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AWARD NUMBER DAMD17-97-1-7301

TITLE: Development of a Novel, Proteinase-Activated Toxin Targeting Tumor Neovascularization

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REPORT DATE: October 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY *(Leave blank)*	2. REP October. 1998	3. REPORT TYPE AND DATES COVERED Annual (30 Sep 97 – 29 Sep 98

4. TITLE AND SUBTITLE

Development of a Novel, Proteinase-Activated Toxin Targeting Tumor Neovascularization

5. FUNDING NUMBERS

DAMD17-97-1-7301

6. AUTHOR(S)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research Command
ATTN: MCMR-RMI-S
504 Scott Street
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

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13. ABSTRACT *(Maximum 200 words)*

In the first year of this grant we have proved the feasibility of the original hypothesis of this grant: that we can re-engineer the proteolytic activation site of alpha toxin such that it is only activated in the presence of proteases which are involved in tissue remodeling during the neovascularization of breast tumors and other solid tumors. We have generated several mutants of alpha toxin in which we have introduced a consensus site for gelatinases A and B. We have explored methods of production of these recombinant toxins and have carried out the complete characterization of one of these mutants, $AT^{PLGIAG396}$. This mutant has the consensus gelatinase recognition sequence PLGIAG in place of the native sequence of K_{396}RSVDS. We have shown that this mutant can be cleaved by purified gelatinases A and B in vitro and exhibits some selectivity for the killing of cells expressing gelatinases versus those which do not. These initial results are encouraging and in the second year of the grant we will begin the characterization of the many other mutants we have generated and will expand our studies to examine other activation site mutants. Just prior to the submission of this report we have partially characterized a second mutant, $AT^{PLGIAG398}$. Unlike the pGEX 4T-2 expressed $AT^{PLGIAG396}$ described above this mutant was expressed in the pET22b+ expression system and retains full hemolytic activity and can be activated *in vitro* by gelatinase B.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer Alpha toxin metalloproteinase	12
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

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Rodney Turner 10/23/98

PI - Signature Date

I. Table of contents

II. Introduction

This work herein describes the initial stages in the development of a unique and innovative therapy for eliminating tumors. Unlike other tumor therapies in which the tumor itself is the primary target, the gelatinase-activated toxins to be developed in this study will primarily impact tumor vasculature, but with the added benefit of attacking invading tumor cells. Since tumor types vary widely in their susceptibility to cancer therapies, treatments based on tumor cell recognition often lead to the development of resistant tumor cells. This is particularly true when the treatment consists of drugs that are susceptible to cellular detoxifying mechanisms. Other therapies based on immunological approaches often require that a unique target be identified on the tumor cell that distinguishes it from a normal cell. The search for tumor-specific markers has been difficult since often times it is not a question of the absence or presence of a marker, but the relative concentration of the marker on tumor cells versus the level on normal cells. In the approach proposed here, we bypass the need to target a unique aspect of the tumor itself, and target the neovascularization of the tumor. The process of angiogenesis (the production of new capillaries) is necessary for the growth of breast tumors and other solid tumors. Targeting the angiogenic process enables us to target a wide variety of tumor types rather than just a single or few tumor types. In addition, tumors cannot become resistant to this form of therapy since it is not a tumor cell antigen or receptor that is the target. A fundamental requirement for angiogenesis is the dissolution of basement membranes, and possibly other extracellular matrix proteins, by the action of metalloproteinases. The fact that MMP inhibitors are such potent anti-angiogenic agents strongly supports this model. We are in a unique position to target the neovascularization of breast tumors for the following reasons: 1) we work on a unique Clostridial toxin which has a high cytolytic activity towards the vascular endothelium, 2) the toxin requires proteolytic activation, and 3) it appears that we can significantly alter the protease activation site of this toxin without affecting its structure or activity.

Neovascularization of tumors requires the localized presence and activation of MMPs, and most likely gelatinase A, to break down the basement membrane for the development of new capillary vessels. Therefore it is possible that we can target this process by the generation of gelatinase A target sequences in the activation site of *Clostridium septicum* alpha toxin. We have also proposed to develop more general, pan-MMP activated toxins with optimal substrate characteristics for cleavage by gelatinase A, but with some susceptibility to other MMPs. Based on studies with broad specificity MMP inhibitors used in several animal models, and based on a large number of localization studies, MMPs are typically not expressed in normal tissues, and, when expressed, appear to be latent. In other words, MMP activity is highly controlled, and occurs during developmental and repair processes, as well as pathologically. The few tissues known to express MMPs normally, including cycling uterus, may be susceptible to MMP-activated toxins. Because the mechanisms utilized by tumor cells to invade and attract a vasculature are common to those used by normal tissues, the problem of absolute tumor specificity is shared by all therapies. We believe that the toxins to be developed in this study offer a unique and effective means of combating tumor growth. It combines the specificity and efficacy of targeting active MMPs with the lethality of a very potent toxin. Treatments would thus be rapid, eliminating the need for a lifetime of therapy with its attendant complications and expense.

III. Body

A. Abbreviations and nomenclature used in this report

AT^{pro}: Native protoxin, protoxin is is the inactive form of alpha toxin and requires proteolytic activation by normal cell proteases such as furin in order to be converted to the cytolytically active toxin, **AT^{act}**.

$AT^{PLGIAG396}$: Alpha toxin in which the native sequence of AT^{pro} starting with residue 396 has been replaced by genetic engineering to now code for PLGIAG. Thus, any toxin designated with an superscripted amino acid sequence followed by a number describes a mutated alpha toxin in which the residues in the superscript replace the residues of the native sequence start with that numbered amino acid in the superscript.

Gelatinase A and B (Gel A and Gel B, respectively): These matrix metalloproteinases have been purified and pre-activated for the *in vitro* assays of there cleavage activity towards the various recombinant forms of alpha toxin which contain consensus gelatinase cleavage sites.

B. Overview of the year 1 effort towards re-engineering alpha toxin to selectively target the process of angiogenesis

Our primary efforts in the first year of this grant have been focused on the generation of substrate sites for the gelatinase A and B proteinases which replace the normal furin activation site of alpha toxin Fig. 1. Gelatinase A and B enzymes are two of the major proteolytic enzymes involved in the process of tissue remodeling of blood vessels involved in the neovascularization of tumors. Since these enzymes are primarily active during tissue remodeling they are intimately involved in the process of angiogenesis (the formation of new blood vessels) and tumorigenesis. Thus, it should be possible to re-engineer alpha toxin so that it is only activated within the blood vessels that are growing into tumors. Towards this end we have generated the alpha toxin derivatives shown in Fig. 1 in which the normal activation site of alpha toxin (RGKR) has been replaced with various target sequences for gelatinases A and B. We are also moving these sites within this region in order to identify the optimal location of this site within the activation domain of alpha toxin in order to optimize cleavage by the gelatinase enzymes. In addition we have also initiated studies into the use if urokinase sites (SGRS) since this enzyme has also been implicated in various cancers and angiogenesis.

```
Native AT        388-PLPDKKRRGKRSVDSLDARLQNEGIRIENI
PLGIAG393                 PLGIAG
PLGIAG394                  PLGIAG
PLGIAG396                     PLGIAG
PLGIAG398                        PLGIAG
PQGIAG396                     PQGIAG
PQGIAG398                        PQGIAG
Urokinase                   SGRS
```

Fig. 1. Position of mutated activation sites with respect to the native activation site for alpha toxin. The amino acid sequence of the native alpha toxin cleavage site is shown in the top line of this figure and the substituted residues for a optimum gelatinase A and B cleavage site are shown positioned below this region. Native alpha toxin is cleaved by furin and related cellular proteases between residues R398 and S399. The recognition sequence for the activation of native alpha toxin is underlined.

In Fig. 1 the native proteolytic activation site is shown for alpha toxin as well as those changes we have generated to alter this site so that it is recognized by gelatinase A and B. The sequence PLGIAG is one of two optimal cleavage sites for the gelatinase enzymes that we are substituting for the native cleavage site. The other site is PQGIAG as is shown in Table 1. We are expanding our studies to include additional sites for other proteases that might be in tumor neovascularization and tumorigenesis. The first of these alternative sites is the urokinase siteSGSR. Urokinase, in addition to the matrix metalloproteinases like gelatinases A and B, has been shown to play a major role in the tissue remodeling during the neovascularization of tumors (1). Therefore, this proteinase site also offers a potential mechanism to specifically activate alpha toxin within blood vessels that are infiltrating tumors. Also, the fact that the sequence is a less dramatic change to the activation site of alpha toxin also makes this an appealing alternative.

Mutant	Cloned	Expression	Purified	Trypsin Activation	Hemolytic	Digested by Gel A	Digested By Gel B
Expressed in pGEX 4T-2							
PLGIAG393	+	NA	NA	NA	NA	NA	NA
PLGIAG394	+	NA	NA	NA	NA	NA	NA
PLGIAG396	+	+	+	+	+	+	+
PLGIAG398	+	+	NA	NA	NA	NA	NA
PQGIAG396	+	-	NA	NA	NA	NA	NA
PQGIAG398	+	+	NA	NA	NA	NA	NA
SGRS	+	-	NA	NA	NA	NA	NA
Expressed in pET22b+							
PLGIAG393	+	NA	NA	NA	NA	NA	NA
PLGIAG394	+	NA	NA	NA	NA	NA	NA
PLGIAG395	+	NA	NA	NA	NA	NA	NA
PLGIAG396	+	+	+	+	+	-	-
PLGIAG398	+	-	NA	NA	NA	NA	NA
PQGIAG396	+	+	NA	NA	NA	NA	NA
PQGIAG398	+	+	+	-	-	NA	NA
SGRS	+	NA	NA	NA	NA	NA	NA
Native AT	+	+	+	+	+	-	-

Table 1. Summary of the alpha toxin mutants which have been generated and their testing status. In the "Mutant" column each substitution described in Fig. 1 is denoted by its sequence and the first amino acid number of alpha toxin which was replaced by this sequence. The second column specifies whether that mutant toxin has been cloned into either pGEX 4T-2 or pET33b+. The third column indicates whether the cloned recombinant has been purified. The fourth indicates whether trypsin can activate the toxin at a downstream activation site which can only be cleaved in vitro by trypsin. The fifth column indicates whether the toxin exhibits hemolytic activity when activated with trypsin and the last two columns indicate whether or not the toxin is cleaved by Gel A or B.

C. Generation and Purification of the recombinant alpha toxin derivatives

1. Generation and expression of alpha toxin recombinants

Some of the recombinant alpha toxin derivatives which contain the various activation site mutations described in Fig. 1 were expressed in two different expression systems as shown schematically in Fig. 2. In the first system the gene for alpha toxin was fused at its 5' end to the gene encoding glutathione-S-transferase (GST). The plasmid expression vector pGEX4T-2 carries the GST gene downstream of the inducible T7 promoter which can be induced by the gratuitous inducing agent IPTG. Thus, we can induce the intracellular expression of the GST-alpha toxin fusion protein to high levels. Typically we can purify 20 mg of these fusion proteins form an 8 liter culture. Purification of each GST-alpha toxin fusion protein is achieved by passing the crude lysate from the bacterial cells over a column packed with glutathione affinity gel. The fusion protein binds to the affinity matrix and the rest of the contaminating proteins pass through. The affinity column is then treated with a solution of 0.3M imidazole to specifically elute the GST-alpha toxin protein. The recombinant alpha toxin is then separated from the GST by treating the fusion protein with the protease thrombin which cleaves a site between the two proteins. The GST is removed by passing the mixture back over a glutathione column and only the free alpha toxin passes through the column. Therefore, this recombinant form of alpha toxin is expressed intracellularly in *E. coli*. It appears that alpha toxin expressed by this approach only retains 10-20% of the activity of the native toxin, probably due to its intracellular expression. However, as will be seen below this form of the recombinant toxins appears to exhibit some selectivity in its activation in the presence of gelatinase A and B.

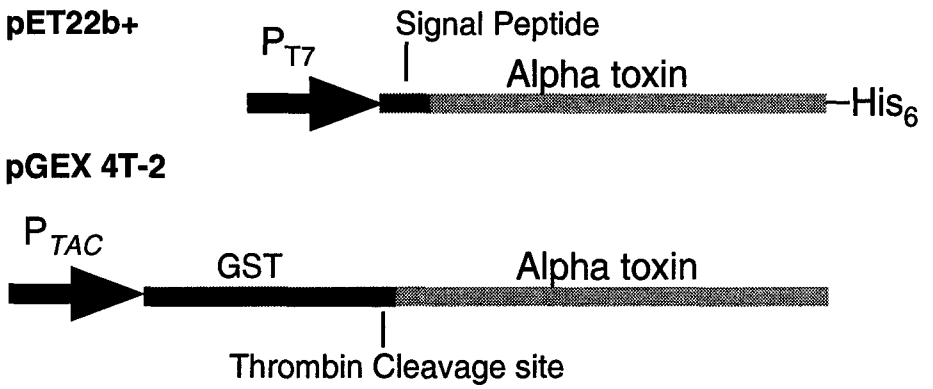

Fig. 2. Plasmid vectors used for the expression recombinant alpha toxin and its derivatives. The vector pET22b+ expressed a form of alpha toxin in which a pelB derived signal peptide was substituted for the native signal peptide of alpha toxin. The signal peptide directed the secretion of alpha toxin to the periplasm of E. coli BLR-DE3. Additionally, this vector fused 6 histidine residues to the carboxy terminus of alpha toxin which were subsequent used to purify the recombinant toxins by cobalt affinity chromatography. In contrast, the vector pGEX 4T-2 fused the alpha toxin directly to glutathione-S-transferase which resulted in the expression of the fusion protein in the cytoplasm of *E. coli*. pET22b+ drives the expression of the alpha toxin gene with the T7 promoter whereas pGEX 4T-2 is driven by the *tac* promoter. Both promoters are inducible with IPTG.

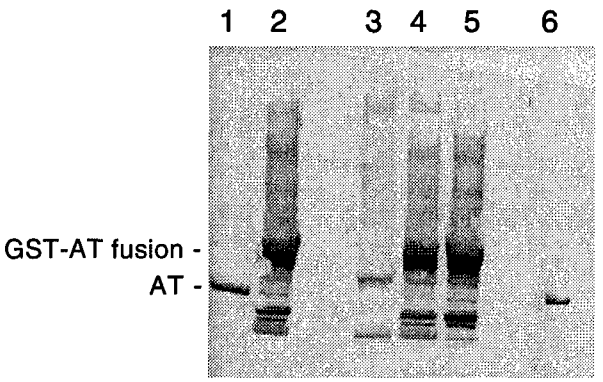

Fig. 3. Examples of the expression of various recombinants of alpha toxin in *E. coli*. The expression of selected GST-alpha toxin mutants in *E. coli* were detected by affinity purified anti-alpha toxin antibody. Lane 1, recombinant alpha toxin expressed in *E. coli* from the pET22b+ vector; lane 2, wildtype toxin fused to GST expressed from the pGEX 4T-2 vector; lane 3, negative control (proteins from *E. coli* carrying the pGEX 4T-2 vector); lane 4, same as lane 2 except the PQGIAG398 mutant was expressed in pGEX 4T-2, ;ane 5, same as lane 2 except the mutant expressing the urokinase site, SGRS was expressed. Fusion PLGAIG which replaced residues 396-411 of native alpha toxin starting at position 396 in alpha toxin

Normally, we have expressed alpha toxin as a secreted protein in *E. coli* since it is normally secreted from *Clostridium septicum*. The second expression system based on the pET22b+ vector (Fig. 2) duplicates this mode of expression. When alpha toxin is cloned into the pET22b+ vector the native signal peptide sequence is replaced by the signal peptide of the pelB protein so that the toxin is secreted into the periplasm of *E. coli*. Additionally, cloning the alpha toxin gene into pET22b+ fuses the carboxy terminus of alpha toxin to a hexahistidine sequence. The hexahistidine sequence facilitates the rapid affinity purification of this form of the toxin by the use of a metal chelate column loaded with Co^{+2}.

Why have we chosen two modes of expression for these recombinant derivatives of alpha toxin? Initially we utilized the pET22b+ system for expression since we have been expressing native alpha toxin from this vector successfully for many years (2, 3). However, some of the initial derivatives if alpha toxin which contained the gelatinase substrate sites PLGIAG at positions 396 and 398 were not expressed well or expression was inconsistent. Therefore, we utilized the pGEX 4T-2 expression system which had been used by us to successfully express an unrelated toxin. When this system was used for the expression of these alpha toxin derivatives it was found to be more reliable and yielded much larger quantities of each recombinant protein.

In spite of good yields of the recombinant toxins when expressed as a fusion in pGEX 4T-2 (Fig. 3) we have found that alpha toxin derived by this means is only 10-20% as active as active as native toxin or the recombinant native toxin expressed in the

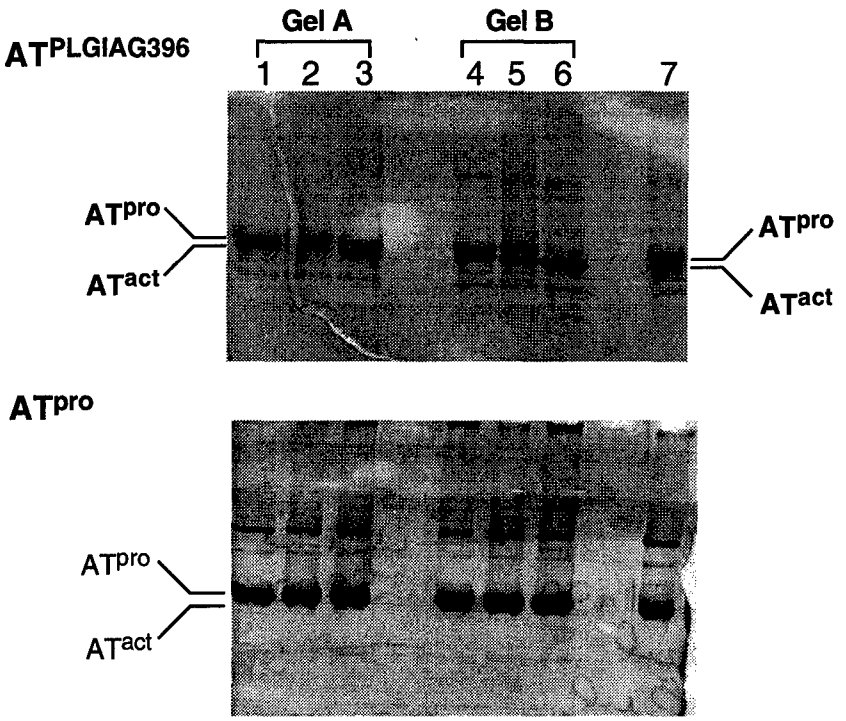

Fig. 4. *In vitro* cleavage of $AT^{PLGIAG396}$ and native AT with gelatinase A and B. 10 µg of purified $AT^{PLGIAG396}$ (upper panel) or AT^{pro} was digested with 10, 100 or 500 ng of gelatinase A (lanes 1-3) or 10, 100 or 500 ng of gelatinase B (lanes 4-6) for 4 hours at 37°C. Lane 7 contained either $AT^{PLGIAG396}$ (upper panel) or AT^{pro} (lower panel) which had been treated the same as the other samples but had not contained any proteinase.

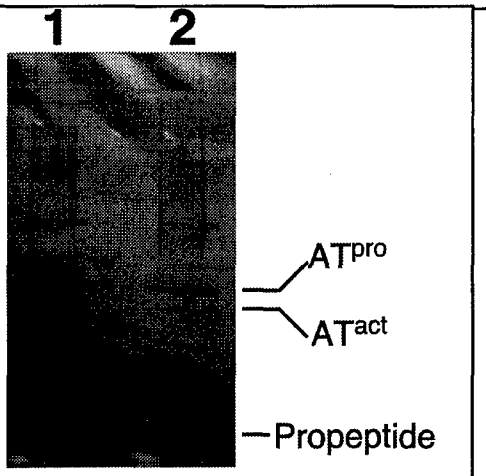

Fig. 5. Proteolytic cleavage of $AT^{PLGIAG398}$ by gelatinase B. 10 µg of $AT^{PLGIAG398}$ was incubated with 500 ng of gelatinase B for 4 hours at 37°C (lane 2). In lane 1 the same toxin was incubated under identical conditions but no enzyme was added.

pET22b+ system. However, as described in the next 2 sections these less active variants exhibit some selectivity towards cells expressing gelatinase A and B. Also, these mutants also appear to be efficiently cleaved by gelatinase A and B *in vitro* with purified the enzymes. Thus, it is possible that the less active toxin derivatives may exhibit a greater selectively in attacking gelatinase producing cells. However, we are just beginning of this endeavor and have only explored a single derivative which shows some promise.

D. In vitro activation of an alpha toxin derivative containing the PLGIAG sequence by gelatinase A and B

In the first year we have primarily been making mutations in the activation site and purifying these derivatives for analysis. To date we have carried one of the derivatives through all of the analysis we originally proposed to test the feasibility of this approach. $AT^{PLGIAG396}$ was expressed as a fusion protein with GST, purified and assayed for its susceptibility to cleavage by gelatinases A and B. The assay consisted of incubating 10 µg of the recombinant alpha toxin or native AT^{pro} with 0.01, 0.1 or 0.5 µg of pre-activated gelatinase A or B (Fig. 4). As can be seen in Fig. 4 gelatinase B cleaves $AT^{PLGIAG396}$ in a dose-dependent manner and gelatinase A cleaves to a lesser extent. Neither enzyme cleaves native AT^{pro} (Fig. 4) showing that it is possible to replace the native site with another protease recognition site and thus generate a new activation site.

When we expressed $AT^{PLGIAG396}$ in the pET22b+ expression system we determined that its hemolytic activity was similar to that of native toxin (by in vitro activation with trypsin) but that the gelatinase enzymes did not cleave this toxin (not shown). We believe that one of the reasons for the inability of the gelatinases to cleave $AT^{PLGIAG396}$ may be because of the presence of the polyhistidine tag located at the carboxy terminus of this construct. We are currently exploring the effect of removing the polyhistidine tag on the ability of the gelatinase enzymes to cleave pET22b+ expressed $AT^{PLGIAG396}$.

Just prior to the submission of this report we isolated and purified another derivative of α-toxin, $AT^{PLGIAG398}$ which appears to retain wildtype cytolytic activity. This mutant was also expressed in the pET22b+ system and unlike $AT^{PLGIAG396}$ that was expressed in pET22b+, it can be cleaved *in vitro* by gelatinase B (Fig. 5). This latest finding is particularly exciting since we proposed in the "Conclusion" section of this report that we believed that moving these gelatinase cleavage sites downstream of position 396 of alpha toxin would possibly enhance cleavage. As it turns out we were apparently correct. Whereas $AT^{PLGIAG396}$ was not cleaved by gelatinase in vitro, $AT^{PLGIAG398}$ is cleaved.

E. Cellular killing by alpha toxin derivative $AT^{PLGIAG396}$

We recently evaluated the $AT^{PLGIAG396}$ recombinant toxin for the ability to selectively kill cells in which gelatinase A and B were active versus cells in which these enzymes were specifically inhibited with the peptide hydroxamic acid based inhibitor GM6001. In this assay 5387 fetal lung fibroblast cells were induced for gelatinase A and B production and were treated with serial 2-fold dilutions of purified $AT^{PLGIAG396}$ in the presence and absence of the GM6001 inhibitor of metalloproteinases. If $AT^{PLGIAG396}$ showed a selectivity for killing the cells in which the gelatinase proteinases were not inhibited with GM6001 it would suggest that $AT^{PLGIAG396}$ was preferentially activated by the gelatinase enzymes. As can be seen in Fig. 6 we observed approximately a 2-fold difference in cell killing in the presence and absence of the inhibitor. These results were consistent when repeated several times. These preliminary data are very exciting and suggest that we can indeed selectively kill cells producing metalloproteinase enzymes by re-engineering the activation site of alpha toxin to be cleaved by these enzymes. Although the selectivity was not high it was consistent with this one mutant. Thus, as stated below we feel that we can now begin the work of optimizing the activation of alpha toxin by gelatinases by changing the location and presentation of the gelatinase activation site in alpha toxin.

IV. Conclusions

The first year of this grant was devoted to the construction and expression of various derivatives of alpha toxin which contained various consensus sequences for the metalloproteinases gelatinase A and B which were substituted for the native activation site of alpha toxin. In addition we also examined various modes of expression of these constructs and discovered that intracellular expression decreased the activity of the toxin significantly but still yielded useful results. We have fully analyzed one of these recombinants, $AT^{PLGIAG396}$ for *in vitro* cleavage with gelatinases A and B and the ability to differentially kill cells which express active gelatinase enzymes. These data confirm our original hypothesis that we could re-engineer the proteolytic activation site of alpha toxin so that preferential cleavage and activation could take place in the presence of metalloproteinases like gelatinases A and B.

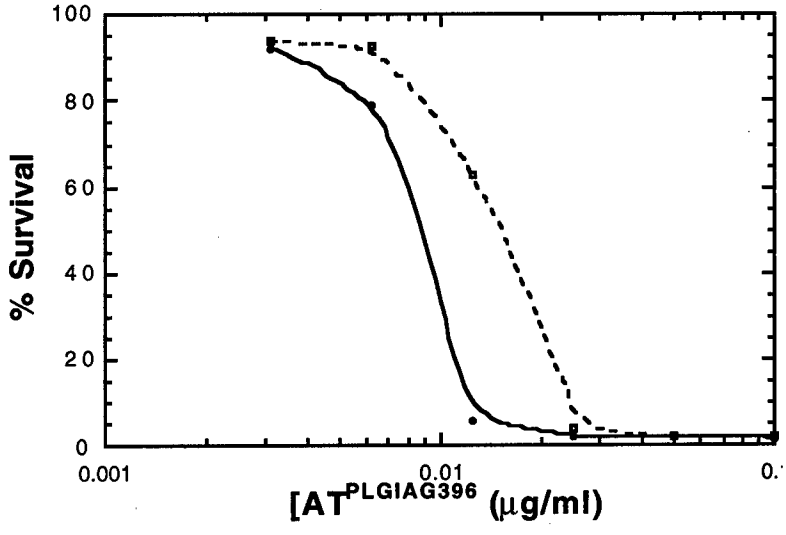

Fig. 6. Cell killing by $AT^{PLGIAG396}$ in the absence and presence of gelatinase inbitor. 5387 fetal lung fibroblast cells which expressed gealtinase A and B were treated with $AT^{PLGIAG396}$ in the absence (O) or presence (□) of the gelatinase peptide inhibitor GM6001.

We have also found that expression of alpha toxin, either the native form or derivatives containing metalloproteinase sites, as a GST fusion protein are generally less active than alpha toxin expressed into the periplasm of *E. coli* (via the pET22b+ expression system). Therefore, one of our first priorities in the second year will be to examine the expression of these constructs in the pET22b+ expression system (Fig. 2). Originally we tried the pET22B+ expression system and obtained variable results. Since then we have had better success with this system and are re-exploring its use for the expression of these recombinant toxin molecules.

Where are going in the next year? The initial results with the $AT^{PLGIAG397}$ mutant have proven that we can re-engineer the specificity of the activation site of alpha toxin so that it is specifically activated in the presence of the matrix metalloproteinases gel A and gel B. Although the selectivity of $AT^{PLGIAG396}$ was not as high as we had hoped it is just the first in a large number of recombinants we will be examining. Based on the first year studies we have several avenues we intend to explore. First, as evident in Table 1 we have constructed several other mutants in which the PLGIAG site and PQGIAG consensus sites have been moved around within the activation site region of alpha toxin. We believe that the location of the gelatinase cleavage site may play an important role in the efficiency of the activation event. It is clear that location of this site in $AT^{PLGIAG396}$, is sub-optimal for recognition and cleavage by the gelatinase enzymes. Therefore we have already begun to generate mutants which move these sites upstream and downstream of this location. Most of the mutants already generated move these sites from residue 393 to 398 (Table 1). However, we believe that moving the site downstream of position 396 may be more desirable since it may make this region more accessible to the gelatinase enzymes. In addition, there remains a native minor activation site in alpha toxin (located at arginine 406) which is cleaved by trypsin *in vitro* that has not been removed yet from the native alpha toxin sequence. We have found it useful to be able to check the activation and cytolytic activity of the mutants containing the gelatinase cleavage sites by *in vitro* activation with trypsin for one reason: it is a convenient way to check that the recombinant toxins are as cytolytically active as native toxin even if the metalloproteinases do not cleave these mutants. However, once we find a site that is optimal for the selective killing of gelatinase producing cells we will change this arginine so that trypsin or other serine proteases cannot activate the toxin at R406 *in vivo*.

We are also beginning to examine the possibility to introduce urokinase sites into alpha toxin. Urokinase has also been shown to be highly active duting the process of angiogenesis, tumor neovascularization and tumor growth. Therefore it offers another means of targeting alpha toxin to the process of tumorigenesis and neovascularization.

V. References

1. **Rabbani, S. A.** 1998. Metalloproteases and urokinase in angiogenesis and tumor progression. In Vivo. 12(1):135-42.
2. **Sellman, B. R., B. L. Kagan, and R. K. Tweten.** 1997. Generation of a membrane-bound, oligomerized pre-pore complex is necessary for pore formation by *Clostridium septicum* alpha toxin. Molec. Microbiol. **23:**551-558.
3. **Sellman, B. R., and R. K. Tweten.** 1997. The propeptide of *Clostridium septicum* alpha toxin functions as an intramolecular chaperone and is a potent inhibitor of alpha toxin-dependent cytolysis. Molec. Microbiol. **25**(3)**:**429-440.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 23 Aug 01

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to the technical reports listed at enclosure. Request the limited distribution statement for these reports be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
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