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13. ABSTRACT *(Maximum 200 words)*

The objectives of the research program were to study the structure-immunogenicity relationships of a hypoglycosylated human tumor-specific mucin common to breast and other adenocarcinomas. Hypo-glycosylation of breast mucin leads to exposure of a tumor-specific epitope (TSE). The structural and immunogenic properties of the TSE were examined using synthetic mucin peptides and recombinant mucin proteins that contain the TSE and/or mutations in potential glycosylation sites surrounding the TSE. One TSE is the pentapeptide PDTRP of the 20 amino acid mucin tandem repeat sequence PDTRPAPGSTAPPAHGVTSA. Immunogenic cross-reactivity with tumor-specific monoclonal antibodies show that antigenicity is maximized with the 40 amino acid MUC1-mtr$_2$. By contrast, the MUC1-mtr$_3$ elicited a 9-fold better cellular response compared to either single or double tandem repeat peptides. The single, double and triple tandem repeat peptides show few elements of preferred conformation, based on ^{1}H-NMR spectroscopy. A model of the distribution of the oligosaccharide side chains along the mucin core protein was developed showing that the carbohydrates may not surround the protein in a uniform "coating". Thus, regions of the core protein are exposed under normal circumstances, yet tumor-specific epitopes remain masked by the sugars in the non-malignant cells.

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FOREWORD

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Table of Contents

Front Cover 1

SF 298 2

Foreword 3

Table of Contents 4

Introduction 5

Body 7

Conclusions 29

References 31

Appendix 33

Introduction. Immunotherapy is a state-of-the-art approach to augment existing cancer therapies of radiation, chemotherapy and surgery. Immunotherapy takes advantage of the specificity of the immune system to recognize and rid the body of tumor cells. Immune cells have been identified which recognize and kill tumor cells in a specific manner [e.g. tumor-infiltrating lymphocytes (TIL); (1-3), as well as in a non-specific manner [e.g. lymphokine activated killer (LAK) cells and natural killer (NK) cells]. Pertinent to this research program, T-cells (4;5) and B-cells (6) specific for breast, ovarian and pancreatic adenocarcinomas have been described. These observations demonstrate that the body does mount an immune response against several adenocarcinomas.

Tumor proteins or tumor antigens associated with the development or expression of the malignant cell often render these cells immunogenic. Pertinent to this proposal is normal self-proteins that are aberrantly expressed or post-translationally modified on the tumor cells compared to the normal cells. Polymorphic epithelial mucin [(7); mucin for this report] is a normal protein that is aberrantly expressed on the tumor cells. As such, mucin becomes a tumor-specific protein.

Mucin is the major glycoprotein of mucous secretions and is normally confined to the luminal surface of the glandular epithelial cells (7;8). The core protein is heavily glycosylated with carbohydrate accounting for up to 80% of the glycoprotein mass (7;8); the sugars O-linked to the protein through serine and threonine (7). The entire mucin core protein has been cloned and sequenced (7). To date, at least 12 different mucin proteins have been described. The hallmark of mucin is tandem repeating sequences. MUC1 is the mucin associated with breast, ovarian, pancreatic and several other adenocarcinomas. MUC1 has the 20 amino acid repeating sequence of P^1DTRPAPGST^{10}APPAHGVTSA^{20} (MUC1-mucin tandem repeat; i.e., MUC1-mtr$_1$). Mucins are structurally polymorphic and generally contain between 40-100 tandem repeat sequences.

The structure of the MUC1-mtr$_n$, where n=1-3, has been partially characterized based on NMR, hydropathicity and structure prediction calculations (9-11). Viscosity measurements suggested that the structure is rod-shaped (10). Residues P^1DTRP^5 are suggested to be poly-proline β-turn elements described as "knob-like" protrusions (10-12). It is the poly-proline β-turn regions of the core protein which are suggested to be within the tumor-specific epitope (7). This model is intriguing in that the positions of all of the O-linked glycosylation sites surround the tumor-specific epitope, which is contained in the β-turn regions. Thus, glycosylation of any, or all, of these residues may result in altered immunogenicity of the tumor-specific epitope.

The extensive branching of the carbohydrate side chains of the mucin glycoprotein inhibits the tumor-specific antigenicity and immunogenicity of mucin. Cancer-associated mucins are aberrantly glycosylated in that the carbohydrate side chains are shorter than those of the mucin produced by normal cells (7). These mucins have unique antigenic epitopes exposed on the core protein that are masked in the fully glycosylated form (7). Other non-tumor-specific epitopes are expressed in both the normal and tumor-associated mucins (7). The presence of tumor-specific epitopes is evidenced by the development of many monoclonal antibodies (mAb) that recognize tumor-specific mucins and apo-mucin, but not the fully glycosylated molecule (7). The epitope recognized by a representative mAb, SM3, is the sequence P^1-P^5 in the tandem repeat sequence (7). This epitope was identified by competition of antibody binding to tumor-specific mucin by synthetic peptides of the mtr (7).

Human cytotoxic T lymphocyte (CTL) cell lines from breast (4), ovarian (5), and pancreatic (4) adenocarcinomas have also been described which recognize mucin-expressing cell lines. One cell line, WD, was derived from the tumor-draining lymph node cells from a

patient with pancreatic adenocarcinoma by continuously stimulating the cells with allogeneic pancreatic tumor cell lines. WD has a $CD3^+$, $CD4^-$, $CD8^+$ phenotype. Pancreatic mtr sequences are identical to the breast mtr (7). This explains why CTL generated against pancreatic carcinoma recognize breast carcinoma (4). The WD CTL did not recognize colon cancer cell lines, as expected, since the primary sequence of colon mucin expressed by cell lines differs from the sequence of the breast mucin peptide (7). These results show specificity of the immune recognition. Also, immunohistochemical staining and Northern blot analysis of pancreatic and breast tumor cell lines with SM3 showed that the cell lines lysed by WD CTL express mucin epitope recognized by the monoclonal antibody (4). Fresh colon cancer tissue expressed the same mucin as breast and pancreatic cancers, i.e. MUC1 (13). The colon cancer cell lines do not express MUC1, but express other genes of the mucin family (MUC3 & MUC4). In addition, renal, gastric, lung and ovarian cells, express MUC1 (7;13). This supports the hypothesis that mucin is a common tumor antigen on multiple adenocarcinomas.

Target cell recognition by these CTL lines is Human Leukocyte Antigen (HLA)-non-restricted. This was shown by the ability of mucin-specific CTL cell lines to recognize and kill non-HLA-matched tumor target cell lines. One explanation for this is that mucin, due to its multiple tandem repeat sequences, is a multivalent antigen. Multiple epitopes on the tumor cell surface not associated with HLA are postulated to bind to, and cross-link, the T cell receptor (TCR), thus leading to CTL activation and target killing (4,14,15). Another explanation for the lack of HLA-restriction is that adenocarcinoma cells do not express HLA-class I molecules (15). By contrast, CTL recognition of target cells bearing a single tandem repeat of mucin is HLA-restricted (16). Barnd et al. (4) and Ioannides et al. (5) postulated that the mucin epitope bound to HLA class I (T-cell epitope) is included in, or adjacent to, the same epitope that is recognized by the SM3 monoclonal antibody (7). This was based on the observation that SM3 bound to cancer cells expressing mucin inhibited the recognition and lysis of the target cell by CTL. The CTL epitope was narrowed to residues P^1-T^{10} that includes the sequence recognized by SM3 (5). Antibodies to sequences outside these 10 amino acids had no inhibitory effect on the lysis of mucin-bearing adenocarcinomas by CTL (5). This suggests that the monoclonal antibody binding site and the T-cell epitope are the same, or near one another. However, it was not clear whether the inhibition of cytolytic activity was due to steric hindrance as a result of antibody binding. The exact sequence of the HLA-bound tumor-specific mucin T cell epitope remains to be determined.

We have used an algorithm (17) to predict the potential HLA class I-bound mucin peptide. This work showed for both the MUC1-mtr_1 and (T3N)-MUC1-mtr_1, a peptide with a mutation in a potential glycosylation site within the tumor-specific mAb site which also elicited tumor-specific T cells (5), the nonamer S^9TAPPAHGV^{17} has the highest predictive value for the potential T cell epitope. This suggests that a mutation in a potential glycosylation site within the mAb tumor-specific epitope does not effect the potential T cell epitope. Experimental evidence has also been published suggesting that this nonamer may be a T-cell epitope (18). This peptide bound to HLA-A1, -A2.1, -A.3 and -A11. The affinity of HLA-A11 for this peptide was close to that found for HLA-bound non-mucin peptides. Since this peptide is "outside" the range of the T cell epitope suggested by Ioannides et al. (5), it is reasonable to conclude that the inhibition of cytolytic activity by SM3 was due to steric hindrance and not to masking of the epitope by the antibody. These results also suggest HLA class I-restricted T cell antigen recognition of tumor-specific mucin.

Multiple approaches have been used to generate mucin tumor-specific CTL. These methods have involved the use of hypoglycosylated mucin, obtained from autologous and allogeneic tumor cells or expressed as a recombinant protein, as the immunogen. For example, mucin has been expressed in a mammalian system using phenyl-N-acetyl-α-galactosaminide (19), an inhibitor of glycosyl transferases involved in the early chain elongation of O-linked

oligosaccharides. However, the initial N-acetylgalactosamine (GalNAc) still modifies the core protein (19). Information presented at the 2nd International Workshop on Carcinoma-associated Mucins by Singal et al. And Reddish et al. have suggested that SM3 binds to MUC1 modified by a single GalNAc, but no published evidence to supports this hypothesis. Also, Finn and co-workers (20) have used a truncated mucin, suggested to be non-glycosylated using antibodies claimed to recognize glycosylated vs. non-glycosylated mucin; however, these mucins were not biochemically proven to be non-glycosylated. Therefore, this raises questions of the utility of this approach for generating mucin-specific CTL. We address the issue of obtaining a reproducibly non-glycosylated of mucin immunogens below and the structure-immunogenicity relationship of tumor-associated MUC1.

Body

Antigenicity and immunogenicity of synthetic and recombinant mucin peptides. Our approach to generating adenocarcinoma-specific CTL from the peripheral blood mononuclear cells (PBMC) of patients with breast or ovarian cancer is to stimulate these lymphocytes with synthetic mucin peptides. Since glycosylation masks mucin tumor-specific epitopes, the synthetic nature of these peptides ensures reproducible non-glycosylation of the immunogen and unmasking of core protein tumor-specific epitopes. The synthetic peptide immunogens, ranging from five to 60 amino acids in length, are described in ***Fig. 1***. Additionally, we have constructed a synthetic gene encoding five tandem repeats of the tumor-specific epitope of human mucin was designed for efficient cloning and expression in *E. coli* (21). The synthetic gene was cloned in the correct reading frame into the maltose-binding protein (MBP)-fusion expression vector pMAL-p2. Bacterial clones containing the mucin synthetic gene insert produced a protein that bound to amylose resin, verifying the presence of the MBP moiety of the fusion protein, and was reactive with monoclonal antibodies which are specific for human mucin, verifying the presence of the mtr_5 polypeptide. Thus, this fusion protein is consistent with the intended recombinant fusion protein, MBP-mtr_5. Furthermore, the fusion protein produced represents a significant fraction of the cellular protein, and is not heavily degraded. This result represents a major advance over the expression of a previously published seven tandem repeat-containing fusion protein which was a human gene expressed in bacteria that was heavily degraded (22). MBP-mtr_5 was purified by affinity chromatography on amylose resin, and for immunological studies. However, in order to perform structural studies, the mucin peptide must be released from its fusion partner. This fusion protein was designed by including a protease Factor Xa site to link the N-terminus of the mucin polypeptide to the C-terminus of MBP. Although the mucin tandem repeat polypeptide does not contain the canonical Factor Xa cleavage site, this polypeptide is digested by the protease, resulting in the loss of the mucin peptide, based on immunocrossreactivity with the mucin core protein-specific mAb HMFG-2 (21). Although we do not fully understand the reason for this outcome, it is possible that Factor Xa has "star" activity on the mucin protein. To overcome this problem, we are in the process of subcloning the mucin cDNA into a fusion protein system with a genenase cleavage site; genenase does not cleave the MBP-mtr_5, and thus should be a suitable expression system for this polypeptide.

All synthetic peptide and bacterial-expressed immunogens contain a monoclonal antibody (mAb) tumor-specific epitope, PDTRP, and are antigenic (***Fig. 1***). Residues flanking the tumor-specific epitope profoundly influence the antigenicity of the peptides; the presence of amino acid residues to the N-terminus of the epitope appear to be more important to the antigenicity of the peptides than do residues to the C-terminus. ***Fig. 1*** shows that the MUC1-mtr_1 is not recognized strongly by the mAb, HMFG-2, but MUC1-mtr_2 cross-reacts with this mAb. Increasing the length of the mucin peptide to three tandem repeats (MUC1-mtr_3) or five tandem repeats (MBP-mtr_5; not shown) does not enhance the antigenicity of the molecule. This suggests that there are no cooperative structural contributions that occur by increasing the length of the polypeptide.

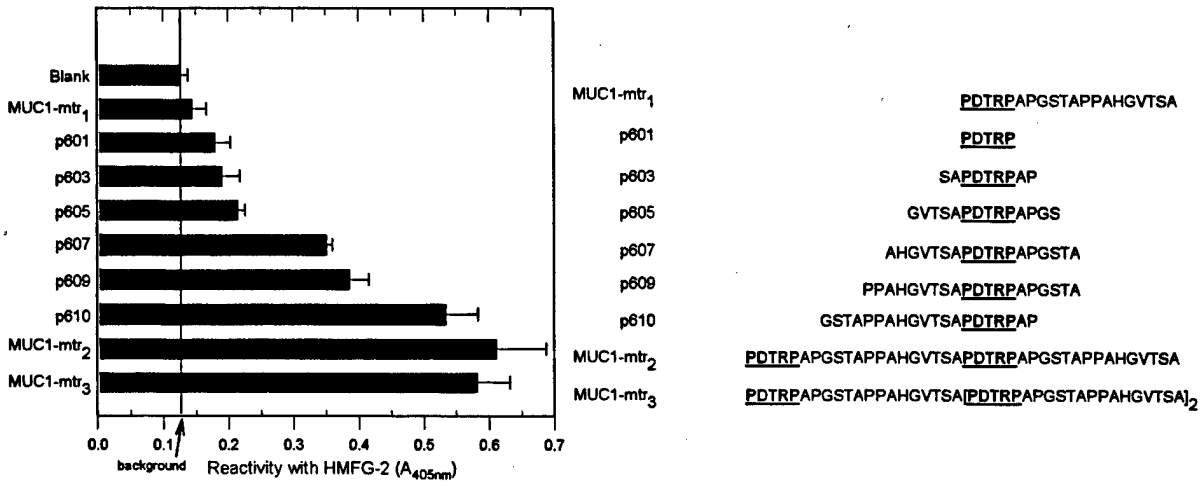

Figure 1. Antigenicity of MUC1-mucin peptides. All wells were coated with the same molar equivalent of the tumor-specific epitope PDTRP (bolded and underlined in right panel). Antigenicity was determined by cross-reactivity with the tumor-specific mAb HMFG-2 by ELISA.

Peptide p609, which contains the mAb epitope in the center of the peptide, cross-reacted with the mAb, but to a lesser extent than did peptide p610, which contains the mAb epitope near the C-terminus of the peptide. Peptide p610 was recognized by HMFG-2 to approximately the same extent as the MUC1-mtr_2. Since the same molar equivalent of each peptide was assayed, we concluded that residues flanking the tumor-specific mAb epitope influences the antigenicity of the molecule, but there are not significant cooperative conformational features added by increasing the number of tandem repeats. Maximal antigenicity is achieved with p610 and the 40 amino acid, double tandem repeat peptide, MUC1-mtr_2. NMR conformational studies are in progress to fully understand the structural basis of this result.

Cellular response to MUC1-mtr_1 peptides. The cellular immune response to the MUC1 mucin peptides differs from the antigenicity of the peptides. Whereas MUC1-mtr_1 reacts poorly with the tumor-specific mAb (***Fig. 1***), the 20 amino acid mucin tandem repeat (mtr) peptide MUC1-mtr_1 + IL-2 stimulated the proliferation of lymphocytes from the PBMCs of a patient with breast cancer. Partial characterization of these cells showed that they were > 95% $CD3^+$, with ~ 60% $CD4^+$, 20% $CD8^+$ cells, and < 3% NK cells ($CD3^-$, $CD56^+$). These lymphocytes showed ~ 25% specific cytolytic activity against the MHC-unmatched MUC1-core protein positive breast tumor cell line MCF-7 at an effector-target cell (E:T) ratio of 20:1 (***Fig. 2***). PBMCs stimulated by anti-CD3 cross-linking + IL-2 did not have significant lytic activity against MCF-7, suggesting that mucin peptide caused the expansion of the CTL that recognize the mucin core protein epitope on MCF-7. Since the effector cells and target cells were not matched, this cytolytic activity implies that target recognition and lysis is MHC-unrestricted. In addition, there was no significant cytolytic activity (< 3%) against the NK target cell line, K562, or the lymphokine-activated killer (LAK) cell target, Raji (not shown), suggesting that the lymphocytes are mucin-specific.

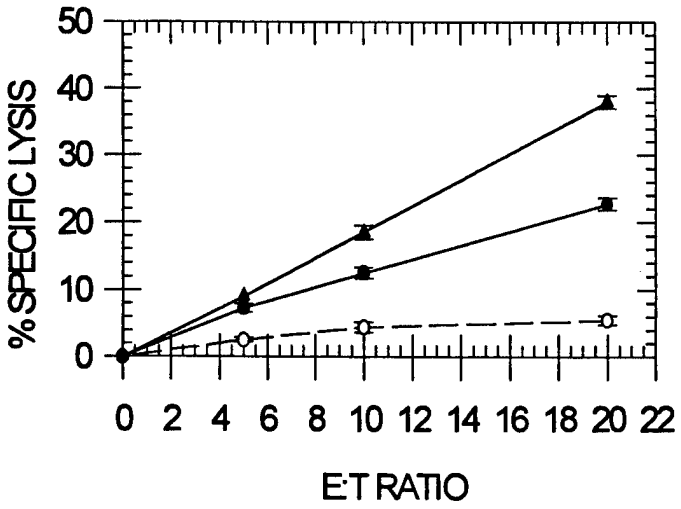

Figure 2. Cytotoxic response of PBMC 1244 from a patient with breast cancer after mucin peptide stimulation. PBMC were stimulated weekly by adding (•) MUC1-mtr$_1$ (1 μg/ml) + 100 IU IL-2, or (▲) (T3N)MUC1-mtr$_1$ (1 μg/ml)+ 100 IU IL-2. (o) CD3$^+$ T cells were isolated from PBMC on day 0 using CD3 microCELLector™ T-25 flasks containing immobilized anti-CD3 mAb. The adherent CD3$^+$ T cells were cultured with 600 IU IL-2. After 22 days in culture, the cytolytic activity of the lymphocytes was measured by ^{51}Cr release assay using 10,000 MCF7 breast cancer target cells per well. Neither effector cells or target cells were HLA-matched: PBMC 1244 was HLA-A11, A24, B38 (w4), B22 (w6), Cw3, DR1(0103)-DRB1*0103, DR13(1301)-DRB1*1301, DR52a-DRB3*0101, DQb5.1-DQB1*0501, DQb6.4-DQB1*0603; MCF-7 is HLA-A02, A10, B18, B44, DR15(1501)-DRB1*1501, DR17(0301)-DRB1*0301, DR51a-DRB5*0101, DR52b-DRB3*0202, DRb2-DQB1*0201, DQb6.3-DQB1*0602. Shown is a representative cytotoxicity experiment, repeated at least twice. Data shown are the mean of triplicate assays ± S.E.

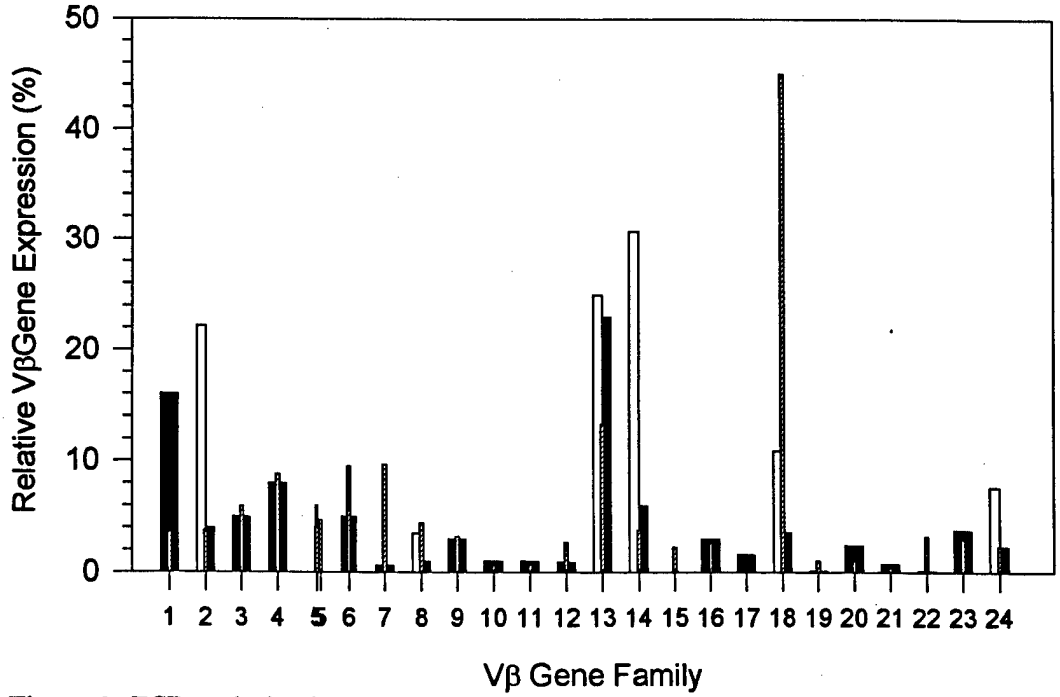

Figure 3. TCR analysis of mucin peptide stimulated PBLC cultures. Vβ gene expression was determined by RT-PCR of mRNA PBMC 1244 after 17 days of stimulation with anti-CD3 + IL-2 (solid bars), MUC1-mtr$_1$ (open bars), or mutated (T3N)MUC1-mtr$_1$ (thin, hatched bars) as described in Fig. 2 above. (On the X axis, Vβ's indicated as two five's and are Vβ 5.1 and 5.2, respectively.)

To test whether a mutation in a glycosylation site would affect the immunogenicity of the mucin peptides, we synthesized a peptide with a conservative mutation of Thr^3 to an Asn since both have the same general size and polarity: (T3N)MUC1-mtr_1. This mutation is in the immunodominant mAb tumor-specific epitope recognized by several tumor-specific mAb (7), and also possibly an epitope recognized by T cells (5). This mutation did not cross-reaction with tumor-specific mAb SM-3 or HMFG-2 (not shown). However, this peptide stimulated lymphocytes that had greater cytotoxicity against MCF-7 cells (***Fig. 2***). Since Thr^3 is an *in vivo* glycosylation site, this result suggests that a conservative mutation of this glycosylation sites may not decrease immunogenicity, but rather possibly enhance the immunogenicity of the peptide. However, additional study of the structure-immunogenicity relationships of MUC1 peptides is needed before we can fully explain this result.

The lymphocytes expanded by both mucin peptides displayed a limited Vβ repertoire. Lymphocytes expanded by MUC1-mtr_1 were predominantly Vβ 2 (22%), Vβ 13 (25%) and Vβ 14 (32%) and other minor Vβs, whereas anti-CD3-stimulated PBMC were predominantly Vβ1 and Vβ13 (***Fig. 3***). However, lymphocytes expanded by the mutated (T3N)MUC1-mtr_1 were Vβ 18 (46%) and Vβ 13 (14%) with other minor Vβ's < 10% each (***Fig. 3***). Thus, mucin peptides stimulate the oligoclonal expansion of lymphocytes with cytolytic activity against mucin-expressing target cells.

Similar results in cytolytic activity and Vβ restriction were also obtained using tumor-infiltrating lymphocytes (TIL) from a patient with ovarian cancer. As with PBMC shown above, TIL stimulated by the mucin peptides showed oligoclonal expansion of CTL that recognize the MHC-unmatched MCF-7 cells, but not NK or LAK targets (not shown). Because cytolytic lymphocytes were expanded from both breast and ovarian patient PBMCs using mucin peptide antigens, and both CTL recognize the mucin-expressing target cell, we believe that the cells are recognizing the mucin core protein.

Increasing the length of the mucin peptides alters its immunogenicity. Our studies have shown that doubling the length of the mucin peptide immunogen to the 40 amino acid, MUC1-mtr_2, did not afford better proliferation or cytolytic activity compared with the single tandem repeat peptide (not shown). However, the 60 amino acid MUC1-mtr_3 stimulated lymphocyte proliferation ~ nine-times better than the MUC1-mtr_1 or MUC1-mtr_2 (not shown). Unstimulated PBMC (***Fig. 4A***) and IL-2-stimulated PBMC (***Fig. 4B***) had no cytolytic activity against the MCF-7 breast cancer cell line, NK or LAK target cells. However, lymphocytes stimulated by MUC1-mtr_3 showed significant cytolytic activity against MCF7 (40 %) (***Fig. 4C***). The peptide-stimulated lymphocytes also possessed ~ 25% specific lysis against K562 and 2.5-fold lower activity (~ 10%) against Raji cells. This latter result may be accounted for by the presence of a small population of NK cells since NK cells recognize K562 ~ 2.5-fold better than Raji cells (***Fig. 4D***). However, the presence of these cells does not significantly contribute to the cytolytic activity against MCF-7 since MCF-7 is a very poor target cell of NK cells (***Fig. 4D***). Thus, we conclude that stimulation of PBMC by MUC1-mtr_3 expands CTL that recognize $MUC1^+$ breast tumor cells. Furthermore, the presence of NK cells in a lymphocyte culture most likely would not be detrimental for immunotherapy, but possibly an asset, since adoptively transferred NK cells infiltrate tumor, albeit at low efficiency (23), and presumably will aid in the eradication of the tumor (24). An alternative explanation for the presence of lytic activity against K562 and Raji cells is that NK T cells ($CD3^+$, $CD8^+$, $CD56^+$) were expanded. This subset of lymphocytes cells show effector properties of both T cells and NK cells which have been observed with mucin-specific CTL, such as HLA-unrestricted killing of targets, and lysis of cells lacking HLA molecules (i.e., K562; ATCC) (25-29).

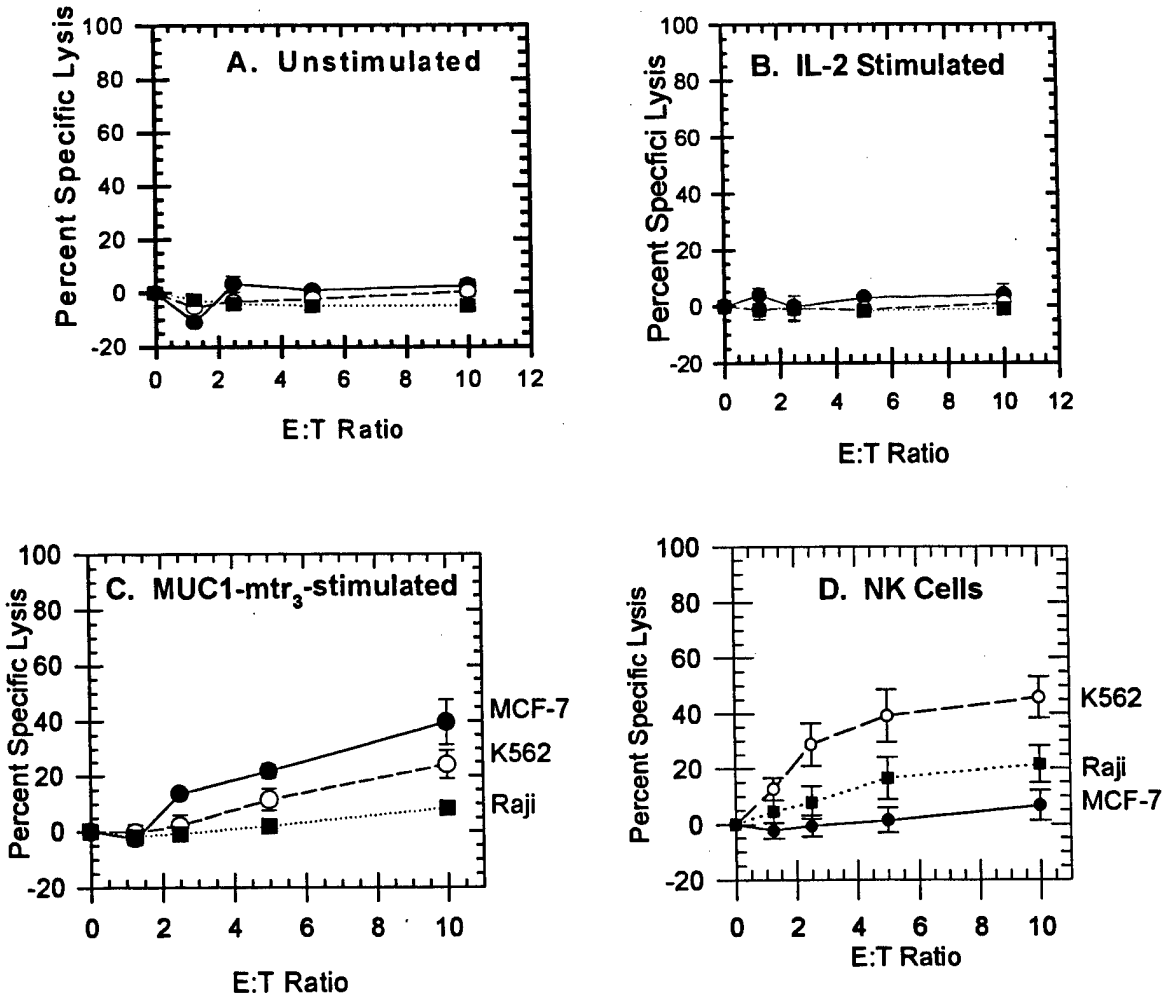

Figure 4. Representative experiment showing tumor-specific cytolytic activity of PBMC from a single donor with breast adenocarcinoma following MUC1-mtr$_3$ stimulation. A portion of the PBMC were cryopreserved on day zero. The remaining cells were used cultured with 100 IU IL-2 alone, or 1 μg/ml MUC1-mtr$_3$ + 100 IU IL-2. Cultures were stimulated weekly with IL-2 or peptide-pulsed irradiated autologous PBMC as APC + IL-2, respectively. Cytolytic activity was determined by ^{51}Cr-release assay on day 28 of culture with effector cells using 10,000 target cells per assay. (E:T, Effector cell to target cell) (A) Unstimulated PBMC frozen on day 0 (with no cytolytic activity) and thawed on day 28 (purified by Ficoll gradient to remove dead cells; cells were >95% viable by trypan blue exclusion prior to assay) and assayed in parallel with IL-2- and peptide-stimulated cells; (B) IL-2-stimulated PBMC, (C) MUC1-mtr$_3$-stimulated lymphocytes, and (D) human NK3.3 cells. Target cells: (•) MCF-7 breast tumor cell line; (○) K562, a NK target cell line; (■) Raji, a LAK cell target.

Finn and colleagues (Hiltbold et al., FASEB J. 12:A888, Abstr. #5142; Barratt-Boyes, et al., FASEB J. 12:A908, Abstr. #5256) presented data at the recent FASEB meeting in San Francisco (April 18-22, 1998) also showing the expansion of MUC1 core protein CTL from a patient with breast cancer. Their approach entailed pulsing purified dendritic cells with the 100 amino acid MUC1-mtr$_5$ peptide. The authors showed ~ 20-25% lysis of target cells at an E:T ratio of 50:1. This is in contrast to our studies which show 25% target lysis at E:T ratio of 20:1 by lymphocytes stimulated with the 20 amino acid MUC1-mtr$_1$, and ~ 40% target lysis of an E:T ratio

of 10:1 using lymphocytes stimulated with the 60 amino acid MUC1-mtr$_3$. Thus, we believe that our approach is simpler than using purified dendritic cells as APC since we achieve at least the same extent of target lysis with a smaller peptide antigen using unfractionated, autologous PBMC as APC.

Development of potential recombinant viruses and ex vivo "expansion of CTL from donors with no clinical signs of cancer. Efficient vaccination was though to require an *in vivo* expression of the antigen from a live virus. Vaccinia virus is widely used to achieve this purpose. However, because this virus can replicate in individuals, this might be harmful in persons who are immunocompromised. Therefore, we began studies to construct recombinant viruses using the fowlpox expression system. This virus, while expressing the transfected gene, does not replicate. Hence, it is a safer approach to potential vaccination. This aim has been hampered by slow progress due to problems of defining conditions for generating recombinants during the course of productive virus infection. In essence, one must find the right conditions for infecting with virus, then introducing DNA in the infected cells in such a way as to not seriously disrupt productive infection but have sufficient intact plasmid DNA present at the correct time to allow recombination during virus replication. We have tried three different modes of transfection (cationic lipids, calcium phosphate precipitation, polybreen) and a variety of timing schemes (duration of infection process before introducing DNA, length of incubation of DNA with Infected cells, length of incubation before screening for plaques, etc.) In recent months, about three dozen picks have been through the screening process try to identify recombinant virus, in some cases through three rounds of screening. We, unfortunately, have not yet obtained a recombinant virus.

While the generation of a recombinant virus was being pursued, we attempted the *ex vivo* immunization of PBMC from healthy male and female donors using synthetic mucin peptides. Here we present initial studies that address whether CTL can be expanded by mucin peptide stimulation from the PBMC of healthy individuals (i.e., not previously diagnosed or treated for any form of cancer, nor been previously exposed to tumor-specific MUC1 mucin). ***Fig. 5*** shows that the PBMC from both a healthy female (***Fig. 5A***) and male (***Fig. 5B***) responded to stimulation by the 40 amino acid MUC1-mtr$_2$, but not the 60 amino acid MUC1-mtr$_3$. This is in striking contrast to the expansion of CTL observed using the PBMC of cancer patients that preferentially responded to the 60 amino acid MUC1-mtr$_3$ (***Fig. 4***). The addition of IL-2 did not significantly alter the incorporation of ^{3}H-thymidine in peptide-stimulated lymphocytes, but rather just increased the background do to its T cell-stimulatory effects (compare ***Figs. 5A*** to ***5C*** and ***5B*** to ***5D*** particularly when no peptide was added). In addition, these proliferation experiments were performed using only 10 IU IL-2, which is at least 10-fold lower than that needed to expand CTL from PBMC of cancer patients (***Figs. 2-4***). Furthermore, the PBMC from the female donor were stimulated by the mucin peptide about 2-fold better than the PBMC from the male donor, in the presence or absence of IL-2. The explanation for this difference in response to mucin peptides between the healthy male and female, as well as between the healthy donors and cancer patients, is not yet clear. These experiments must be repeated with several different donors before these results can be ascribed to being a general phenomenon or biological variance between individuals. In addition, we have been able to maintain these cell lines in culture for over 4 months. Thus, we will pursue to continue our experiments with these cells, as well as, enrolling other donors to study the response of PBMC from healthy donors and cancer patients to mucin peptides, and the generation of recombinant viruses to express tumor-specific antigens may not be necessary.

Fig. 6A shows that lymphocytes expanded by MUC1-mtr$_2$ stimulation of PBMC from healthy individuals possessed cytolytic activity against both MCF-7 and K562, but little to no LAK

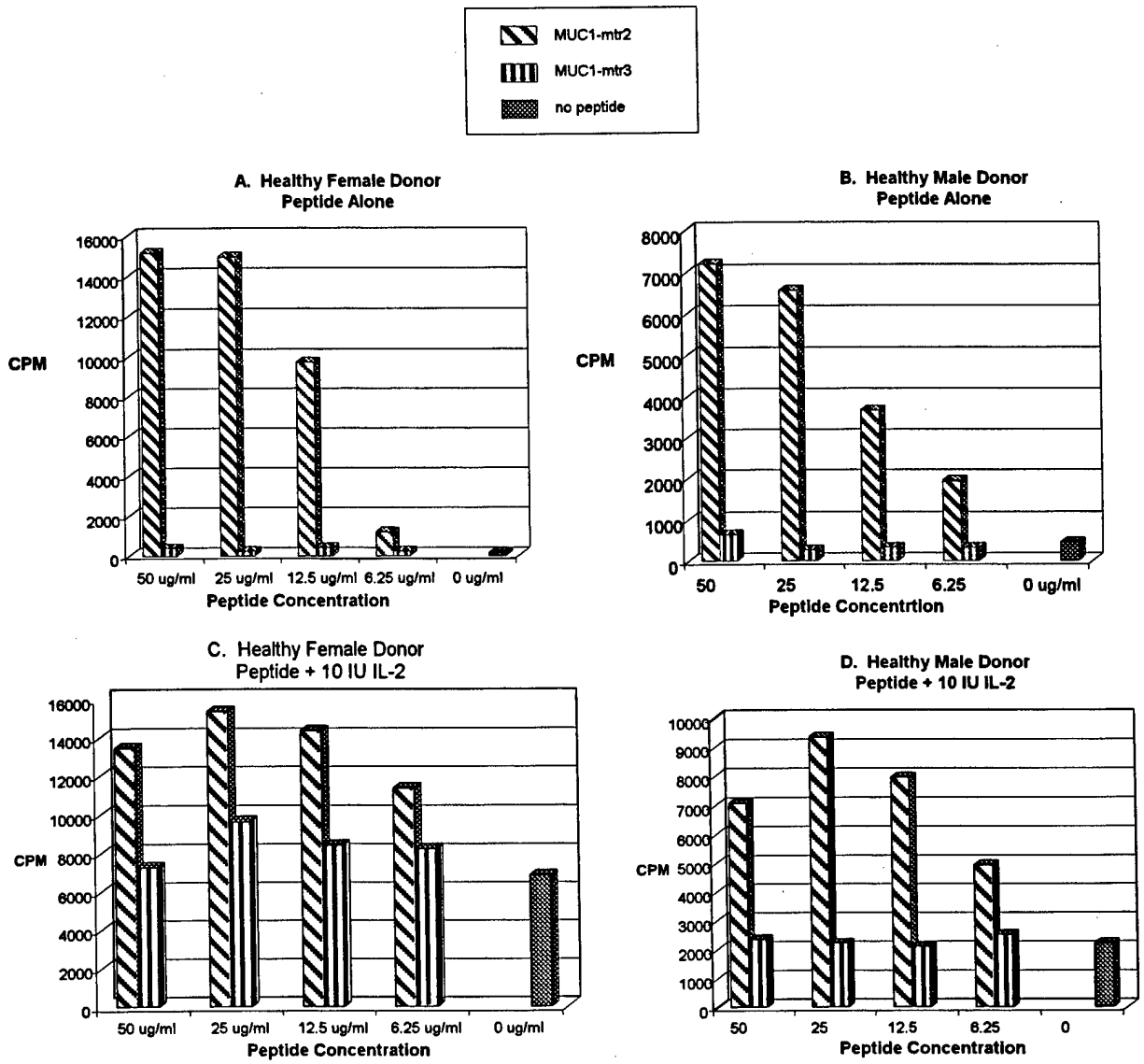

Figure 5. Proliferation of lymphocytes from healthy female and male donors in response to MUC1 mucin peptides in the presence and absence of exogenous IL-2. PBMC were obtained from a healthy 31-year-old female (with no previous pregnancies) and a healthy 37-year-old male. 1 x 10^5 PBMC (responder cells) were cultured with 2.5 x 10^4 irradiated, autologous PBMC as APC pulsed with the peptide indicated. Each panel describes the donor and culture conditions. PBMC shown in Panels A & B (without IL-2) were pulsed with ^{3}H-thymidine on day 6 and harvested on day 7. PBMC shown in Panels C & D (+ 10 IU IL-2) were pulsed with ^{3}H-thymidine on day 3 and harvested on day 4. CPM on the Y-axis indicates the radioactivity of ^{3}H-thymidine incorporated.

activity. In addition, unstimulated lymphocytes died when cytokines and peptides were omitted from the cultures showing that peptide antigen is necessary for the expansion of the cytolytic lymphocytes (not shown). Since both MCF-7 and K562 were lysed about equally by these lymphocytes, it is possible that this peptide stimulates the generation of NK T cells.

Domenech et al. (18) have also presented preliminary evidence similar to ours. This group stimulated lymphocytes from several healthy individuals that were enriched in $CD8^+$ T cells with T2/HLA-A11 APC pulsed with the putative mucin T cell epitope peptide $S^9TAPPAHGV^{17}$. The expanded $CD8^+$ cells lysed T2/HLA-A11 target cells pulsed with the same peptide, but with variable levels of cytolytic activity. Although these CTL were not assayed against other MUC1-expressing target cell lines, this experiment complements our preliminary work suggesting that it may be feasible to immunize humans with mucin peptides to elicit a cellular immune response.

Inclusion of cytokines, such as IL-12, to "drive" the lymphocytes to a $CD8^+$ phenotype can produce dramatic results. We stimulated PBMC from a healthy male with IL-12 (***Fig. 6B***) in parallel with the same PBMC (shown in ***Fig. 6A***). Cultures that contained IL-12 had higher cytolytic activity against MCF-7 and K562 cells than those cultures that did not contain the cytokine (***Fig. 6B***), yet produced very low levels of possible LAK activity against Raji cells. This result suggests that IL-12 is important for optimizing CTL activity (also compare with the cytolytic activity from the PBMC of a cancer patient without IL-12; ***Fig. 4C***). Under similar culture conditions, TIL stimulated with IL-2 + IL-12 resulted in $CD8^+$ CTL with approximately equal cytolytic activity against MCF-7 and K562 (29). These results are consistent with our findings using mucin peptides and again suggestive of NK T cells, particularly since IL-12 synergizes with IL-2 to induce NK T cells (29). These studies are not yet sufficient to conclude that we have expanded tumor-specific CTL or NK T cells. Thus, we are in the process of characterizing the phenotype of the lymphocytes, and sorting them to study their tumor-specific cytolytic activity, against MHC matched and unmatched, mucin-expressing and mucin-negative target cell lines. We will also need to repeat these experiments with CTL expanded from cancer patients in the presence of IL-12. However, based on these *in vitro* results, we believe that the subset of lymphocytes expanded by our stimulation protocol may be an efficient cell population for the potential immunotherapy against cancer.

CTL clone by MUC1 mucin peptide stimulation. By limiting dilution cloning, we have expanded a population CTL from the PBMC of a healthy male donor. These cells were cloned from two rounds of limited dilution. These cells are 99% $CD3^+$ $CD8^+$. There was no appreciable expansion of $CD4^+$ Th cells or NK cells. Although we have not yet fully characterized the cytolytic activity of these lymphocytes, we believe that testing the cytolytic activity of these cells will yield tumor specific CTL since they originated from the same CTL having tumor-specific cytolytic activity as we showed in ***Figs. 5 & 6***.

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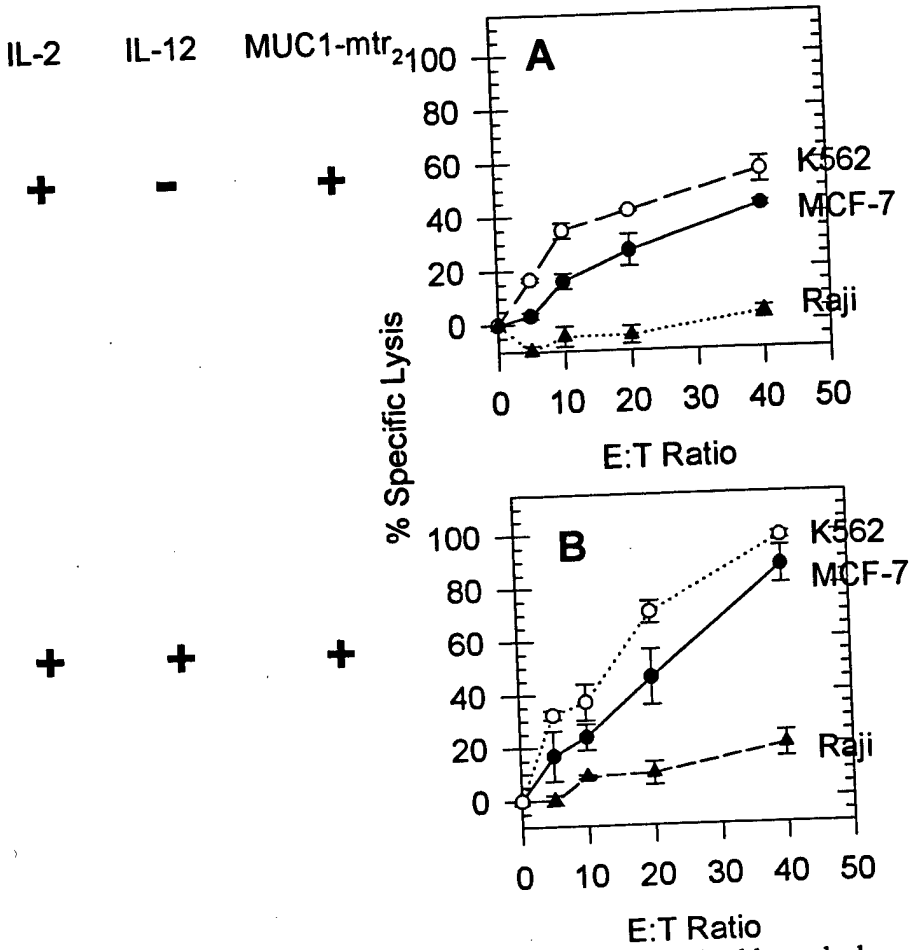

Figure 6. Cytolytic activity of lymphocytes expanded from a healthy male donor using MUC1-mtr$_2$. 1 x 10^5 PBMC from a 37 year old healthy male donor were stimulated with 2.5 x 10^4 irradiated autologous PBMC pulsed with 25 μg/ml MUC1-mtr$_2$ as APC for 7 days with 10 IU IL-2 and in the absence (**upper panel A**) or presence of 2 ng/ml IL-12 (**lower panel B**). Lymphocytes were purified by Ficoll-Hypaque density centrifugation. Cytolytic activity was determined by ^{51}Cr-release assay using 10,000 (•) MCF-7, (o) K562, or (▲) Raji target cells listed in each panel. The data represent a single experiment performed in triplicate.

Conformation of MUC1-mtr$_1$ peptides. Peptides, such as the 20 amino acid MUC1-mtr$_1$, generally assume a random, non-ordered conformation. With the observations that the 40 amino acid MUC1-mtr2 stimulates CTL from healthy individuals, whereas the 60 amino acid MUC1-mtr$_3$ does not, and MUC1 mucin is suggested to begin to assume an ordered conformation whereas peptides of lesser length are random, we also studied the conformational preferences of human mucin. As we have reported previously, we have made the sequential proton assignments for the MUC1-mtr$_1$ and (T3N)-MUC1-mtr$_1$ (***Fig. 7***) in water based on COSY, TOCSY and NOESY ^{1}H-NMR spectroscopy. We have observed reasonable dispersion of backbone NH and α-CH resonances. The temperature dependence of the backbone NH resonances was also examined over the range of 0-35 °C. Temperature coefficients were of intermediate value and show little evidence for a single stable secondary structure (not shown). However, three residues (R^4, S^9, and G^{16}) show less temperature dependence. This suggests that these residues remain in a more stable conformation relative to the other amino acids. Based on NMR studies in d^6-DMSO (11) a β-turn was proposed as a major conformational feature of native mucin peptide. Initial

NMR studies in aqueous phosphate buffer at pH 6.8 (11), the single native mucin peptide yielded broad, overlapped NH signals, and these workers concluded that the native repeat peptide is largely disordered in solution. More recently, better-resolved 2D spectra (11) also indicate a largely unordered structure with evidence for "knob-like" domains.

Our data obtained at pH 4 show good dispersion of backbone NH and α-CH resonances in both the native and mutant peptides. This supports the case for the presence of elements of secondary structure. Our data also provide clear evidence that no large global changes occur upon substitution of the uncharged polar Thr side chain [-CHOH-CH_3] with the uncharged polar Asn side chain [-CH_2-$CONH_2$]. Only local shift changes are seen: the T^3 NH is replaced by the N^3 NH and concomitantly the R^4 NH moves upfield. The remaining amino acid resonances have similar shifts and similar temperature behavior. Since there is little evidence for a single stable secondary structure, it may well be that more than one tandem repeat sequence is needed to stabilize a more ordered polypeptide. However, a sufficient population of preferred peptide conformers may exist in a single tandem repeat to elicit the immunogenic response.

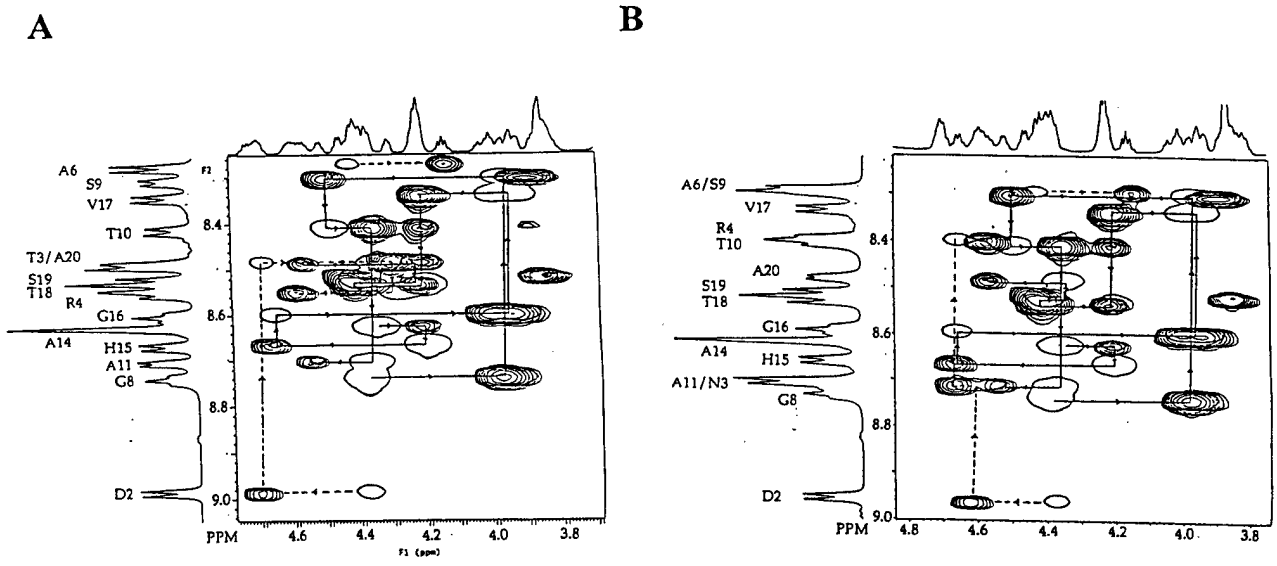

Figure 7. ^{1}H-NMR network for backbone assignments of (A) MUC1-mtr_1 and (B) (T3N)MUC1-mtr_1 illustrated on overlaid plots of TOCSY and NOESY spectra. NMR spectra were obtained in water at 5°C.

While our detailed NMR efforts have focussed on the single 20 amino acid tandem repeat peptide, MUC1-mtr$_1$, we have obtained our first NMR observations for the 40 amino acid, double tandem repeat peptide, MUC1-mtr$_2$ and the 60 amino acid MUC1-mtr$_3$. The His^{15} and His^{35} C2H proton signals are found to be equivalent in chemical shift in MUC1-mtr$_2$, MUC1-mtr$_3$ and to resonate at the same value as the His^{15} in MUC1-mtr$_1$. Since these protons are not exchangeable with solvent water protons, we can compare the intensities of the NH signals of MUC1-mtr$_1$ and MUC1-mtr$_2$ by scaling relative to the number of histidine protons. ***Fig. 8*** shows the NH resonances at 5 and 35°C, respectively. The bulk of the NH resonances at both temperatures are found to behave similarly to the His C2H signals, i.e., the shifts of the second repeat are coincident with the shifts of the first repeat unit. However, a closer examination of the 5°C spectra reveals three new NH resonances in MUC1-mtr$_2$ not observed in MUC1-mtr$_1$, and that the resolved Asp^2 NH signal 8.96 ppm integrates to only one proton in both the single and double tandem repeat peptides. This implies that Asp^2 and Asp^{22} of MUC1-mtr$_2$ are not coincident in chemical shift. Moreover, at 35°C, loss of NH intensity due to exchange with solvent is apparent mainly at the Asp^2 NH in both mucin peptides.

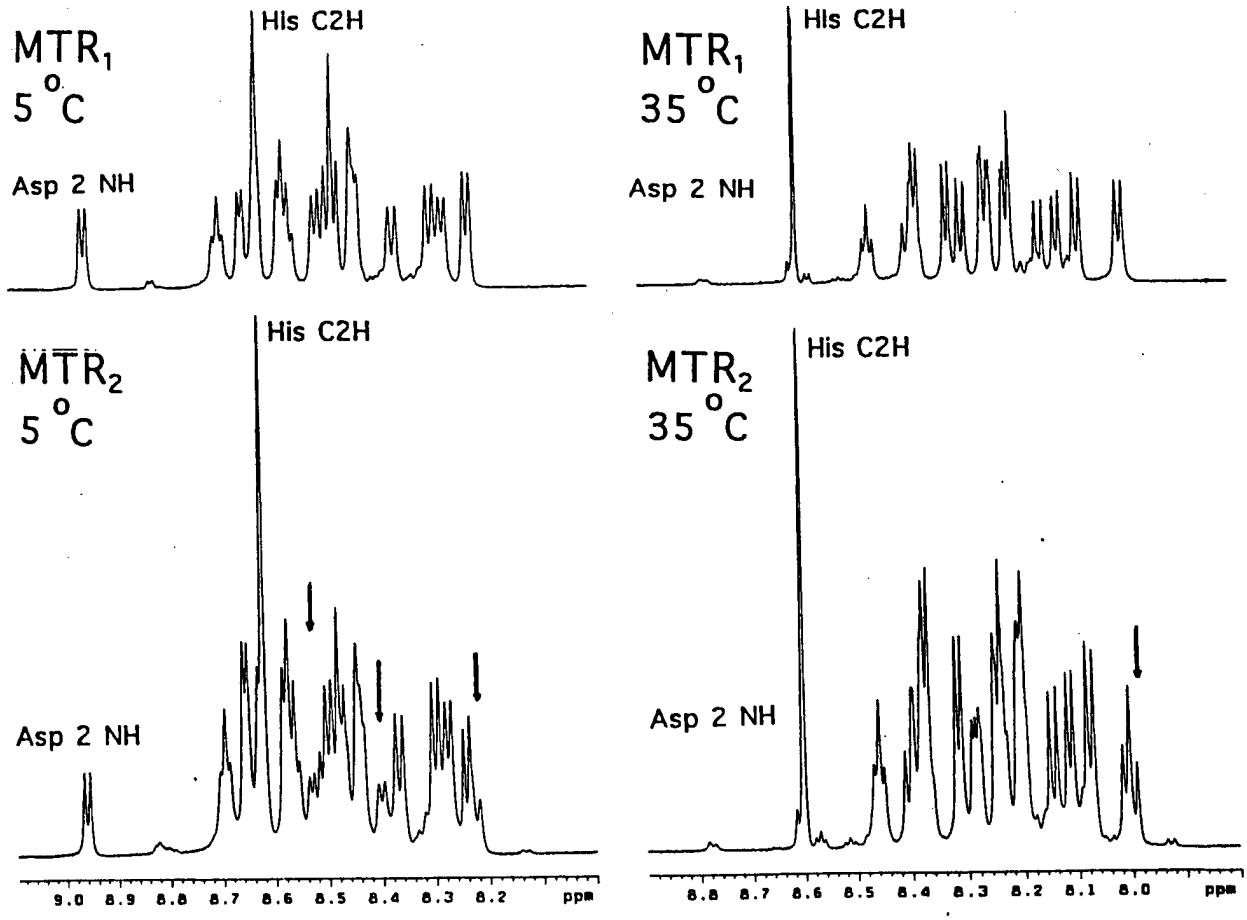

Figure 8. Comparison of the amide NH region of MUC1-mtr$_1$ (top panel) and MUC1-mtr$_2$ (bottom panel). NMR spectra were obtained in water at 5°C and 35°C.

We have obtained NMR observations with the MUC1-mtr$_3$. ***Fig. 9*** compares the spectra of the single (***Fig. 9***, *upper panel*), double (***Fig. 9***, *middle panel*) and triple (***Fig. 9***, *lower panel*) mucin tandem repeat peptides at pH 4 and 5°C. The spectra were normalized to the His C2H at full scale. The Asp^2 NH proton (8.9 ppm) shows a ratio of 3:2:1 (monomer: dimer: trimer). MUC1-mtr$_2$ and MUC1-mtr$_3$ have not previously been studied at this detailed level and with this excellent dispersion of backbone protons, and further work should provide new insights into the conformational preferences of these tandem repeats.

Surface Analysis of Native and Deglycosylated Mucin. X-ray photoelectron spectroscopy (XPS) is a surface sensitive analytical technique that measures the binding energy of electrons in atoms and molecules. The binding energy can be related to the molecular bonding or oxidation state of an element in the outermost layer of a material (<6 nm). Thus, XPS is able to identify chemical species present on the surface of a molecule. The quantitative XPS results of C, O and N for amino acids, simple carbohydrates and peptides of human mucin have been previously determined. Using this technology, we published a model (***Fig. 10***) describing the arrangement of carbohydrate along the core protein of MUC1 mucin (30). This model shows that the oligosaccharide side chains form patches of carbohydrate along the core protein, with regions of core protein not covered by carbohydrate under normal circumstances.

Development of a SCID/hu breast tumor model. To test the *in vivo* efficacy of tumor-specific CTL to cause the regression of established tumor, or prevent the establishment of new tumor, an animal model must be developed. The immunodeficient SCID mouse, which lacks functional T and B cells, is the model that we have begun to pursue. At the preparation of this final report, we have transferred MCF-7 human breast cancer cells subcutaneously in the nuchal region of SCID mice. These tumors grow slowly and are approximately 1-2 mm based on palpation. This is agreement with published observations (31). Because the size of these tumors remains small, it is necessary to wait until the tumors reach a larger size to test the ability of the expanded tumor-specific CTL to cause regression of the mass.

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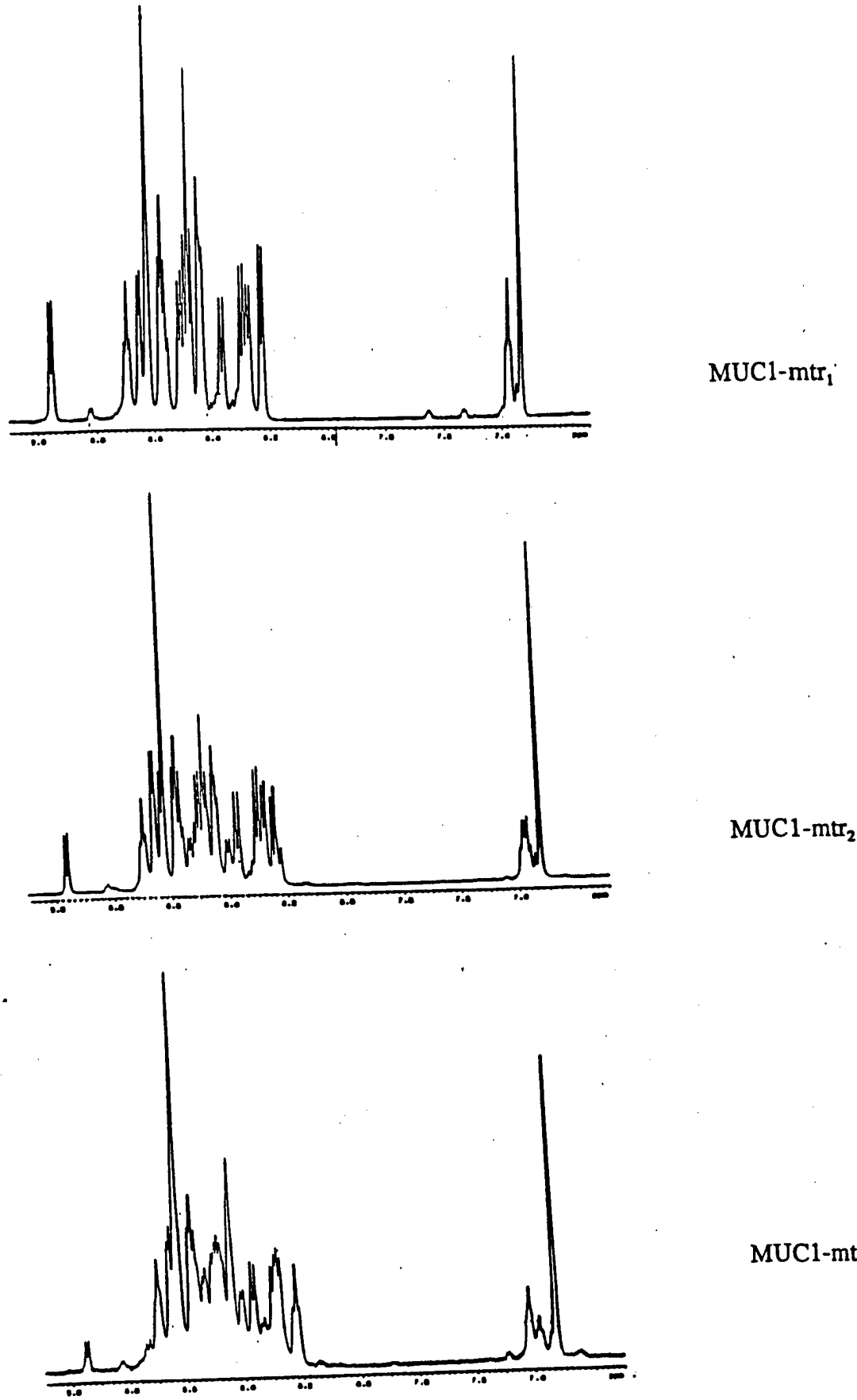


Figure 9. Comparison of the ^{1}H-NMR spectra for the single (*upper panel*), double (*middle panel*) and triple (*lower panel*) MUC1 mucin polypeptides.

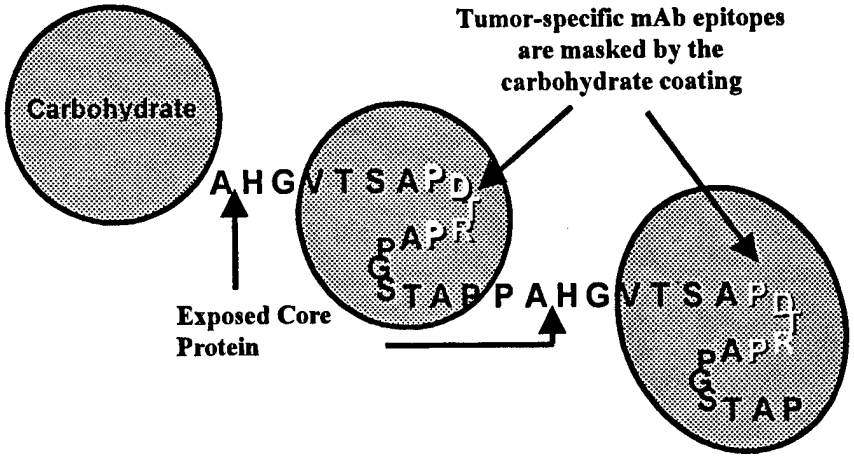
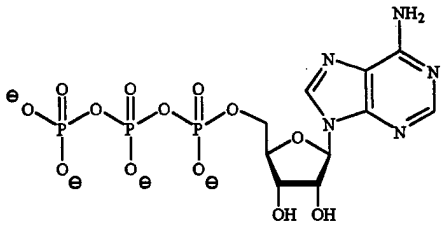


Figure 10. Model of MUC1-mucin [from Russell et al (30)]. This model shows patches of carbohydrate (filled circles) masking regions of core protein from immunological recognition, and regions of core protein that are normally exposed.

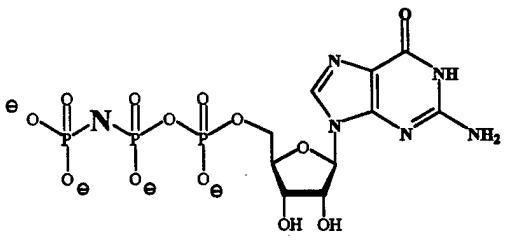
Extracellular regulation of T and B cells. In the development of the tumor-specific CTL and EBV-B cells as APC, we identified a novel transmembrane enzyme, ecto-ATPase, that is expressed by activated, but not resting, T cells ($CD4^+$ and $CD8^+$), B cells, and NK cells (32). The hydrolysis of ATP by this enzyme is essential for the activation of lymphocyte effector cell functions (32). With the utilization of adoptive immunotherapy to treat cancer (1;33) the regulation and maintenance of lymphocyte effector cell functions is key to clinical success. This laboratory is interested in studying factors, such as ecto-ATPase, that may augment a weak immunological response for the potential adoptive immunotherapy against cancer. Thus, we believe that understanding the role of immunoregulatory enzymes in the regulation of immune effector cells will give further insight into the development of agonists and antagonists for developing therapeutic interventions for immunotherapy protocols, and also the regulation of immune-related diseases, for the treatment of patients under care in the VA hospitals.

Ecto-ATPase is a transmembrane enzyme that hydrolyzes extracellular ATP to ADP and inorganic phosphate (P_i). This enzyme is expressed by activated $CD8^+$ CTL (32;34;35), $CD4^+$ Th lymphocytes (32;36), B cells (37-39), and NK cells (34;40), but not by non-activated cells or naïve splenic B and T cells [reviewed in (32)]. Ecto-ATPase is a 66 kDa enzyme that forms disulfide-linked dimers and trimers (32;37). The K_m for ATP is ~ 40-80 μM (41). The detailed structural and kinetic properties have been reviewed elsewhere (32;42). Normally, serum ATP concentrations are in the low micromolar range, but under acute conditions, such as hemolysis and cell damage, or secretion from activated lymphocytes (43;44), local concentrations up to 1 M can be transiently reached. Thus, physiological conditions are sufficient to support ecto-ATPase activity.

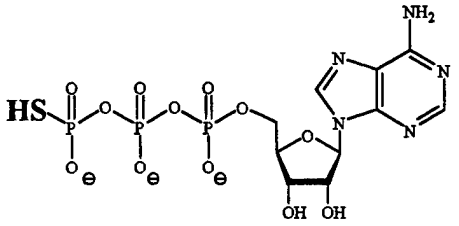
We have recently identified a role for ecto-ATPase in the regulation of lymphocyte effector function using the reversible ecto-ATPase-specific antagonists AMPPNP and ATPγS (***Fig. 11***) and the irreversible, ecto-ATPase-specific affinity label, 5'-FSBA (***Figs. 11 & 12***). Since extracellular nucleotides (43) and 5'-FSBA (45) do not cross the cell membrane, and



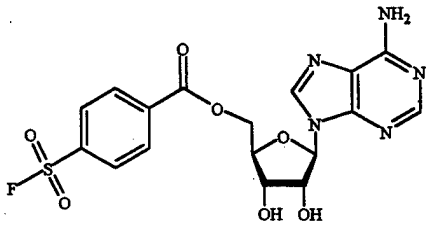
ATP: Adenosine 5'-triphosphate



AMPPNP: 5'-Adenylylimidodiphosphate



ATPγS: Adenosine 5'-O-(3-thiotriphosphate)



5'-FSBA: 5'-p-(Fluorosulfonyl)benzoyladenosine

Figure 11. *Comparison of structures of ATP, AMPPNP, ATPγS and 5'-FSBA*

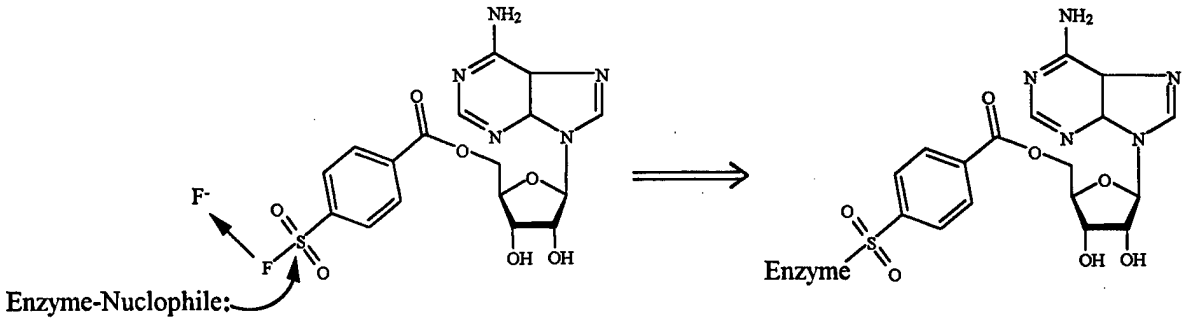


Figure 12. 5'-FSBA binds to the ATP binding domain (*left panel*). A nucleophilic amino acid side chain in this region displaces the fluoro group (*left panel*) and forms a covalent attachment (*right panel*).

since our cell lines do not express non-specific phosphatases, ecto-kinases (32), or other ATP-binding proteins (KE Dombrowski, JA Kapp, GA Weisman, unpublished observation), the inhibition of observed biological activities are a specific result of the inhibition of ecto-ATPase.

Inhibition of ecto-ATPase by both the reversible and irreversible antagonists resulted in the complete inhibition of NK cell natural cytotoxicity and antibody-dependent cellular cytotoxicity (32;41;46) and $CD8^+$ CTL cytolytic activity (47). We have shown using an NK cell model system that inhibition of ecto-ATPase by 5'-FSBA did not inhibit effector cell:target cell conjugate formation (41;46), yet the terminal event in signal transduction, granule release, was inhibited (46). Thus, ecto-ATPase regulates effector function by most likely acting at a step down-stream of E:T conjugate formation, yet upstream of granule release. We have also shown that inhibition of ecto-ATPase inhibits the mobilization of calcium in NK cells (32), suggesting that ecto-ATPase may play an important role in regulating NK cytolytic activity through calcium signaling.

We also examined whether the ecto-ATPase antagonists inhibited the effector function of ecto-ATPase-expressing $CD4^+$ T cell lines (32) and clones from transgenic mice expressing OVA-specific, MHC class II-restricted TCR (47). Secretion of IL-2 and IFNγ in response to OVA was sensitive to the ecto-ATPase antagonist in Th1 cells whereas IL-4 secretion by Th2 cells was resistant to the antagonist. In Th0 clones, ecto-ATPase antagonists inhibited secretion of IFN-γ but not IL-4. By contrast to this differential effect on cytokine secretion pathways, proliferation of both IL-2- and IL-4-secreting hybridomas require ecto-ATPase activity. These results suggest that ecto-ATPase plays a critical role in some, but not all, effector functions of $CD4^+$ T cells. Although the signaling pathways for lymphokine secretion by T cells are very complex and not completely characterized, there is evidence that lymphokine production by Th2 cell lines is relatively independent of Ca^{2+} flux whereas IL-2 production by Th1 cell lines is highly dependent upon Ca^{2+} signals (48;49;49). Thus, we speculate that secretion of IL-4 by activated T cells may be resistant to ecto-ATPase inhibitors because activation of this cytokine gene is relatively independent of Ca^{2+} fluxes. However, we do not yet know whether the inhibition of calcium flux involves the initial mobilization of intracellular calcium, or is limited to the influx of extracellular calcium, and whether inhibition of calcium flux will be observed in $CD8^+$ T cells.

Fig. 13A shows that 5'-FSBA inhibits ecto-ATPase activity expressed by the EBV-transformed B cell line 32993 in a dose-dependent manner with > 80% inhibition of enzyme activity at 1 mM. Inclusion of the ecto-ATPase antagonist AMPPNP in the modification reaction to occupy the ATP binding site protects > 80% of ecto-ATPase against inhibition by 5'-FSBA. This shows that 5'-FSBA modifies ecto-ATPase at or near the ATP binding site. Furthermore, ***Figs. 13B&C*** show that 5'-FSBA is incorporated only into the 120 kDa ecto-ATPase, and that AMPPNP blocks its incorporation (***Fig. 13D***). Thus, we conclude that 5'-FSBA is specific for ecto-ATPase in 32993 B cells.

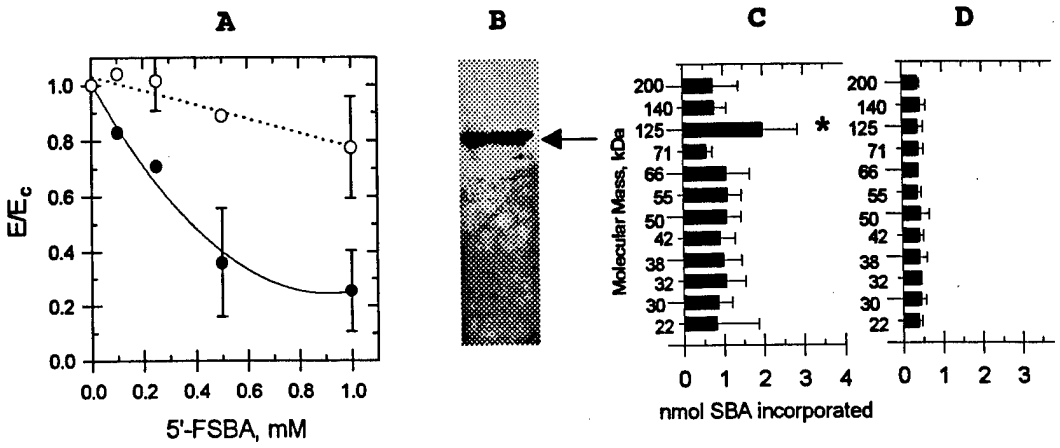


Figure 13. 5'-FSBA is a specific inhibitor of ecto-ATPase. ***(A) Inhibition of ecto-ATPase by 5'-FSBA***. EBV-B cell line 32993 was incubated with the indicated concentrations of 5'-FSBA (•) or 5'-FSBA plus 1 mM AMPPNP (o) for 1 hr. Following the reaction, the cells were pelleted, washed and resuspended in fresh RPMI 1640. Ecto-ATPase activity was determined by the release of $^{32}P_i$ from [γ-^{32}P]ATP (37;40;47). Residual activity was calculated from the ratio of the measure enzymic activity for the reaction mixture at the times indicated (E) to the measured enzyme activity for the corresponding control reaction (Ec). ***(B) Western analysis using anti-ecto-ATPase polyclonal antibodies***. Membrane proteins from 32993 cells were separated by SDS-PAGE under non-reducing conditions and transferred to PVDF membrane for Western blot analysis using anti-ecto-ATPase polyclonal antibodies (37), showing that ecto-ATPase has a molecular mass (Mr) of ~ 120 kDa. ***Panels C and D: Modification of 32993 cells by (C) [2-^{3}H]5'-FSBA or (D) [2-^{3}H]5'-FSBA plus 1 mM AMPPNP***. Cells were modified by 1 mM [2-^{3}H]5'-FSBA (0.6×10^{12} cpm/mol) or 1 mM [2-^{3}H]5'-FSBA plus 1 mM AMPPNP for 1 hr, washed and membrane fraction prepared. Membrane proteins were separated by SDS-PAGE under non-reducing conditions. Serial-2 mm slices were counted for incorporation of the radioactive sulfonylbenzoyladenosine (SBA) moiety, and show that only the 120 kDa slice that contains ecto-ATPase contain radioactivity that is statistically different from background, which was eliminated by blocking with AMPPNP.

* indicates significant incorporation of [^{3}H]5'-FSBA into the band corresponding to ecto-ATPase.

Spontaneous calcium uptake is a property of 32993 B cells (***Fig. 14A***). Addition of exogenous ATP did not enhance the uptake of Ca^{2+} (not shown) suggesting that the influx is at maximal velocity under culture conditions. 5'-FSBA and the reversible ecto-ATPase antagonist, ATPγS, abrogated this influx (***Fig. 14A***). Furthermore, depletion of extracellular ATP by the addition of an excess of ecto-ATP-diphosphohydrolase, which sequentially cleaves ATP to AMP, inhibited calcium uptake. This shows that Ca^{2+} uptake is dependent on ATP hydrolysis by ecto-ATPase, and also verifies that the activity of the antagonists is specific for ecto-ATPase.

The functional consequences of calcium influx were also examined. ***Fig. 14B*** shows that chelation of extracellular Ca^{2+} inhibits the proliferation of these transformed B cells. The growth rate of EBV-B cells treated with the ecto-ATPase antagonists 5'-FSBA (***Fig. 14C***) or 1 mM ATPγS (***Fig. 14D***) also decreased by >90%. The decrease in the rate of growth was not due to cell death since the cells remained >95% viable at each time point (not shown). No DNA fragmentation (apoptosis) of the 5'-FSBA-modified cells was observed as compared to the unmodified controls (not shown). Preincubation of the cells with ATPγS for 30 min., followed by removal and washing, restored both enzyme activity and cell growth (***Fig. 14E***). However, preincubation of the cells with ATPγS for 60 min., followed by removal and washing, restored only enzyme activity (***Fig. 14E***). This result suggests that there is a threshold time period of ecto-ATPase inhibition, after which, the inhibition of biological effects (e.g., proliferation) is irreversible. Although the inhibition of calcium influx by 5'-FSBA was circumvented by the Ca^{2+} ionophore, ionomycin (***Fig. 15A***), neither the addition of ionomycin, ionomycin plus mitogen, nor mitogen alone, overcame the inhibition of cell growth caused by 5'-FSBA (***Fig. 15B***). Thus, ecto-ATPase has a profound effect on intracellular pathways controlling cell growth.

DNA replication was examined by measuring the incorporation of ^{3}H-thymidine into unmodified control and 5'-FSBA-modified 32993 B cells. ***Fig. 16A*** shows that control cells incorporated the radioactive nucleoside into *de novo* synthesized DNA, but radioactivity incorporated into the DNA of 5'-FSBA-modified cells was nearly completely inhibited. Inhibition of DNA synthesis was not a result of limited intracellular availability of [^{3}H]thymidine since 5'-FSBA inhibition of ecto-ATPase did not inhibit the uptake of the isotope (***Fig. 16B***). We conclude that 5'-FSBA inhibition of ecto-ATPase, in part, targets DNA replication. We also examined whether *de novo* protein synthesis was effected by 5'-FSBA. DMF did not significantly affect the rate of *de novo* synthesis of proteins in the cell-free translation assay. Also, *de novo* protein synthesis was not inhibited by 5'-FSBA (***Fig. 16C***). We conclude from these studies that inhibition of the rate of cell growth by ecto-ATPase antagonists occurs at the level of calcium flux and DNA synthesis, but not protein synthesis.

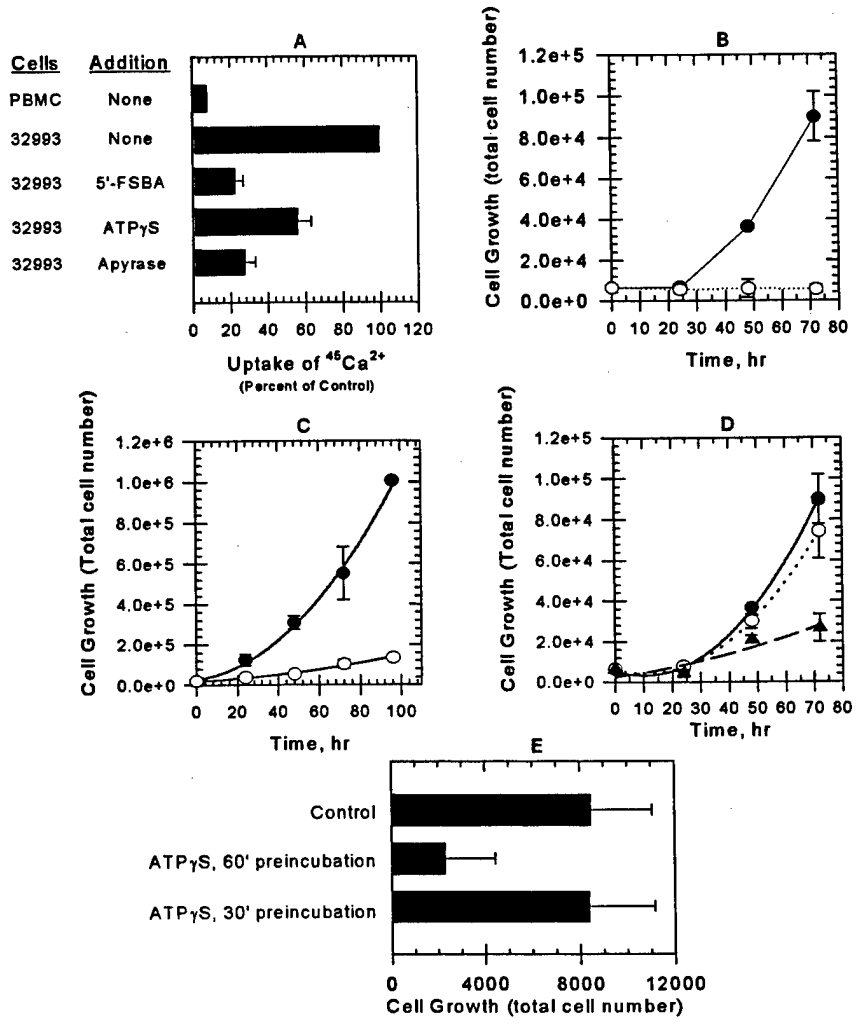


Figure 14. (A) Uptake of extracellular Ca^{2+} by unstimulated human PBMC and immortalized EBV-32993 B cells. PBMC were obtained from the buffy coat fraction of human blood. Prior to assay, cells were modified by 1 mM 5'-FSBA for 1 hr and washed as described above. To assay for calcium uptake, cells were suspended in 10 mM HEPES buffer, pH 7.4, containing 10 mM glucose, 1 mM $MgCl_2$, 3 mM KCl, 140 mM NaCl. ATPγS (1 mM) and apyrase (3 U) were added to the before the addition of the isotope and remained present during the assay. Uptake of calcium was determined by incubating cells with 3 μCi $^{45}Ca^{2+}$ at 37 °C for 10 min, pelleting and washing three times with the assay buffer without isotope. The entire cell pellet was counted for uptake of radioactivity. ***(B) Effect of chelation of extracellular Ca^{2+} on proliferation of 32993 cells.*** EBV-B cells 32993 were cultured in RPMI-1640 containing 10% fetal calf serum in the (○) presence or (●) absence of 2 mM EGTA to chelate extracellular calcium. At the times indicated, an aliquot of cells was withdrawn and the total cell number determined by cell counting and vital dye exclusion. The viability of the cells was > 95% at each time point. ***(C) Effect of 5'-FSBA on cell growth.*** 32993 B cells were incubated for 1 hr at 37 °C with (○) 1 mM 5'-FSBA and (●) control cells incubated in RPMI-1640 containing 2.5% DMF, pelleted, washed three time with 1 ml of RPMI 1640 to remove unbound 5'-FSBA. The cells were seeded at 1 x 10^4 cells/well and grown in RPMI-1640 containing 10% fetal calf serum for the times indicated. The total cell number and viability were determined by cell counting and vital dye exclusion at each time point. The viability of the cells was > 95% at each time point. ***(D) Effect of ATPγS on cell growth.*** 32993 B cells were seeded at 2 x 10^4 cells/well containing (●) no ATPγS, (○) 0.1 mM ATPγS, or (▲) 1.0 mM ATPγS. The cells were cultured, and the total cell number and viability were determined as described above. The viability of the cells was > 95% at each time point. ***(E) Effect of ATPγS preincubation time on EBV-B cell growth.*** 32993 B cells were preincubated for 30 or 60 min. with the reversible ecto-ATPase antagonist ATPγS (1 mM) at 37 °C, pelleted and washed free of the nucleotide antagonist. All cell groups were seeded at 2 x 10^3 cells/well and cultured as described above. Cell growth and viability were determined 24 hr after seeded by vital dye exclusion. The viability of the cells was > 95%.

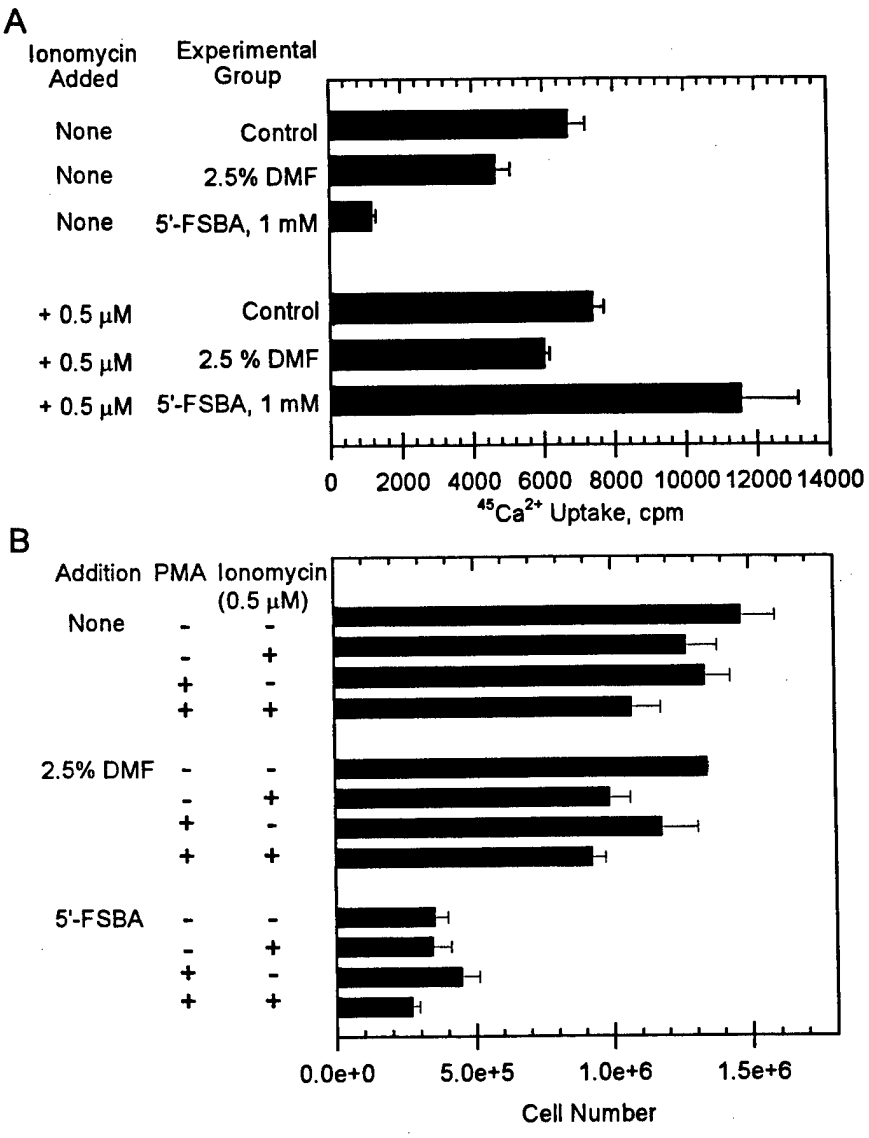


Figure 15. Effect of calcium ionophore and mitogen on reversing the inhibitory effects of 5'-FSBA on EBV-transformed 32993 B cells. ***(A) Effect of ionomycin on calcium uptake of 5'-FSBA modified 32993 B cells***. Untreated control cells, 2.5% DMF-treated control cells and 5'-FSBA-modified cells were assayed for the uptake of extracellular calcium as described above in the absence and presence of 0.5 μM ionomycin. ***(B) Effect of ionomycin and PMA on reversing the inhibition of 32993 B cells growth by 5'-FSBA***. Untreated control cells, control cells treated with 2.5% DMF, and 5'-FSBA-modified cells were cultured for 48 hours in the presence and absence of PMA and/or ionomycin. Cell growth and viability were determined by trypan blue exclusion.

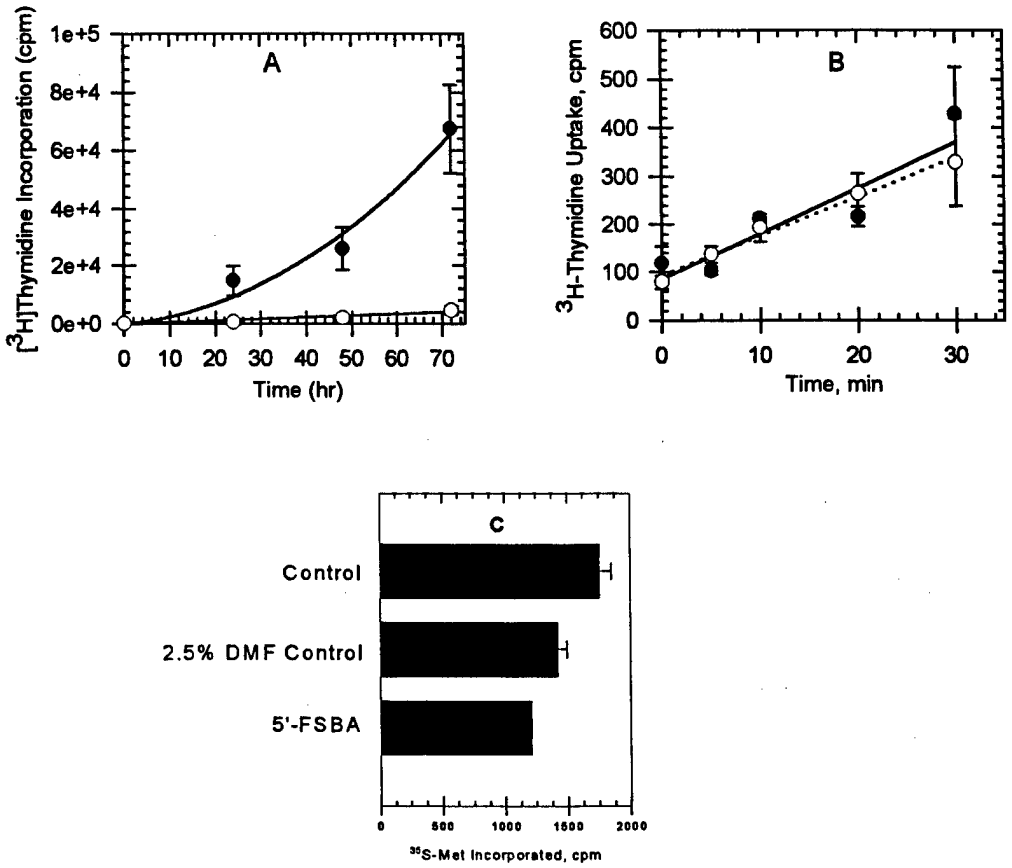


Figure 16. Effect of 5'-FSBA inhibition of ecto-ATPase on several cellular metabolic pathways. Incorporation of ^{3}H-thymidine into de novo DNA synthesis. EBV-B cells 32993 were modified in the presence (●) or absence (O) of 1 mM 5'-FSBA as described in Fig. 3. The cells were grown for the times indicated, pulsed with 1.5 μCi ^{3}H-thymidine and harvested after 16 hr using a PHD cell harvester (Cambridge Technology). The radioactivity incorporated into DNA was determined by liquid scintillation. Shown is a representative experiment of at least three independent determinations, each performed in triplicate. Data shown is the mean ± SD.

(B) Uptake of extracellular thymidine by (●) control cells treated with 2.5% DMF in RPMI-1640 and (O) 5'-FSBA-modified EBV-B cells. The uptake of ^{3}H-thymidine was determined by incubating 1 x 10^{4} cells with 1.5 μCi of the radioactive nucleoside at the times indicated. At each time point, the cells were pelleted and washed free of extracellular radioactivity. The radioactivity incorporated into the whole cell pellet was determined by scintillation counting. Shown is a representative experiment of at least three independent determinations, each performed in triplicate.

(C) Effect of 5'-FSBA on protein synthesis in a cell-free translation assay. A cell-free homogenate was prepared from untreated control cells, cells treated with 2.5% DMF, and 5'-FSBA-modified EBV-B cells. The homogenates were pulsed for 10 min with 1.5 μCi of [^{35}S]Met at 37 °C. *De novo* protein synthesis was assayed by the incorporation of [^{35}S]Met into TCA precipitable proteins.

Effect of ecto-ATPase antagonists on human breast and ovarian tumor cells. We have performed initial experiments showing that ecto-ATPase is expressed by ovarian (***Figs. 17 & 18***) and breast (*not shown*) tumor cell lines. We have also shown that the ecto-ATPase-specific antagonist ATPγS is an effective inhibitor of the enzyme expressed by ovarian tumor cells (***Fig. 18***). This inhibitor is also effective at inhibiting the proliferation of the ovarian (***Fig. 18***) and breast (***Fig. 19***) tumor cells. Although these initial results show that the inhibition of ecto-ATPase correlates with the inhibition of tumor proliferation, they must be repeated with several ecto-ATPase specific antagonists before we can conclude that the hydrolysis of extracellular ATP is necessary for the proliferation of tumor cells. However, these initial results are encouraging that this enzyme may provide a novel target for the control of tumor progression.

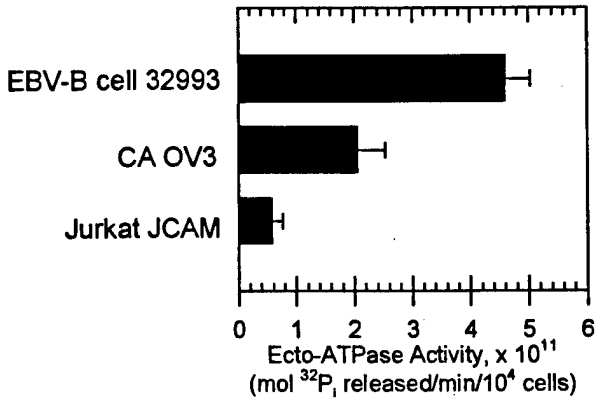
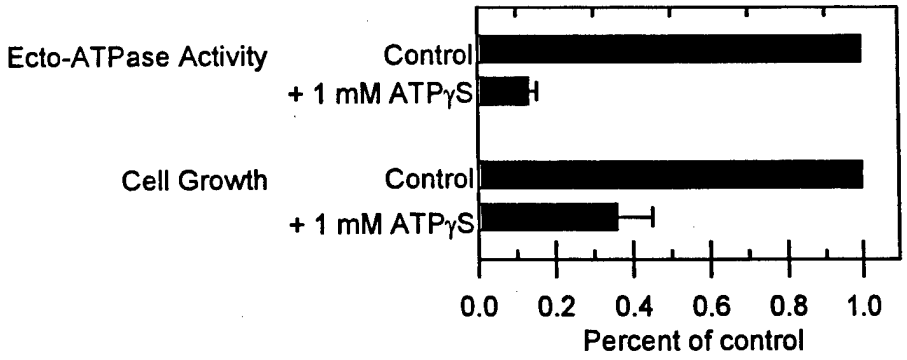


Figure 17. Ovarian cancer cell line CA-OV3 expresses ecto-ATPase. Ecto-ATPase activity was determined by the release of $^{32}P_i$ from [γ-^{32}P]ATP as we described previously (37;40;47)

Figure 18. ATPγS inhibits ecto-ATPase activity and growth of CA-OV3 ovarian adenocarcinoma cells. Ecto-ATPase activity was determined in the absence (control) or presence of 1 mM ATPγS. Cell growth was determined by cell counting by trypan blue staining. Cells were > 90% viable.



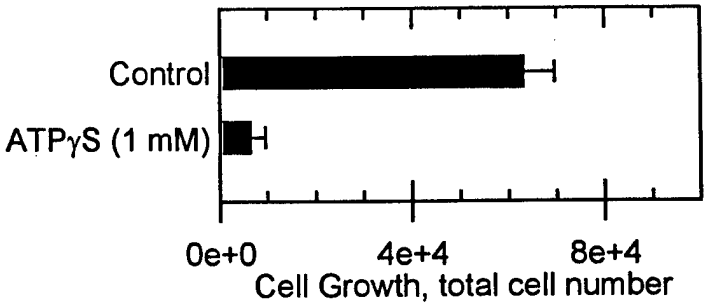


Figure 19. Ecto-ATPase antagonists inhibit growth of breast adenocarcinoma cell line MCF-7. Cells were seeded at 1 x 10^4 cells/well in the absence (control) or presence of 1 mM ATPγS. The growth of MCF-7 cells was determined as described in ***Figs. 17 & 18***. Cell viability was > 90% by trypan blue exclusion.

Conclusions

The goal of this research was to understand the structure-immunogenicity relationships of the tumor-specific mucin. Our accomplishments for each of the technical objectives of this proposal are summarized below.

Technical Objective 1. *Construct DNA coding for native and mutated mucin peptide immunogens.* We have completed this aim by using synthetic mucin peptide immunogens, up to 60 amino acids long (MUC1-mtr$_3$), with both native sequences and with mutations in potential glycosylation sites. Synthetic peptides offered a more economical approach to testing peptide immunogens rather than cloning. However, because the product yield of automated solid-phase protein decreases substantially above 60 amino acids (e.g. three tandem repeats of mucin), we have designed and expressed from bacteria a recombinant mucin polypeptide containing five tandem repeat elements in a non-degraded form.

We initially proposed to construct and express mucin immunogens from recombinant vaccinia viruses. Because this virus will replicate in humans, and therefore, it may present a health hazard. Therefore, we have redirected our efforts to expressing the recombinant mucin polypeptide via fowlpox viruses. These viruses are capable of initiating infection of mammalian cells, but the infection is not productive, i.e., the virus does not replicate. Thus, fowlpox viruses have the potential of providing safe vaccines by producing antigens intracellularly (facilitating antigen presentation) when the inserted gene is designed to be expressed prior to replication. We have successfully grown fowlpox virus (not shown) and work is currently in progress to obtain the recombinant mucin cDNA-containing virus. Task 2 was slowed due to the necessary change in recombinant virus expression system, but progress is being made to completing this goal. We anticipate that a recombinant virus expressing a MUC1-mucin polypeptide immunogen will be obtained. However, we observed that persons may be immunized *ex vivo* with synthetic MUC1 mucin peptides, thus circumventing the need for a recombinant mucin-expressing virus.

Technical Objective 2. *Determine the humoral and cellular immunogenicity of peptide immunogens.* We have completed this objective by showing that the humoral antigenicity of the MUC1 mucin peptides is maximized at 40 amino acids (i.e., two tandem repeats), and therefore does not necessitate a longer, or full length, molecule. By contrast, the cellular immunogenicity of these peptides is somewhat different than the humoral antigenicity. Whereas mucin tandem repeat polypeptides as small a 20 amino acids (one tandem repeat unit) are weakly antigenic against mAb, these peptides elicit the reproducible expansion of tumor-specific CTL from the PBMC of donors with breast cancer. Also, a conservative mutation of a potential glycosylation site in a tumor-specific epitope is effective in eliciting both humoral and cellular immunogenic responses that are tumor-specific; this result makes feasible the ability make similar mutations in other potential glycosylation sites. The expansion of mucin-specific T cells is oligoclonal, and work is in progress to clone these human CTL. Antigen recognition of the expanded CTL at the level of effector function is not HLA-restricted, but may be MHC-dependent for expansion. This stimulation of PBMC with synthetic peptides represents an advance in current methodologies using tumor, or transformed dendritic cells (30) as the immunogen in that an unlimited supply of the synthetic immunogen can be obtained, whereas use of autologous tumor is limited to only the amount of tumor obtained and transformation of dendritic cells is a cumbersome process.

Increasing the length of the MUC1 immunogen to two tandem repeats did not enhance the cellular immunogenicity of this peptide. This is consistent with NMR structural studies showing that MUC1-mtr$_1$ and MUC1-mtr$_2$ have predominantly a non-ordered conformation. However, a significant population of peptide conformers may exist in a single tandem repeat to elicit the immunologic response. By contrast to the single and double tandem repeat peptides

the 60 amino acid MUC1-mtr$_3$ is suggested to have an ordered knob-like conformation (11). Consistent with this idea, the MUC1-mtr$_3$ reproducibly expands tumor-specific CTL 9-fold better than either the single or double tandem repeat peptides. Furthermore, we expanded on our initial goals by showing the healthy men and women without any previous history of cancer elicit a cellular immune response to the mucin peptide immunogens. This suggests that these peptides may serve as potential vaccines against cancer. By defining the humoral and cellular immunogenicity of MUC1 peptides, this objective was completed.

Technical Objective 3. *Characterize the glycosylation of MUC1 mucin.* Through surface analysis, we developed a model that describes a patch-work arrangement of the oligosaccharide side chains along the core protein. This objective was completed.

Technical Objectives 4 and 5. *Characterize the detailed secondary and tertiary structure of the peptide immunogens, and correlate the protein structure with immunogenicity.* We hypothesized that increasing the length of the peptide immunogen would induce the formation of order secondary structure, and thereby enhance the immunogenicity of the peptide. However, this hypothesis was not supported by our experimental findings showing that the immunogenic peptides do not assume any stable conformation. This suggests that antigen processing and presentation, as well as, target cell recognition are independent of the conformation of the antigen. Task 4 to determine the conformational dependence of mucin peptide immunogens and develop a model of MUC1 mucin has been completed. We described that, contrary to published reports (with poorly resolved NMR spectra (11)), our results obtained with highly resolved NMR spectra that there appears to be no conformational preference of MUC1 peptides up to 60 amino acids in length. As a result of this, other spectroscopic approaches such as IR spectroscopy, are not necessary to pursue to further define structural domains. This objective was completed.

Technical Objective 6. *Clone a human CD8$^+$ CTL.* We showed that peptide stimulation of human PBMC expanded an oligoclonal population of tumor-specific CD8$^+$ CTL and CD4$^+$ Th cells. By limiting dilution cloning, we have obtained a clone of human CD8$^+$ CTL using autologous APC. This objective was completed.

Technical Objective 7. *Begin developing a SCID/hu mouse model system.* This objective was to begin testing the efficacy of mucin peptide-stimulated CTL to cause rejection of established tumor and as a prophylaxis against the establishment of tumor. We have established human breast tumor in SCID mice, and thus have developed the model to test the efficacy of our expanded CTL. However, the in vivo testing of these CTL has not been accomplished. However, our goal was to begin the development and testing of the CTL, and with regard to this, this aim has also been successful and completed.

Novel findings. In the course of any research, novel observations are often made while pursuing the aims. This research was no exception. While developing tumor-specific CD8$^+$ CTL for the potential immunotherapy and vaccination against cancer, and EBV-transformed B cells as potential APC to generate the CTL, we observed that these activated lymphocytes expressed a transmembrane-bound enzyme, ecto-ATPase, that is induced upon cell activation. In addition, in our study of tumor cell biology, we made correlations between our observations on lymphocytes and tumor cells, and have observed that tumor cells also express this enzyme. Resting, and non-stimulated cells do not express this enzyme. The cellular activity, including proliferation, is dependent on the hydrolysis of extracellular ATP by ecto-ATPase. Thus, this enzyme offers a new target for the control of immune function. With the utilization of adoptive immunotherapy to treat cancer (33;50) the regulation and maintenance of lymphocyte effector cell functions is key to clinical success.

In conclusion, we have been highly successful in achieving our goals to define the structure-immunogenicity relationships of tumor-specific MUC1 mucin on this Career Development Award. The majority of our hypotheses directing this work were supported by our experimental observations, while other hypotheses were not. In addition, novel observations were made that may give us further insight into the treatment and prevention of cancer and diseases of the immune system. These observations are actively being pursued through new grant applications. The Principal Investigator is greatly appreciative of the US Army for their funding of this research program to allow him to develop a career in cancer research.

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Appendix

Breast Mucin Tumor-specific Epitopes for Cancer Immunotherapy

Grant No: DAMD17-94-J-4161

The following material is a listing of the publications and presentations that have resulted from funding from the above referenced Department of the Army grant:

Publications:

1. Dombrowski, K.E., Brewer, K.A., Maleckar, J.R., Kirley, T., Thomas, J.W. & Kapp, J.A. (1997) Identification and Partial Characterization of the EctoATPase Expressed by B-Lymphocytes. Arch. Biochem. Biophys., 340, 10-18.
2. Russell, B.G., Moddeman, W.E., Birkbeck, J.C., Wright, S.E., Millington, D.S., Stevens, R.D. & Dombrowski, K.E. (1997) Surface Analysis of Human Mucin by X-ray Photoelectron Spectroscopy: A model for the distribution of carbohydrate along the core protein. Biospectroscopy 4, 257-266.
3. Wright, S.E., Kilinski, L., Talib, S., Lowe, K.E., Dombrowski, K.E., Lebkowski, J.S. & Philip, R. (1998) Cytotoxic T-lymphocytes Induced by Native and Glycosylation Site-mutated MUC1 Mucin Peptides from Humans with Adenocarcinomas. J. Immunother. Revised manuscript resubmitted.
4. Dombrowski, K.E., Ke, Y. & Kapp, J.A. (1997) Role of EctoATPase in Lymphocyte Effector Function in EctoATPases: Recent progress in structure and function (Plesner, L., Kirley, T.L. & Knowles, A.F., eds.) Plenum, NY, Ch. 24, pp 197-207.
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Abstracts and Presentations:

1. Dombrowski, K.E. & Kapp, J.A. (1996) Possible Role of EctoATPase in Lymphoid Cell Function. First International Workshop on EctoATPases. Mar del Plata, Argentina, August 26-30.
2. Dombrowski, K.E. & Kapp, J.A. (1996) Role of EctoATPase in the Salvage of Extracellular Nucleotides. First International Workshop on EctoATPases. Mar del Plata, Argentina, August 26-30.
3. Dolby,N., Dombrowski, K.E., & Wright, S.E. (1996) Design and Expression of a Synthetic Mucin Epitope Polypeptide in Escherichia coli. Fourteenth Annual Texas Regional Immunology Conference, San Antonio, TX, November 15-17.
4. Wright, S.E., Lowe, K.E., Talib, S., Kilinski, L., Dombrowski, K.E., Lebkowski, J.S., Philip, R. (1997) Antigen-Specific Cytotoxic T-Lymphocyte (CTL) Response Induced by Tumor-Specific MUC1 Mucin Peptide from Humans with Adenocarcinomas. Keystone Symposum: Cellular Immunology and the Immunotherapy of Cancer - III, Silverthorne, CO. Abstr. #238.