cells.
2) Using the indirect binding assay, 410.4 showed no bound rhICAM-1 but GZ.Hi, displayed both a high level of human MUC1 and increased rhICAM-1 adhesion.

2.4 Specificity of rhICAM-1 binding to GZ.Hi

Objective:

B27.29 Ab (anti-MUC1) and tumor-associated affinity purified MUC1 were used to confirm the specificity of rhICAM-1 binding to the membrane-bound MUC1 on GZ.Hi cells by blocking the adhesion.

Materials and Methods:

mAb*s* and protein

anti-ICAM-3 (ICR-3) was a gift from ICOS Corp.. Bovine collagen was purchased from Celtrix. Affinity purified tumor-associated MUC1 was derived from the ascites of a single breast cancer patient, and donated by Biomira Inc.. 164B-FITC, B27.29 and rhICAM-1 were used.

Cell and Reagents

The GZ.Hi cell lines and growth medium were used as previously indicated in section 2.2.

Inhibitory Binding Assay

1) Anti-MUC1 Ab inhibition

Confluent GZ.Hi cells were washed in PBS, and 100 ul/well B27.29 antibody at 140ug/ml was added to GZ.Hi together with an equimolar amount of rhICAM-1. The concentration of rhICAM-1 used was identical to the previous (see section 2.2), the mixture was incubated at 37°C for 90 min. Subsequently, cells were washed in PBS, and incubated with 164B-FITC for 40 min on ice. After washing, cells were fixed before being analyzed

by flow cytometry. Addition of anti-ICAM-3 Ab (ICR-3) and collagen were controls for the B27.29 and tumor MUC1 inhibition experiments respectively. All controls were used at the same concentration as those of the inhibition tests. Each experiment was run in triplicate and repeated three times; *bar*, SD. Inhibition was determined from analysis of histograms indicating the amount of bound rhICAM-1 detected by 164B-FITC.

2) Soluble MUC1 inhibition

200 ul rhICAM-1 at 70 ug/ml of rhICAM-1 was pretreated overnight at 37 °C with affinity purified tumor MUC1 before incubating with GZ.Hi. Tumor MUC1 used at 0.19 uM is equivalent to 1500 U/ml, which is considered clinically relevant (2), and this optimal concentration was determined from the MUC1 inhibition curve (Figure 16). For the calculation of the amount of MUC1 mucin, the conversion formula 1 BR unit = 50 ng MUC1 mucin was used. The remainder of the protocol is similar to that outlined above. Pre-treatment of rhICAM-1 with collagen before incubation with GZ.Hi served as a control condition.

Results:

Addition of an irrelevant Ab (ICR-3) and protein (collagen) showed a similar level of the rhICAM-1 binding to GZ.Hi cells (Figure 17) as the baseline positive. The bound rhICAM-1 to GZ.Hi was almost undetectable when an equimolar concentration of B27.29 (anti-MUC1) was added simultaneously with rhICAM-1 to GZ.Hi cells. The tumor derived soluble MUC1 also had a similar inhibitory effect as B27.29 Ab (Figure 17).

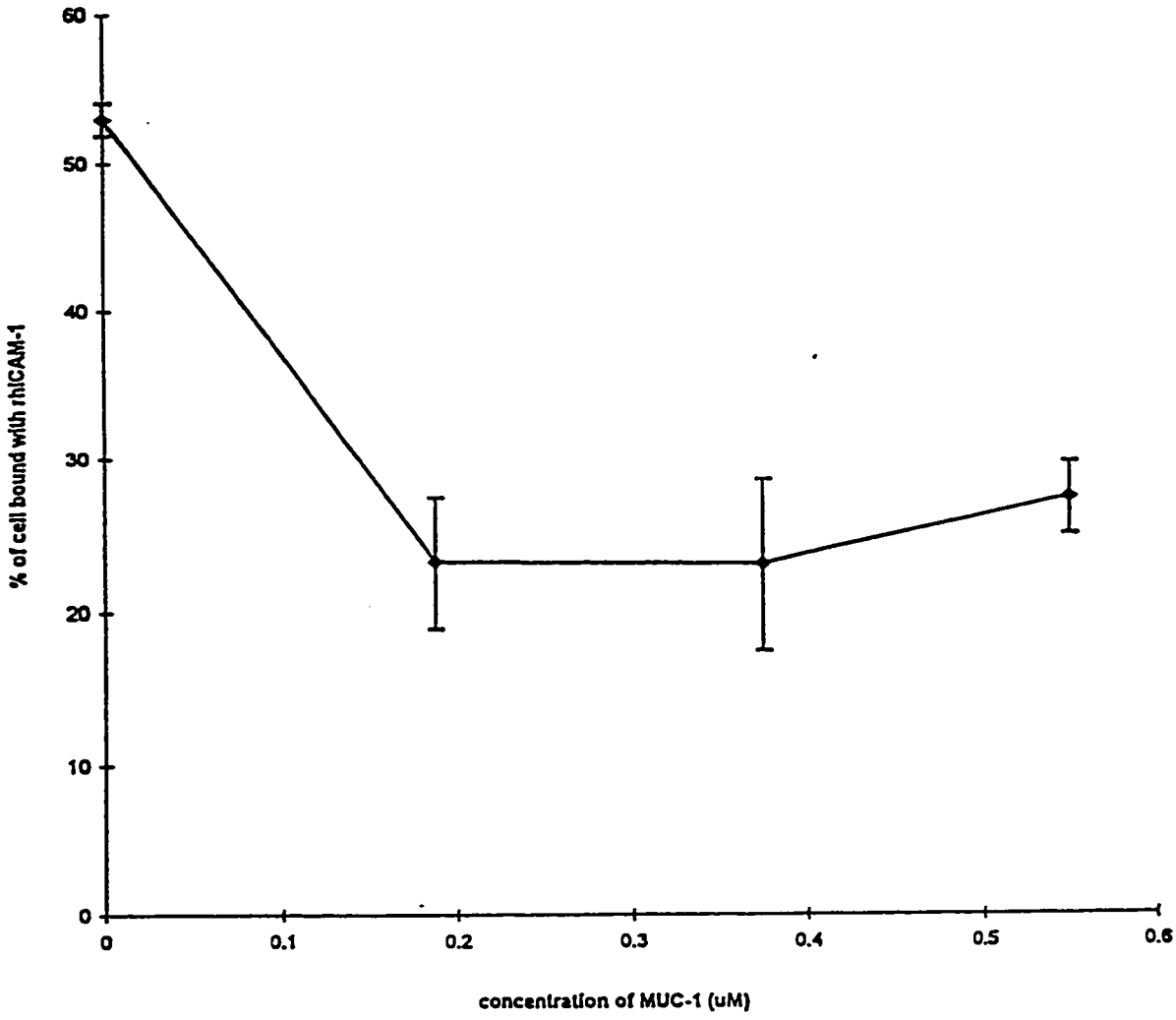
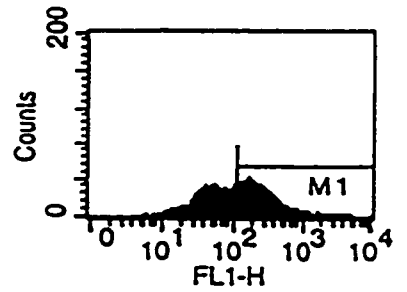


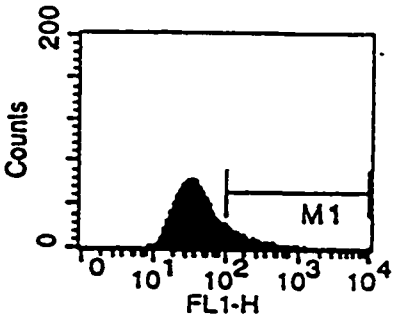
Figure 16. Titration of tumor-associated MUC1 on the inhibition of rhICAM-1 and MUC1 adhesion. There is an abrupt decrease in the amount of bound rhICAM-1 when tumor derived MUC1 is added simultaneously. The inhibition is maximal at the lowest titration point used (MUC1 concentration of 0.2 uM) indicating that the binding sites on ICAM-1 are fully saturated.



a) rhICAM-1 binding

Total Events: 10000

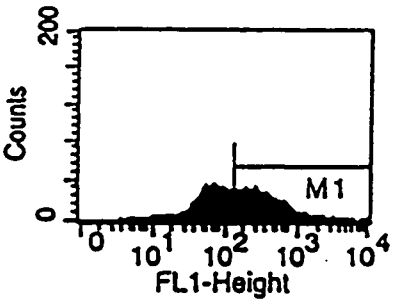
Events	% Total	Mean	Peak Ch
10000	100.00	199.94	153
4821	48.21	356.32	153



b) 164B-FITC control

Total Events: 10000

Events	% Total	Median	Peak Ch
10000	100.00	34.60	24
1369	13.69	156.79	102

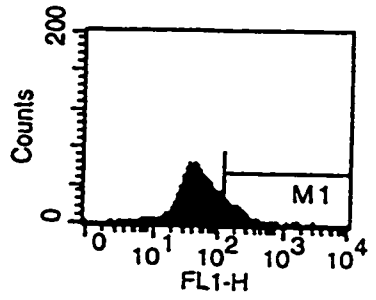


c) ICR-3 inhibition control

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	302.96	48
5049	50.49	538.11	176

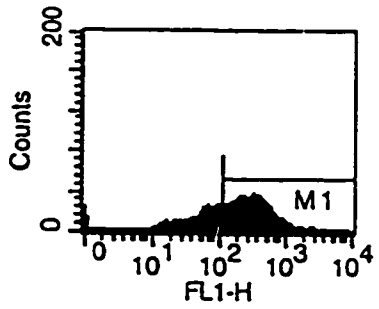
Figure 17. Flow cytometric analysis of B27.29 Ab and tumor MUC1 inhibition on rhICAM-1 to MUC1 adhesion. Incubating rhICAM-1 simultaneously with tumor MUC1 (f) or pretreating rhICAM-1 with B27.29 (anti-MUC1) (d) inhibited rhICAM-1-MUC1 adhesion, compared to the untreated rhICAM-1 binding (a). Controls of (c) ICR Ab (anti-ICAM-3) and (e) collagen for B27.29 and tumor MUC1 inhibition tests respectively display histograms similar to the rhICAM-1 binding to MUC1 (a). 164B-FITC control (b) shows no endogenous ICAM-1 expression.



d) B27.29 inhibition

Total Events: 10000

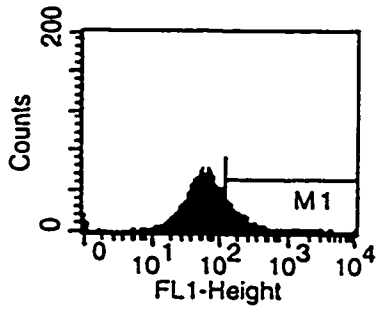
Events	% Total	Mean	Peak Ch
10000	100.00	79.84	34
1359	13.59	268.54	145



c) collagen inhibition control

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	300.28	1
6027	60.27	463.66	310



f) tumor MUC1 inhibition

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	122.73	49
1776	17.76	445.04	140

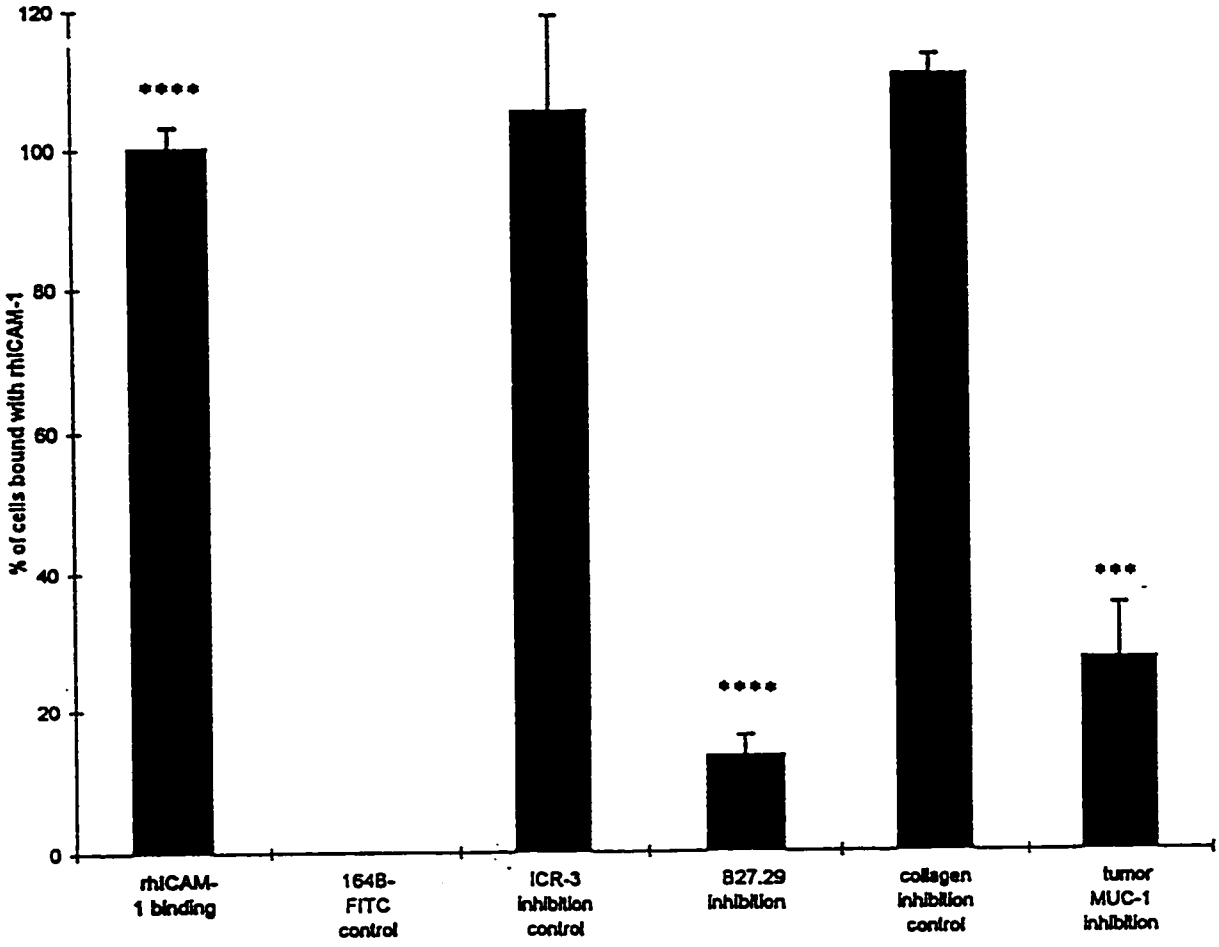


Figure 18. B27.29 Ab and tumor MUC1 inhibition on rhICAM-1 binding to GZ.Hi cells. Both B27.29 (anti-MUC1) and tumor MUC1 significantly decrease rhICAM-1 binding to GZ.Hi cells to the basal level. Results are normalized so that the maximum amount of bound rhICAM-1 is 100% and 164B-FITC control (background flourescence) is 0 %. The data were subjected to a two tailed t-test (Dunnett's test is from Release 6.12, 1989-1996 by SAS Institute Inc.). Dunnett's test tests if any treatments are significantly different from a single control. * , α = 0.05; ** , $\alpha = 0.01$; ***, $\alpha = 0.001$; ****, $\alpha = 0.0001$.

2.5 Inhibitory effect of MUC1 synthetic peptides on rhICAM-1 binding to GZ.Hi

Objective:

To determine the minimum amino acid sequence and number of tandem repeats necessary for inhibiting the rhICAM-1 and MUC1 binding on GZ.Hi cells.

Materials and Methods:

mAbs, Cell and Reagents

Please refer to section 2.2

Human MUC1 synthetic peptides

A library of MUC1 peptides : BP-007, -017, -041, -065, -094, -115, -116 and -147 ranging from 9 to 24 amino acids (Table 1), as well as a 24 amino acid HLA.A2.1 sequenced control peptide, BP1-087, were gifts from Biomira Inc.. Peptides 5DS4 and 6DS5 corresponding to 20 and 120 amino acids of MUC1 protein (Table 1) were kindly provided by Dr. J. Hilgers, Free University Hospital, The Netherlands.

Inhibitory Binding Assay

The same protocol as section 2.4 was used. MUC1 synthetic peptides or the HLA control peptide were preincubated overnight at 37°C with 14 ug rhICAM-1. The peptide-rhICAM-1 mixture was then added to the GZ.Hi cells on the tissue culture plate. The optimal concentration of control BP1-087 peptide (24 aa), MUC1 synthetic peptides BP-065 (24 aa) and 6DS5 (120 aa) were determined from titration (Figure 19, 20 and 21

respectively) before experiments. The tumor derived MUC1 has an estimated molecular weight fifty to one hundred times that of the synthetic peptides. Since Agrawal et al. indicated that considerable molar excess was required for peptide inhibition of MUC1 induced anergy, the concentration of the peptide used in the titration and inhibition experiments represent significant molar excess. 740 uM was used for all MUC1 peptides and control peptide but 6DS4 MUC1 peptide was used at 74 uM (determined from figures 19 -21).

peptide	# of a.a.	sequence	% inhibition	SD
MUC1 20 mer motif	20	PDTRPAPGSTAPPAHGVTSA		
6DS5	120	VTSAPDTRPAPGSTAPPAHG x 6	73	8
BP-065	24	TAPPAHGVTSAPDTRPAPGSTAPP	0	0
5DS4	20	APDTRPAPGSTAPPAHGVTS	22	9
BP-017	18	SAPDTRPAPGSTAPPAHG	30	9
BP-007	16	GVTSAPDTRPAPGSTA	14	11
BP-115	11	DTRPAPGSTAP	8	4
BP-116	11	TRPAPGSTAPP	0	0
BP-041	9	GVTSAPDTR	12	11
BP-094	9	TSAPDTRPA	9	10
BP-147	9	STAPPAHGV	0	0

Table 1 Sequences of human MUC1 synthetic peptides and the corresponding mean % level of inhibition of MUC1-rhICAM-1 binding.

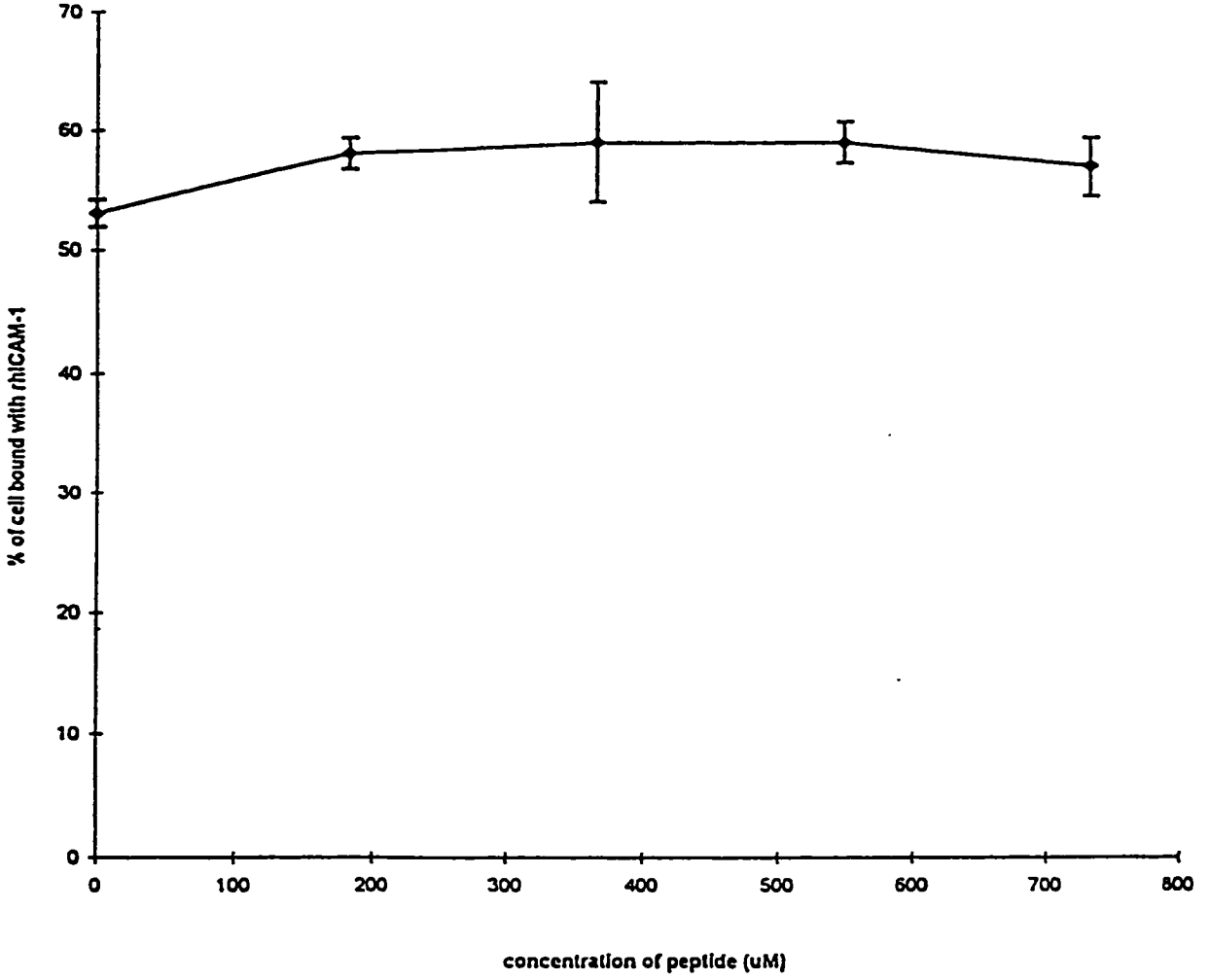


Figure 19. Titration of HLA control peptide on inhibiting the rhICAM-1 and MUC1 adhesion. Increasing the concentration of control peptide does not alter the rhICAM-1 - MUC1 adhesion.

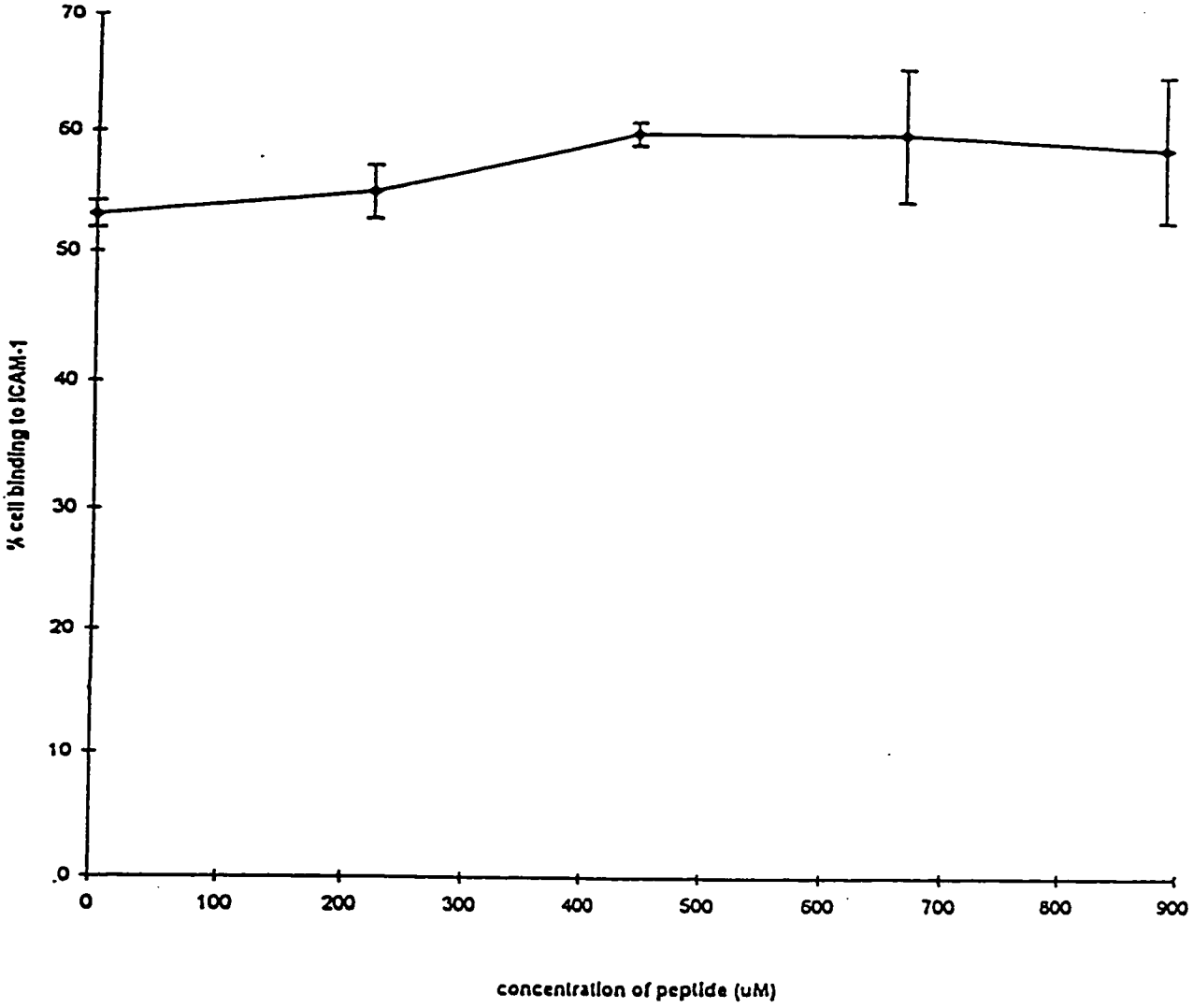


Figure 20. Titration of BP-065 MUC1 synthetic peptide on inhibiting the rhICAM-1 and MUC1 binding. BP-065 peptide does not have an inhibitory effect on rhICAM-1 - MUC1 adhesion, even when the peptide concentration is increased.

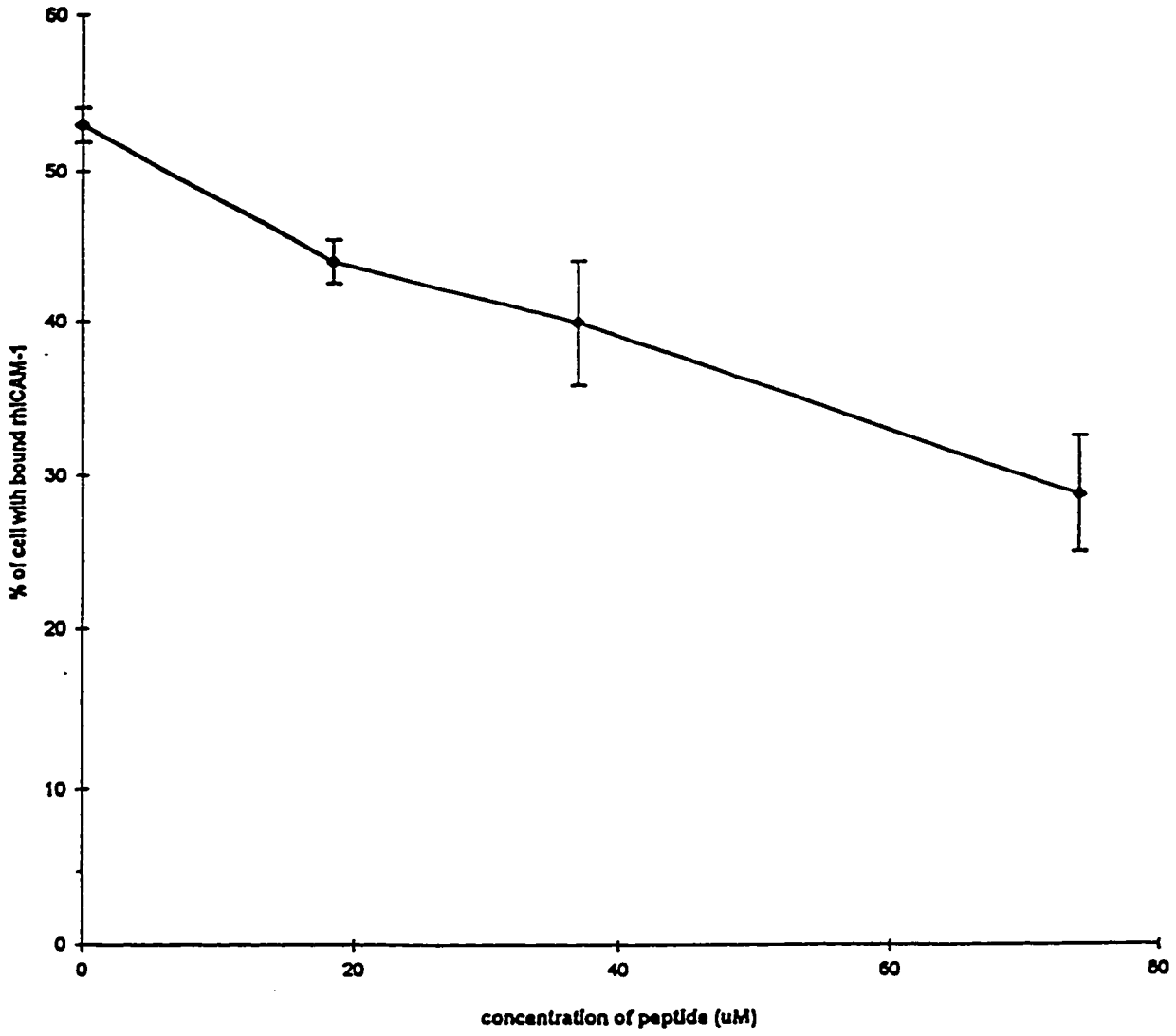
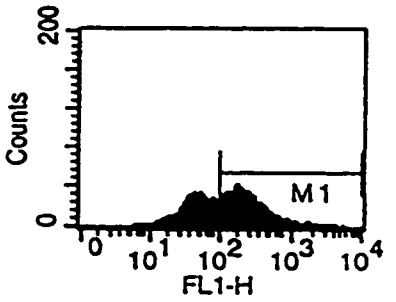


Figure 21. Titration of 6 repeats MUC1 synthetic peptide on inhibition of rhICAM-1 and MUC1 binding. At 74 uM, 6 repeats reduces the number of GZ.Hi cells bound with rhICAM-1 significantly.

Results:

All of the short MUC1 peptides, 9 to 24 amino acids, were used at a saturating concentration of 740 uM (Figure 20) which is ten times higher than the concentration used for the 6 tandem repeat peptide (6DS5) (Figure 21). Peptides BP-116, -065 and -147 had no inhibition on rhICAM-1 and MUC1 binding when compared to the control peptide. Peptides BP-115, -007, -094, 5DS4, -041 and -017 displayed a low-level inhibition (Figure 23). The most efficient MUC1 synthetic peptide tested to inhibit the rhICAM-1 binding to GZ.Hi cells was the 6 tandem repeats peptide (6DS5), which exhibited almost the same inhibitory effect as tumor derived MUC1.

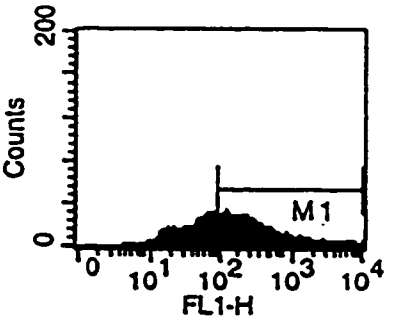
For peptides equal to or less than 24 amino acids, there is no correlation between the length of the peptide and the low-level of inhibition (Table 1). When the sequence of the peptides is compared to the inhibition data, those peptides which exhibited moderate levels of inhibition eg. BP-017, -041, -094, -007 and 5DS4, all share a common epitope (**SAPDTR**) (Table 1). Whereas BP-115 peptide, which contains **DTRPA** sequence, had a minor level of inhibition. The inhibition results of the short peptides suggests that **DTR** is the minimum sequence necessary to inhibit rhICAM-1 binding, and higher levels of inhibition could be achieved with a preceding **SAP** sequence, especially without a preceding threonine eg. **TSAP**. Interestingly, the 24 mer peptide (BP-065) which contains the **SAPDTR** epitope behind a redundant **TAPP** sequence shows no inhibitory effect (Table 1).



a) rhICAM-1 binding

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	199.94	153
5493	54.93	325.51	153

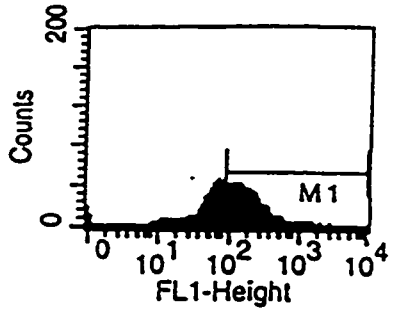


b) control peptide

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	465.39	166
5849	58.49	762.51	166

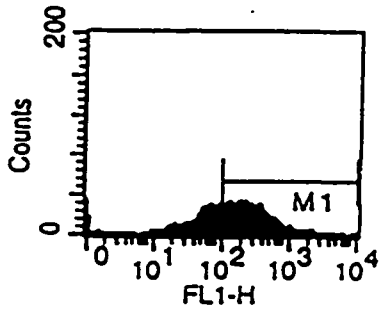
Figure 22 Flow cytometric analysis for MUC1 synthetic peptides inhibition on rhICAM-1 and MUC1 adhesion. For all of the above flow cytometric histograms rhICAM-1 bound to MUC1 transfected cells is detected by FITC labeled anti-ICAM-1 Ab. The gate for each series was set so that the unstained cells are excluded. In (a) the rhICAM-1 is added without pre-incubation with a test protein and there is a bimodal profile where 55% of cells show the presence of bound rhICAM-1. This is unaltered by pre-incubation of the rhICAM-1 with a control peptide (24 aa, HLA-A2.1) (b), peptides lacking the minimum "**DTR**" sequence, BP-116 (c) and BP-147 (e) or the 24 aa MUC1 peptide, BP-065 (d) which contains a redundant "header" **TAPP** sequence. Low levels of inhibition are seen with BP-115 (f) which contains the minimum **DTR** sequence but lacks a preceding SAP.



c) BP-116

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	264.98	139
5371	53.71	448.01	139

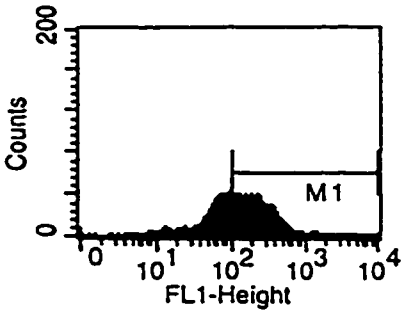


d) BP-065

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	252.39	1
5856	58.56	397.87	118

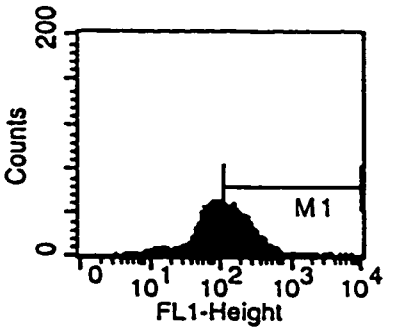
Low to moderate levels of inhibition are seen with BP-007 (g), BP-094 (h) , 5DS4 (i), BP-041 (j) and BP-017 (k) which contain both the minimum **DTR** sequence and the preceding **SAP** but have an antecedent threonine. The highest inhibition by a one tandem repeat peptide was shown by BP-017 which started with the **SAPDTR** sequence. The 6 tandem repeat peptide (6DS5, l) showed levels of inhibition comparable to that of tumor derived MUC1 (m). The profile of the histograms in (l) and (m) show loss of a positive staining modal peak. These results are summarized in the following bar graph (Figure 23).



c) BP-147

Total Events: 10000

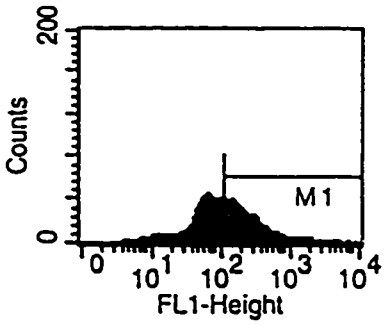
Events	% Total	Mean	Peak Ch
10000	100.00	269.81	116
5779	57.79	425.87	116



f) BP-115

Total Events: 10000

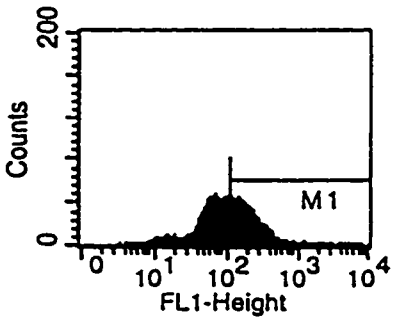
Events	% Total	Mean	Peak Ch
10000	100.00	165.38	78
4905	49.05	277.08	133



g) BP-007

Total Events: 10000

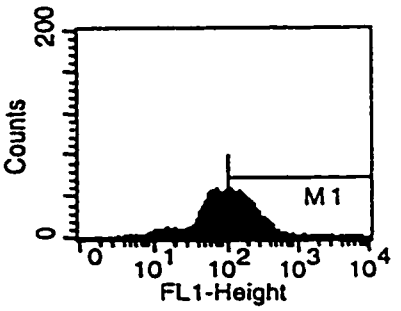
Events	% Total	Mean	Peak Ch
10000	100.00	217.35	52
4526	45.26	411.03	129



h) BP-094

Total Events: 10000

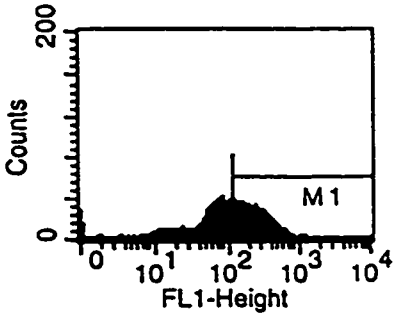
Events	% Total	Mean	Peak Ch
10000	100.00	182.60	56
4566	45.66	329.07	143



i) 5DS4

Total Events: 10000

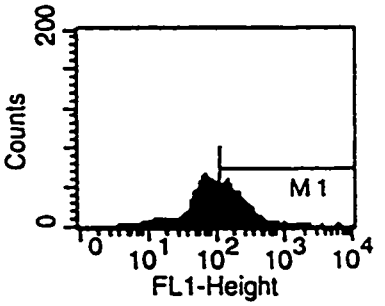
Events	% Total	Mean	Peak Ch
10000	100.00	182.60	56
4789	47.89	318.70	143



j) BP-041

Total Events: 10000

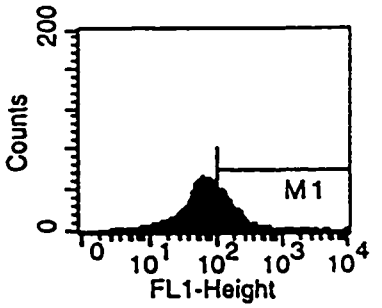
Events	% Total	Mean	Peak Ch
10000	100.00	252.17	1
4862	48.62	462.97	143



k) BP-017

Total Events: 10000

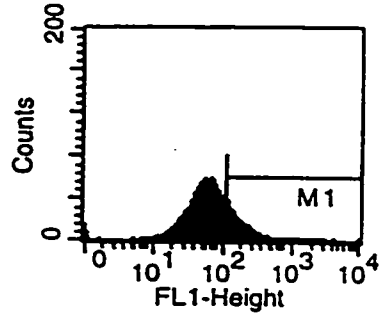
Events	% Total	Mean	Peak Ch
10000	100.00	156.38	69
4052	40.52	299.03	125



l) 6 repeats

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	167.95	77
2883	28.83	447.84	115



m) tumor MUC1

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	122.73	49
1937	19.37	417.36	111

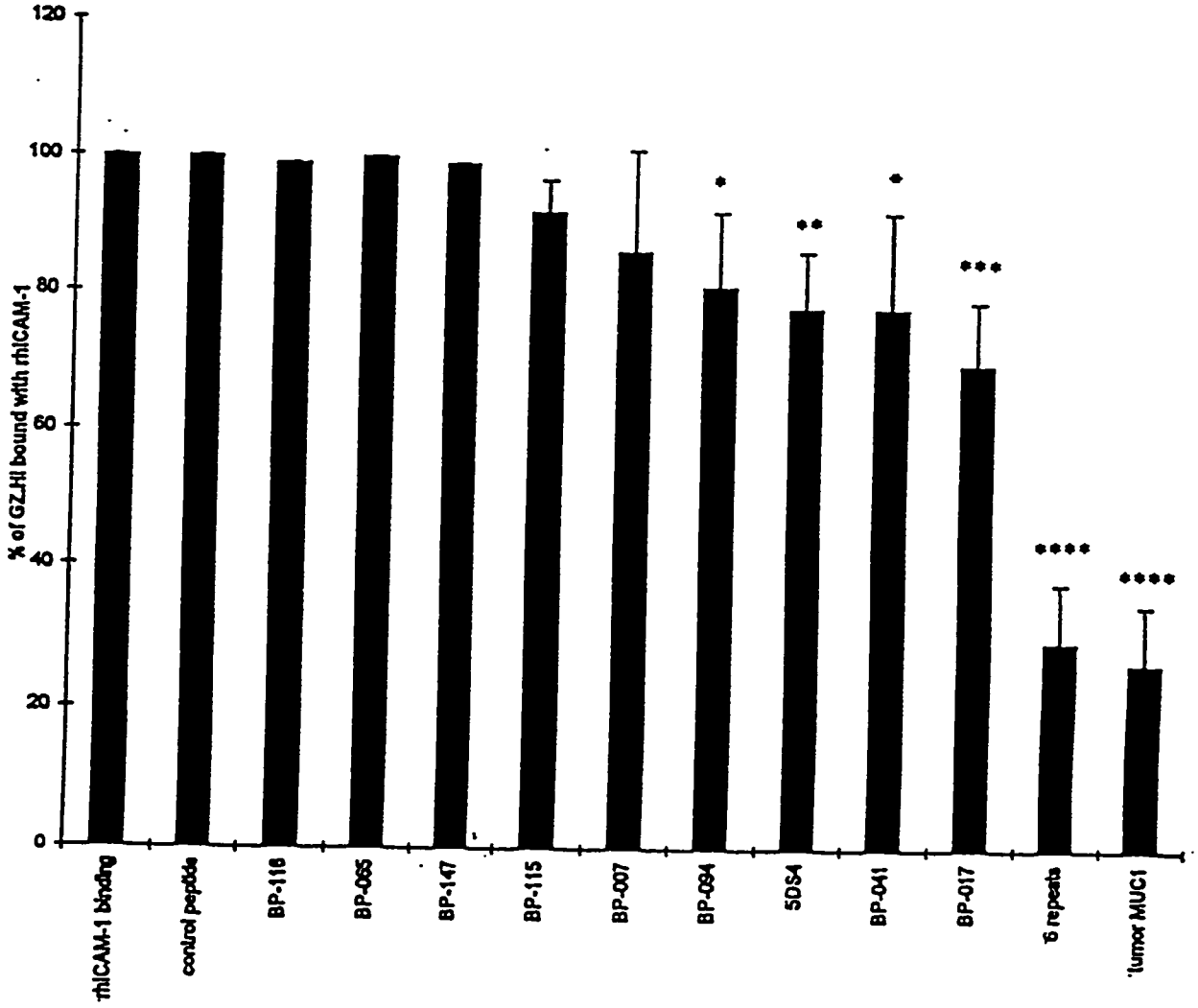


Figure 23. MUC1 synthetic peptides inhibition on the binding of rhICAM-1 to MUC1 on GZ.Hi cells. The data were subjected to a two tailed t-test (Dunnett's test is from Release 6.12, 1989-1996 by SAS Institute Inc.). Dunnett's test tests if any treatments are significantly different from a single control. * , $\alpha = 0.05$; ** , $\alpha = 0.01$; ***, $\alpha = 0.001$; ****, $\alpha = 0.0001$. (See Figure 22 for legend and Table 1 for peptide sequence).

2.6 Antigenicity of MUC1 synthetic peptides

Objective:

ELISA was used to verify the antigenicity of the MUC1 synthetic peptides, which did not inhibit the rhICAM-1 and MUC1 binding.

Materials and Methods:

mAbs

VU4H5 (MUC1 synthetic peptide specific) Ab, which recognizes the **PDTR** sequence, was a gift from Dr. Hilgers. B27.29 Ab is specific for **SAPDTRPA** epitope, and was donated by Biomira Inc.. Goat anti mouse conjugated with horseradish peroxidase was donated by Dr. Carol Cass, Cross Cancer Institute.

MUC1 synthetic peptides

Peptide 5DS4 (20 aa) from Dr. Hilgers and BP-065 (24 aa) from Biomira Inc. were used for ELISA, and BP1-087 (24 aa), a HLA sequenced peptide was used as a control (Table 1).

ELISA (Figure 24):

Microtiter plates (Falcon) were coated overnight at 4°C with the peptides (100 ul/well, 2.5 ug/ml in PBS). The plates were aspirated and washed twice with PBS , and blocked with 1% BSA (Sigma) in PBS for 1 hr at 37°C. After washing twice with PBS, the plates were incubated with anti-MUC1 mAb (B27.29) or MUC1 peptide specific Ab (VU4H5) (100

ul/well at 10 ug/ml in PBS) overnight at 4°C. After washing six times, peroxidase conjugated goat anti mouse Ab at 1:20000 in PBS was added to the plates (100 ul/well), and incubated for 1 hr at 37°C. Subsequently, the plates were washed six times. Staining was developed by adding 100 ul of TMB mixed with 2 ul of 30% hydrogen peroxide. The TMB was prepared by dissolving 1 mg of 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB) (Sigma) in 10 ml 0.05 M phosphate-citrate buffer at a pH of 5.0. The reaction was developed for 15 min in the dark, and was stopped with 25 ul of 2M H_2SO_4. Five min later, the optical density (O.D.) was measured at 450nm in an ELISA plate reader.

Results:

BP-065 peptide was almost three times as reactive as 5DS4 peptide based on the O.D. value, which is directly proportional to the amount of MUC1 synthetic peptides recognized by the peptide specific Ab, VU4H5 (Figure 25). The O.D. value of the control peptide, BP1-087, was equivalent to the "no primary Ab" control. The low O.D value of the 5DS4 peptide by VU4H5 (Figure 25) is similar to that obtained by the Hilgers lab (Dr. D. Schol, personal communication) due to the terminal location of the immunogenic epitope (see Table 1).

Using the MUC1 Ab B27.29 (Figure 26), only the BP-065 peptide was detected at low but statistically significant levels. However, the reactivity profiles of the two peptides are now comparable in contrast to that seen with the VU4H5 Ab. This pattern of recognition is similar to the inhibition profile (Figure 23), perhaps indicating that ICAM-1 and B27.29 may have similar requirements for MUC1 ligation.

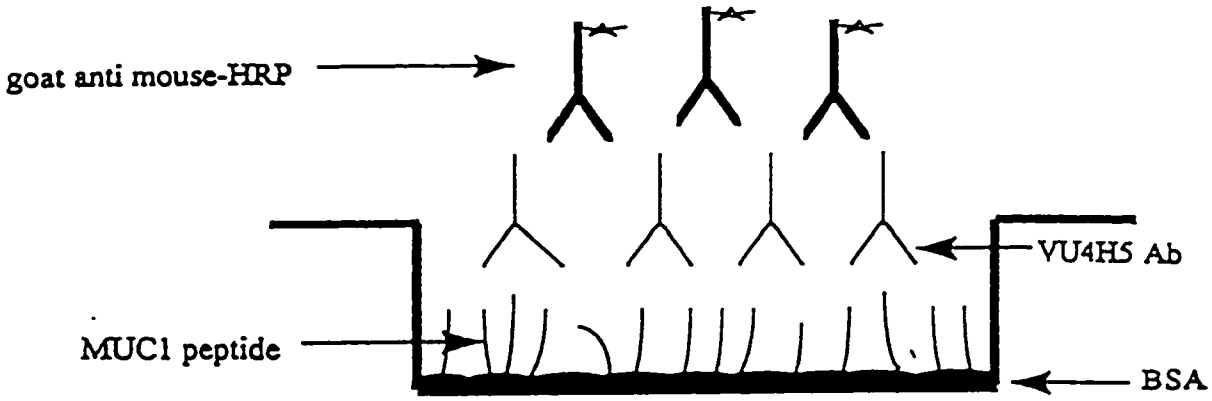


Figure 24. ELISA. Wells were coated with MUC1 peptides, and blocked with BSA. The primary Ab was a monoclonal mouse Ab specific for MUC1 , and this was followed by a secondary Ab of goat anti mouse conjugated with HRP. The presence of bound secondary Ab was detected by a colorimetric reaction and quantitated.

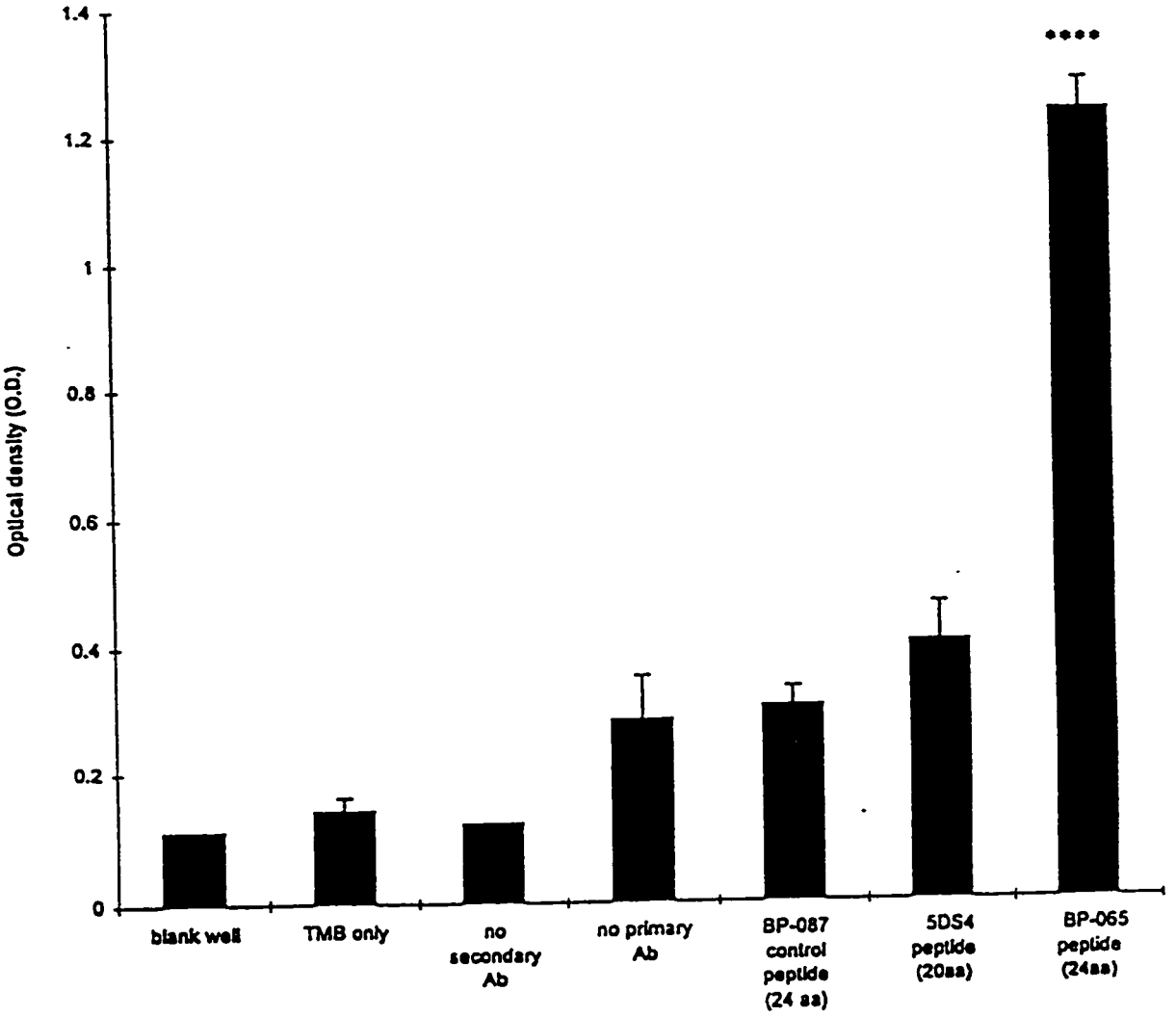


Figure 25. Using ELISA to test the antigenicity of MUC1 synthetic peptides, which did not inhibit rhICAM-1 and MUC1 adhesion. VU4H5 (peptide specific) was used as a primary Ab. BP-065 peptide from Biomira Inc. was detected by VU4H5 efficiently but 5DS4 from Dr. Hilgers showed a minimal detection due to the terminal location of the immunogenic epitope. The data were subjected to a two tailed t-test (Dunnett's test is from Release 6.12, 1989-1996 by SAS Institute Inc.). Dunnett's test tests if any treatments are significantly different from a single control. * , $\alpha = 0.05$; ** , $\alpha = 0.01$; ***, $\alpha = 0.001$; ****, $\alpha = 0.0001$.

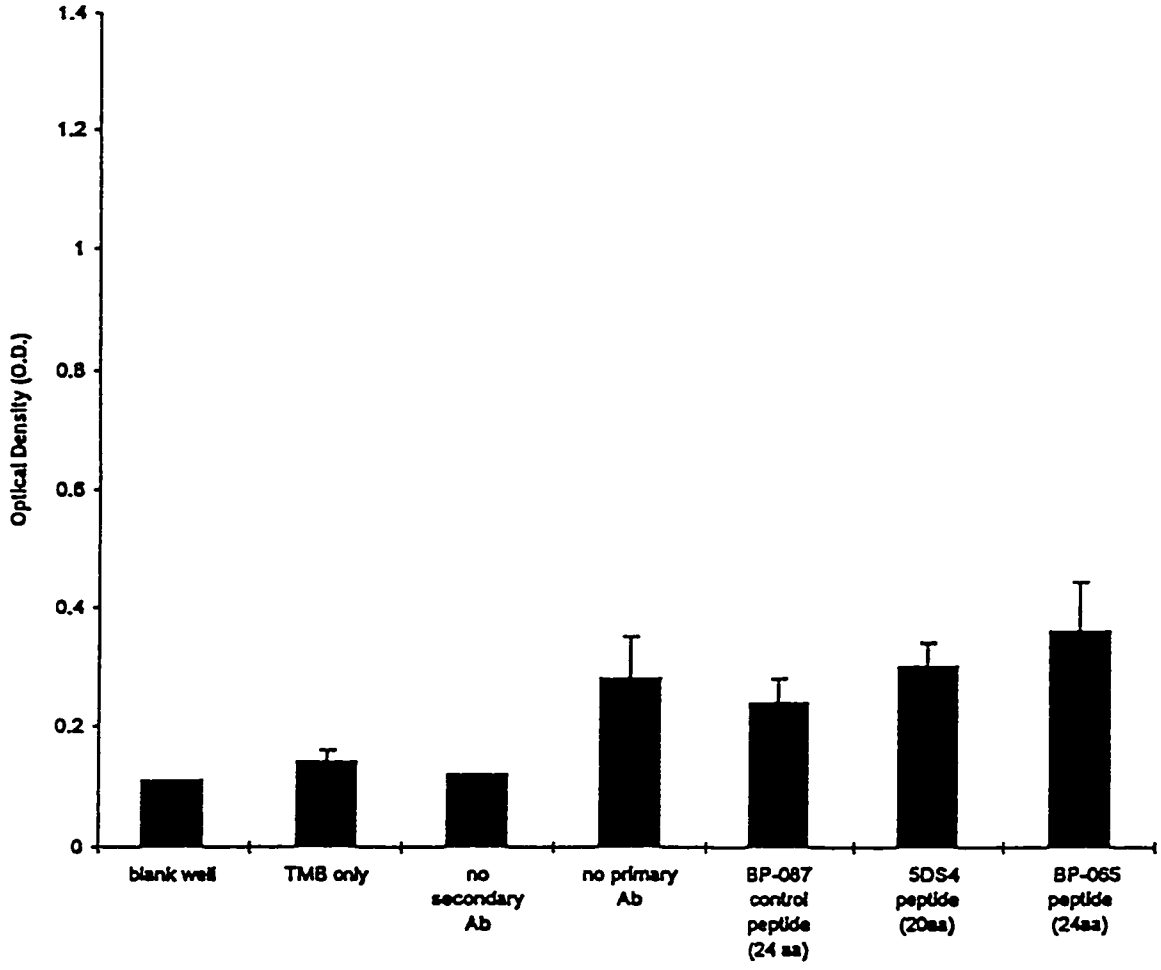


Figure 26. Using ELISA to test the antigenicity of MUC1 synthetic peptides, which did not inhibit rhICAM-1 and MUC1 adhesion. B27.29 (anti-MUC1) was used as a primary Ab. Both BP-065 and 5DS4 showed a low level of detection.

2.7 Summary of 2.4 - 2.6

1) Anti-MUC1 Ab (B27.29) and native tumor-derived MUC1 specifically blocked rhICAM-1 and MUC1 adhesion.
2) The 120 aa (6 repeats) MUC1 synthetic peptide had the same inhibitory effect as tumor MUC1 but synthetic peptides less than or equal to 24 aa in excess molar concentration could not accomplish the same inhibition.
3) The antigenicity of short peptides, which showed no inhibitory effect on rhICAM-1 and MUC1 binding, was shown by a MUC1 peptide specific Ab, VU4H5 using ELISA to ascertain the peptide sequence.
4) B27.29 Ab did not detect the short MUC1 peptides as efficiently as VU4H5.

2.8 rhICAM-3 binding to GZ.Hi

Objective:

To investigate the adhesion of rhICAM-3 and membrane-bound MUC1 on GZ.Hi cells.

Materials and Methods:

mAb*s* and protein

monoclonal anti-ICAM3 Ab (ICR 3-7, 9) as a primary Ab, and rhICAM-3 protein were donated by ICOS Co.. Goat anti mouse conjugated with RPE was used as a secondary Ab. Anti-ICAM-1 (18E3D) and bovine collagen were controls for ICR Ab*s* and rhICAM-3 respectively.

Cell and Reagents

GZ.Hi was used and maintained in the same medium as section 2.1.

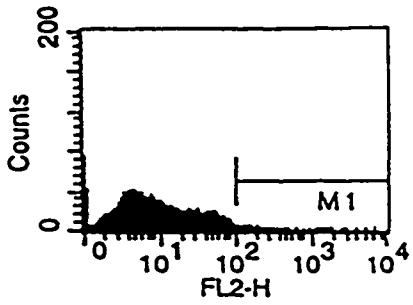
Indirect Binding Assay

The same protocol in section 2.2 was followed (Figure 10). rhICAM-3 was used at the same concentration as rhICAM-1 (200 ul of 70 ug/ml). rhICAM-3 bound to GZ.Hi cells was detected by the primary Ab ICR (anti-ICAM-3) at 50 ul/test, 1/500 dilution of 1mg/ml, followed by the secondary Ab goat anti mouse conjugated with RPE at 50 ul/test, 1/250 dilution of 1mg/ml. The control protein collagen and 18E3D (anti-ICAM-1) were used at a concentration equivalent to the test.

Results:

The GZ.Hi cells were phenotyped for baseline expression of ICAM-3 using a panel of anti-ICAM-3 Ab. Representative histograms are shown in Figure 27, and summarized in Figure 28. ICAM-3 expression on GZ.Hi cells was undetectable by all ICR (anti-ICAM-3) Abs, equivalent to the anti P-selectin control.

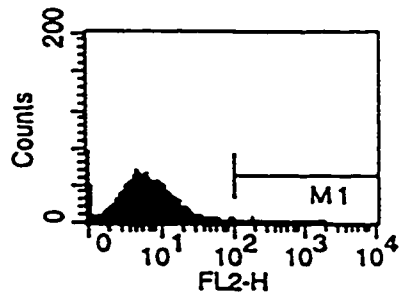
MUC1 expression on the surface of GZ.Hi cells was confirmed and quantitated for each experiment. After co-incubation with rhICAM-3, there was no rhICAM-3 bound to the cells detected by any of the anti ICAM-3 Abs (ICR) (Figure 27), comparable to the collagen and 18E3D controls. Representative histograms are presented in Figure 29 and summarized in Figure 30. These results indicate that rhICAM-3 does not bind to human MUC1 as does rhICAM-1.



a) anti P-selectin control

Total Events: 10000

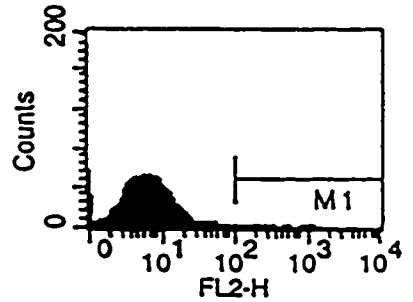
Events	% Total	Mean	Peak Ch
10000	100.00	50.47	1
383	3.83	951.36	104



b) ICR-2 Ab

Total Events: 10000

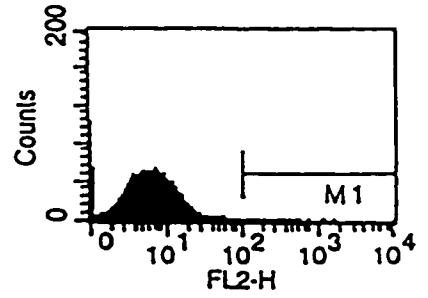
Events	% Total	Mean	Peak Ch
10000	100.00	21.70	1
260	2.60	494.60	100



c) ICR-3 Ab

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	20.45	1
170	1.70	740.84	162

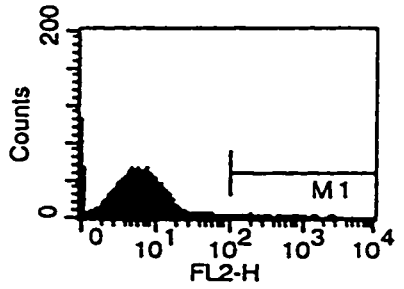


d) ICR-4 Ab

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	20.66	1
209	2.09	603.46	98

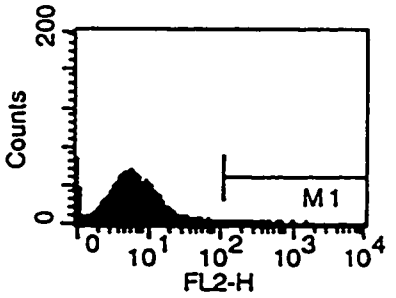
Figure 27. Flow cytometric analysis for ICAM-3 expression on GZ.Hi cells. GZ.Hi cells were stained with a panel of anti-ICAM-3 Ab*s* (b-h). The gate was established using exclusion of an irrelevant Ab (a). The test histograms (b-h) show a uniform uni-modal negative population indicating an absence of endogenous ICAM-3.



c) ICR-5 Ab

Total Events: 10000

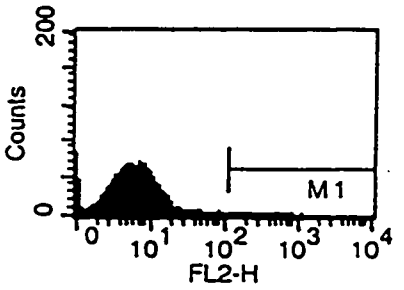
Events	% Total	Mean	Peak Ch
10000	100.00	22.07	1
259	2.59	533.67	182



f) ICR-6 Ab

Total Events: 10000

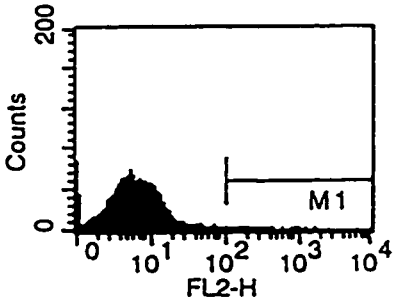
Events	% Total	Mean	Peak Ch
10000	100.00	18.10	1
153	1.53	643.04	139



g) ICR-7 Ab

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	16.82	1
188	1.88	474.73	109



h) ICR-9 Ab

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	16.40	1
142	1.42	595.05	112

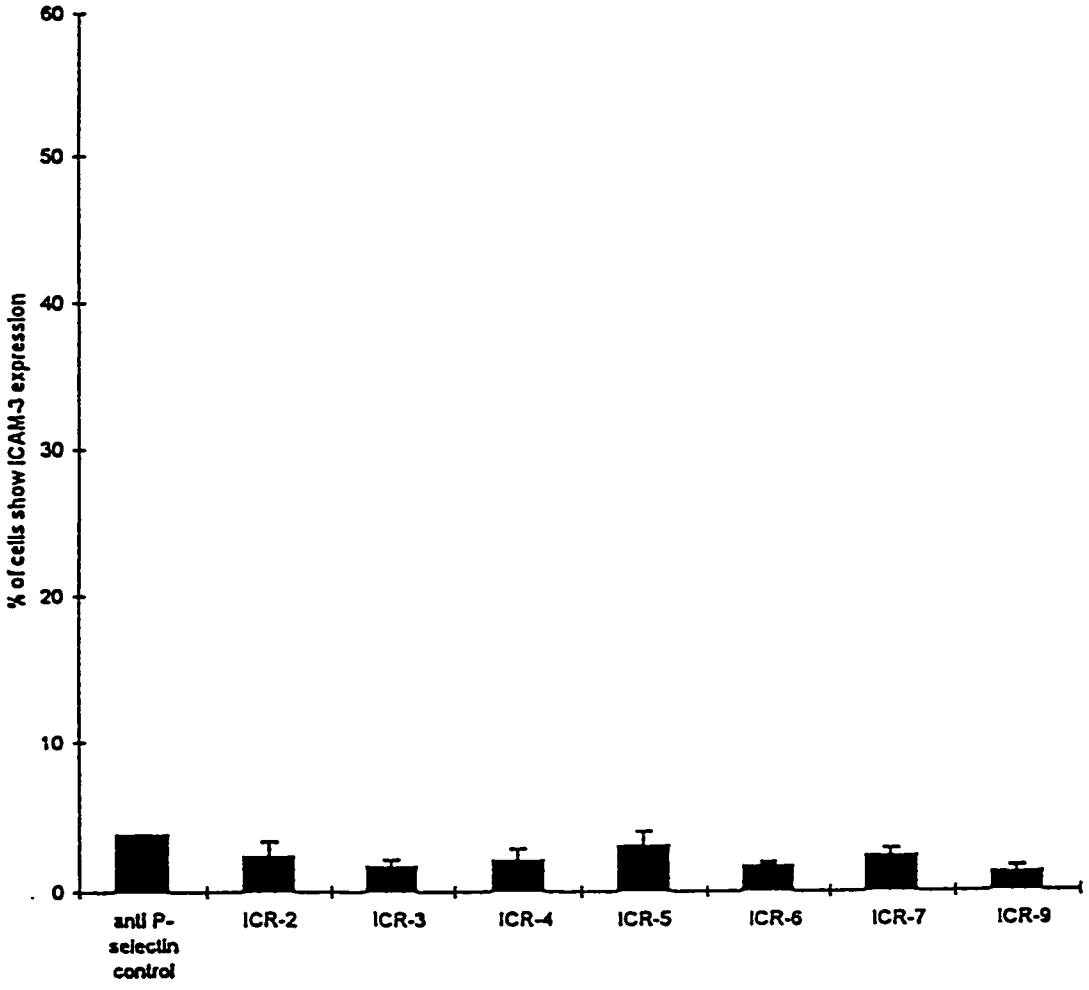
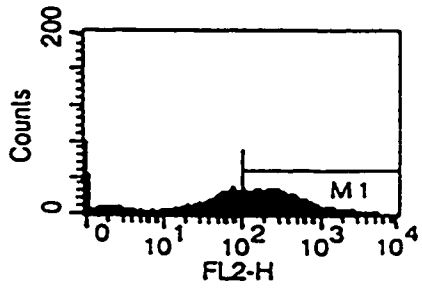


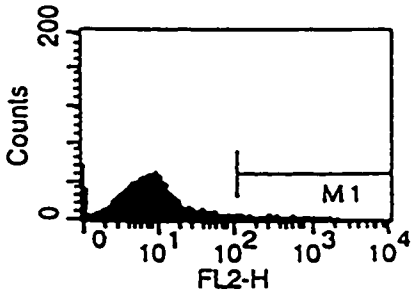
Figure 28. ICAM-3 expression of GZ.Hi cells. No ICAM-3 was expressed by the cells when stained with a panel of anti-ICAM-3 Abs (ICR).



a) human MUC1 expression on GZ.Hi cells

Total Events: 10000

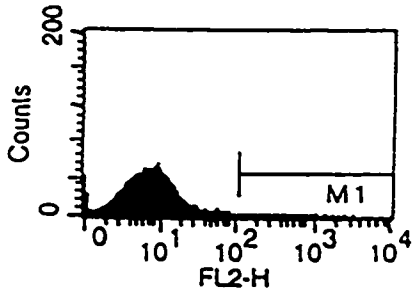
Events	% Total	Mean	Peak Ch
10000	100.00	271.17	1
5371	53.71	468.64	163



b) collagen control

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	30.39	1
381	3.81	523.82	110

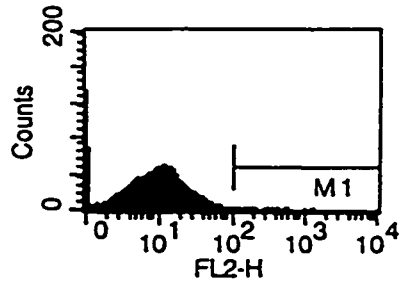


c)18E3D control

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	34.22	1
253	2.53	973.82	220

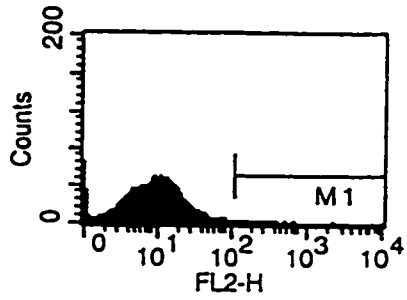
Figure 29. Flow cytometric analysis of the rhICAM-3 binding to GZ.Hi cells. Bound rhICAM-3 was detected by a panel of anti-ICAM-3 Abs (ICR). In each series of tests, the MUC1 expression of the GZ.Hi cells is confirmed using B27.29 (a). Control tests substituting an irrelevant protein, collagen (b) or irrelevant Ab (c) show a low level of background. A panel of anti-ICAM-3 Ab (d-j) shows a similar unimodal negative population as the control, indicating the absence of bound ICAM-3. Results are summarized in Figure 30.



d) ICR-2 Ab

Total Events: 10000

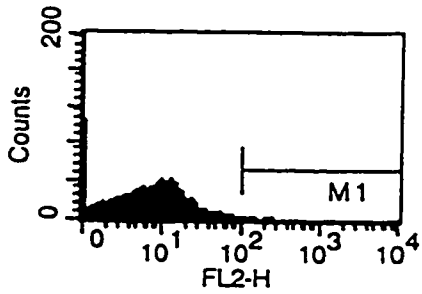
Events	% Total	Mean	Peak Ch
10000	100.00	22.25	1
155	1.55	644.64	108



c) ICR-3 Ab

Total Events: 10000

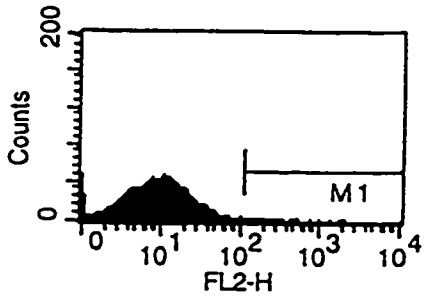
Events	% Total	Mean	Peak Ch
10000	100.00	26.59	1
148	1.48	1026.29	9910



f) ICR-4 Ab

Total Events: 10000

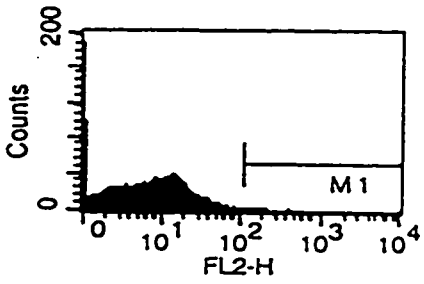
Events	% Total	Mean	Peak Ch
10000	100.00	13.33	1
68	0.68	348.77	104



g) ICR-5 Ab

Total Events: 10000

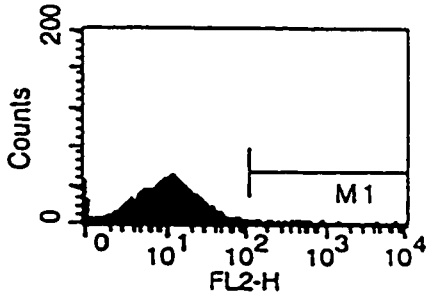
Events	% Total	Mean	Peak Ch
10000	100.00	24.73	1
179	1.79	682.50	179



h) ICR-6 Ab

Total Events: 10000

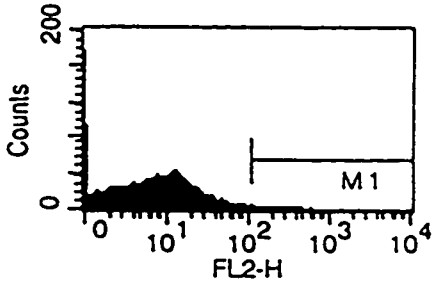
Events	% Total	Mean	Peak Ch
10000	100.00	12.80	1
64	0.64	338.35	109



i) ICR-7 Ab

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	33.98	1
145	1.45	1424.45	9910



j) ICR-9 Ab

Total Events: 10000

Events	% Total	Mean	Peak Ch
10000	100.00	17.54	1
186	1.86	325.80	121

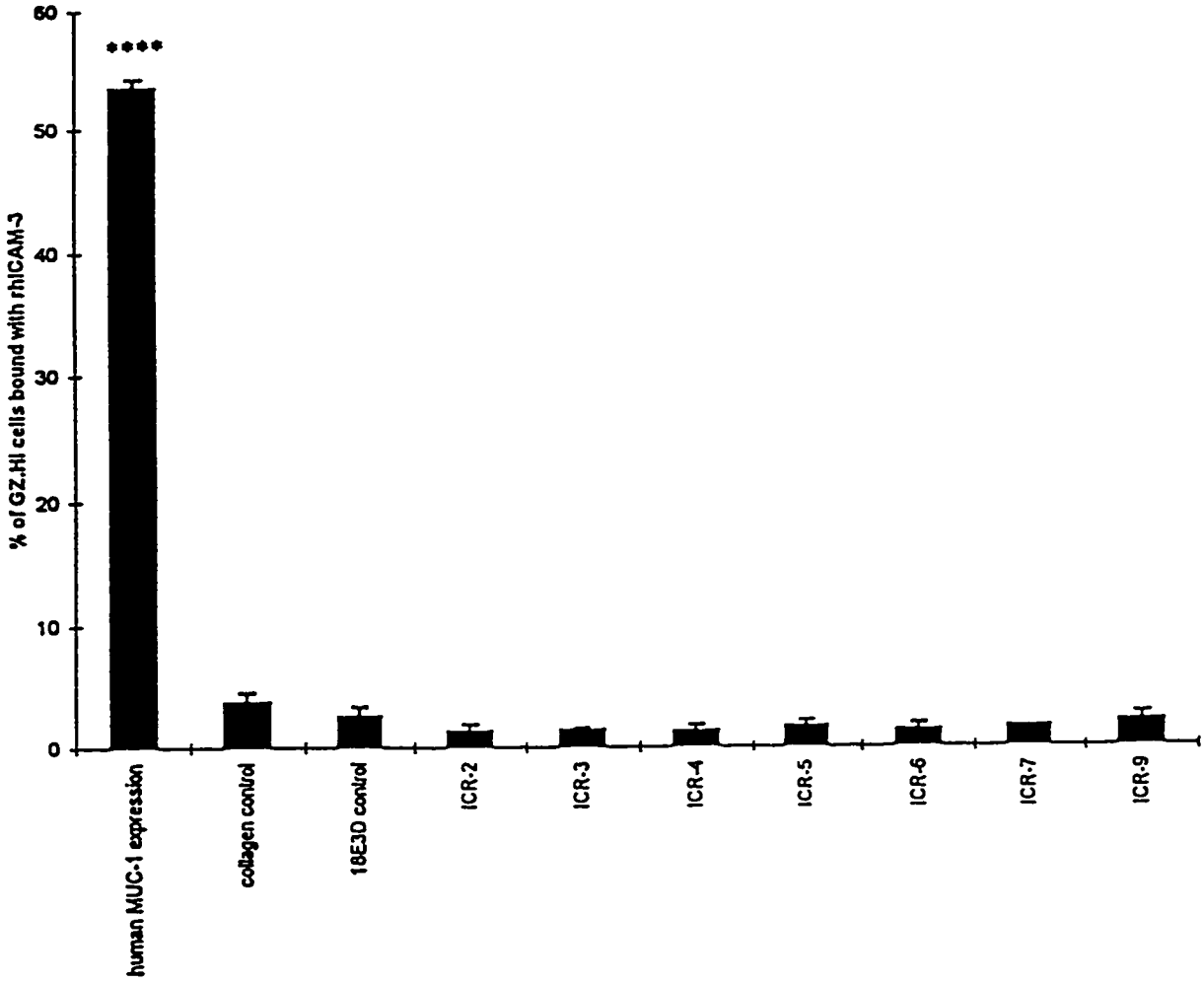


Figure 30. No rhICAM-3 binding on GZ.Hi cells. No bound rhICAM-3 to GZ.Hi cells was observed, when compared to the collagen and 18E3D controls. The data were subjected to a two tailed t-test (Dunnett's test is from Release 6.12, 1989-1996 by SAS Institute Inc.). Dunnett's test tests if any treatments are significantly different from a single control. * , $\alpha = 0.05$; ** , $\alpha = 0.01$; ***, $\alpha = 0.001$; ****, $\alpha = 0.0001$.

Chapter 3

DISCUSSION AND CONCLUSION

3.1 Introduction

Tumor metastasis is a major cause of morbidity in cancer patients. The metastatic process requires a number of adhesive interactions between the tumor cells and the host extracellular matrix and endothelial cells. In breast cancer, the underglycosylated MUC1 molecule has been demonstrated to be a novel ligand for ICAM-1 (1), perhaps providing a mechanism for the tumor cells to metastasize. MUC1 synthetic peptides could be a possible agent to control the metastatic activity of cancer cells, without interfering with the normal immune functions. This thesis examines the ability of MUC1 synthetic peptide to inhibit ICAM-1 and MUC1 adhesion.

3.2 Review and Discussion of Experimental Data

As demonstrated in section 2.1, there is no detectable level of the known ICAM-1 counter-receptors such as CD43, LFA-1 and Mac-1 on either the 410.4 or GZ.Hi cells (Figure 8). Endogenous ICAM-1 expression is also absent. The only ligand for ICAM-1 is human MUC1 on GZ.Hi, which is a human MUC1 transfectant sub-clone of 410.4. In section 2.2, 410.4 cells which do not express human MUC1 showed undetectable levels of adhesion to dimeric rhICAM-1 (Figure 12). However, the antibiotic selected GZ.Hi cells which show a consistent elevation of human MUC1, displayed a high level of adhesion to rhICAM-1 (Figure 14). The possibility that rhICAM-1 is binding to an uncharacterized or cross reacting ICAM-1 ligand of mouse origin is excluded by the use of the parental cell line 410.4. In order to rule out the possibility that the human immunoglobulin portion of rhICAM-1 is adhering to the GZ.Hi cells non-specifically, human IgG conjugated with

FITC was used for testing the non-specific binding to 410.4 and GZ.Hi cells. Figures 12 and 14 show that both cell types have no hIgG-FITC binding.

As described in section 2.3, the specificity of the rhICAM-1 and MUC1 binding is confirmed by inhibiting the adhesion with an anti-MUC1 Ab (B27.29) and tumor-associated MUC1. Figure 17 shows that both B27.29 and tumor MUC1 significantly reduce the binding to levels equivalent to the control protein 164B-FITC. This experiment confirmed that in the indirect binding assay, rhICAM-1 is recognizing human MUC1 on the surface of GZ.Hi cells.

Before we tested the inhibitory effect of MUC1 synthetic peptides, titrations were performed to determine the optimal concentration for peptide inhibition. As seen in figures 16 and 21, the relationship between increasing amounts of soluble tumor MUC1 or the 120 amino acid MUC1 peptide and the amount of MUC-1 - ICAM-1 binding is inversely proportional. This indicates that pre-incubation of rhICAM1 with MUC1 or a synthetic peptide decreases subsequent rhICAM-1 binding in a dose-dependent fashion. In figures 19 and 20, titrations show that increasing the concentration of a control peptide (BP1-087) or a 24 aa MUC1 synthetic peptide (BP-065) did not affect the rhICAM-1 - MUC1 binding at any of the concentrations tested. Subsequently, all MUC1 synthetic peptides ranging from 9 to 24 aa were used at a constant concentration, 740 uM/test which was ten times as much as the effective inhibitory concentration of the 120 aa MUC1 peptide (6DS5). A few of the short peptides displayed a low-level inhibition (Table 1). But only

the 120 aa peptide (6DS5) greatly reduced the rhICAM-1 and MUC1 adhesion, having the same inhibitory effect as B27.29 Ab and tumor MUC1.

In order to rule out the possibility of inaccurate peptide synthesis, which might be responsible for the failure of the short peptides to inhibit rhICAM-1 and MUC1 binding, we chose two short MUC1 peptides : BP-065 and 5DS4 synthesized in different laboratories, and verified their antigenicity by ELISA as described in section 2.6. Both peptides contain the antigenic epitope **PDTR**, which is recognized by the peptide specific VU4H5 Ab. Both peptides were detected by the Ab. Results in figures 25 and 26 are expressed in optical density (O.D.), which is directly proportional to the reactivity of the VU4H5 and B27.29 Abs to the peptides. VU4H5 shows a strong reaction to the BP-065 MUC1 peptides whereas B27.29 shows only a low level of reactivity with this peptide. This suggests that both B27.29 and ICAM-1 may recognize an epitope requiring several repeats (74). The difference in O.D. between the two MUC1 peptides detected by VU4H5 in figure 25 can be explained by the location of the **PDTR** epitope in the peptides. The epitope at the beginning of 5DS4 peptide could minimize the binding of the peptide specific Ab, while an epitope located in the middle of BP-065 is ideal for Ab recognition.

For peptides equal to or less than 24 amino acids, there was no correlation between the length of the peptide sequences and the low-level of inhibition (Table 1). When the sequence of the peptides is compared to the inhibition data, those peptides which exhibited moderate levels of inhibition eg. BP-017, 041, 094, 007 and 5DS4, all share a common epitope (**SAPDTR**) (Table 1). Whereas BP-115 peptide, which contains **DTRPA**

sequence, had a minor level of inhibition. The inhibition results of the short peptides suggest that **DTR** is the minimum sequence necessary to inhibit rhICAM-1 binding, and higher level of inhibition could be achieved with a preceding **SAP** sequence. Interestingly, the 24 mer peptide (BP-065) which contains the **SAPDTR** epitope behind a redundant **TAPP** sequence showed no inhibitory effect (Table 1).

It is possible that the absence of an inhibitory effect of the short peptides and the efficacy of the 6 repeat peptide is due to their structural conformation. It has been previously demonstrated that the tertiary structure of MUC1 is dependent on the number of tandem repeats. Peptides of one repeat have a random coil structure in solution, but peptides with three repeats or more have a stable, ordered, rod-shaped conformation (68). Superimposed on the rod-shaped structure, peptides with greater than or equal to three tandem repeats possess a knob-like structure in each repeat protruding from the peptide backbone. The antigenic site (**PADTR**) is located at the tip of the knobs (67). In breast cancer cells, the antigenic knob is exposed due to underglycosylation of MUC1 mucin (69).

In section 2.4, B27.29 efficiently blocks rhICAM-1 and MUC1 adhesion by binding to the membrane-bound MUC1 on GZ.HI cells. But B27.29 was very inefficient at detecting short peptide sequences by ELISA (Figure 26). It is possible that B27.29 also requires a tertiary structure on MUC1 for binding. These findings support the interpretation that both ICAM-1 and B27.29 anti-MUC1 Ab recognition of MUC1 are dependent on the number of tandem repeats of the MUC1 protein.

There are several other lines of evidence to support the importance of MUC1 tertiary conformation in molecule recognition. It has been proposed that the tertiary conformation of MUC1 presents this epitope for T-cell receptor recognition (12). The recent description of the induction of T-cell anergy by cancer-associated MUC1 also demonstrates that the six tandem repeats peptide has the same inhibitory effect as tumor-associated MUC1 on T-cell proliferation (2). Peptides less than or equal to one tandem repeat do not reduce T-cell proliferation, indicating that the induction of T-cell anergy is directly proportional to the number of tandem repeats present in the peptides. The reactivity of anti-MUC1 antibodies from breast cancer patients (70) is also dependent on the length of tandem repeats in synthetic peptides. It is of interest that the most common formulation of MUC1 peptides and in cancer vaccines to generate an anti-MUC1 response is 5 repeats in length (28). It is highly likely that in these situations, there is also a conformational requirement for MUC1 as a functional immunogen.

The rhICAM-1 used in this thesis is present in a dimeric form, which mimics the physiological ICAM-1. The LFA-1 and ICAM-1 interaction shows a higher affinity with an ICAM-1 dimer than a monomer (60). It is possible that the ICAM-1-MUC1 recognition is also dimeric dependent. This is currently being examined in our lab.

ICAM-3 is a related immunoglobulin family adhesion molecule which shares some homology in the extracellular domains with ICAM-1. It is not expressed on normal

endothelium but increases on tumor endothelium (65), and therefore it is possible that ICAM-3 may bind to tumor MUC1, providing another mechanism for breast cancer metastasis. Hence, in the experiments outlined in section 2.8, the adhesion between rhICAM-3 and MUC1 was examined. The GZ.Hi cells were first phenotyped and shown to be negative for endogenous ICAM-3 expression using a number of anti-ICAM-3 (ICR*s*). Figure 28 shows that, unlike rhICAM-1, rhICAM-3 did not bind to any cell surface molecules on GZ.Hi cells. The sequences and structural similarity between ICAM-1 and -3 resides primarily in domains two and five. Previous work (1) has shown that anti-ICAM-1 Ab*s*, which block the LFA-1 binding site on ICAM-1 are poor inhibitors of MUC1-ICAM-1 adhesion. It follows from these observations that MUC1 binds to ICAM-1 through a molecular binding site different from that recognized by LFA-1 (75).

3.3 Conclusion :

The results presented in this thesis demonstrate that a 120 amino acid MUC1 synthetic peptide can block the ICAM-1 and MUC1 binding, whereas peptides less than or equal to 1 repeat (20 amino acids) cannot accomplish a similar inhibition. As the number of tandem repeats determines the tertiary structure of the MUC1 peptide, it is possible that a conformational peptide is important for inhibiting ICAM-1 and MUC1 binding. Studies of the MHC unrestricted recognition of native MUC1, as well as tumor MUC1 induction of T cell anergy (2) also show the significance of the number of tandem repeats in MUC1 immunomodulatory effects (68) . This suggests that MUC1-ICAM-1 binding may also underlie these phenomena. As such, a conformationally appropriate MUC1 peptide offers a potential strategy for immunotherapy or anti-metastatic therapy.

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IMAGE EVALUATION
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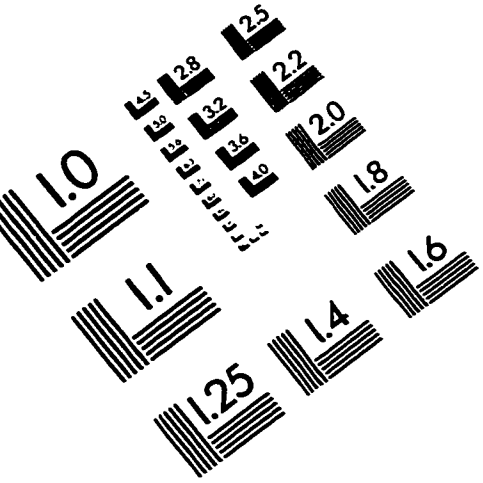

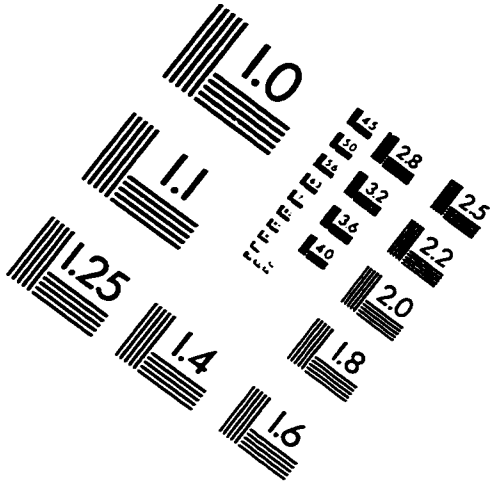

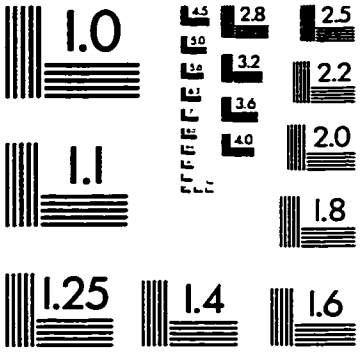

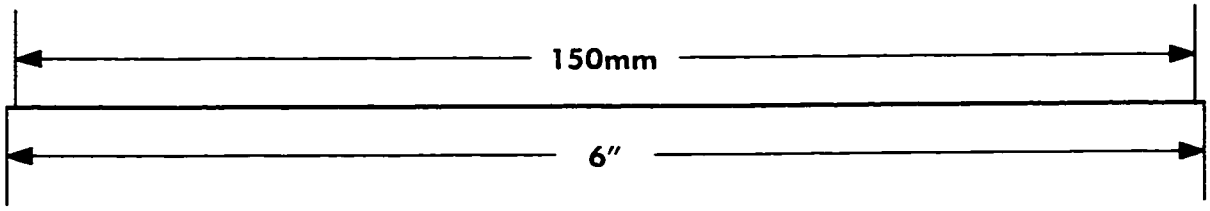

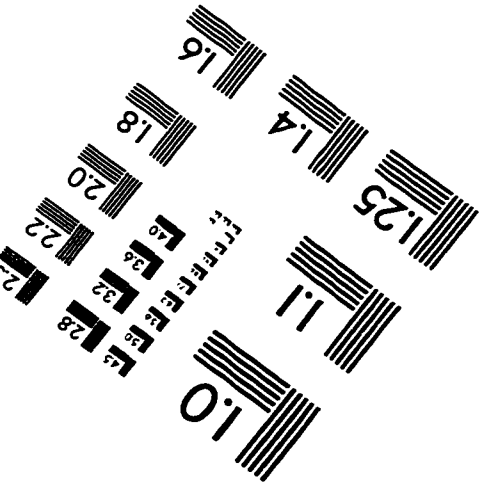

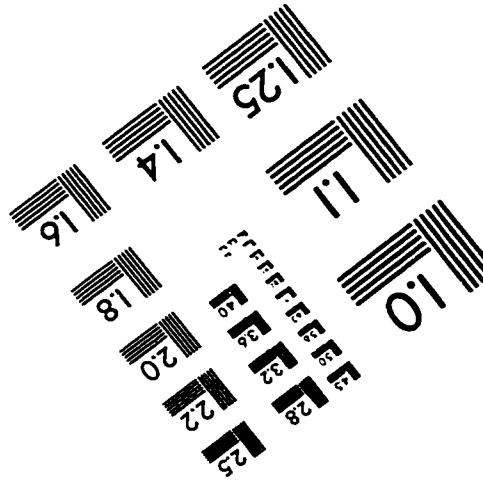

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