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POSSIBLE APPLICATION OF BIOTECHNOLOGY TO THE DEVELOPMENT OF BIOLOGICAL AGENTS BY POTENTIAL ENEMIES

by William E. White, Ph.D.
RESEARCH DIRECTORATE

June 1987

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Aberdeen Proving Ground, Maryland 21010-5423

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Biological agents, including both vegetative organisms and toxins, have posed a considerable threat to the military in the past. New developments in biotechnology, including recombinant DNA, hybridomas, fermentation, and genetics, provide the scientific basis for developing new biological agents and for modifying existing ones. Agents posing little threat in the past may become major concerns in the future. Biotechnology might be used to increase toxicity, to produce large quantities of a material, or to alter the method of delivery. This report begins with a brief description of different disciplines that collectively constitute biotechnology and then describes how selected agents could be modified. The author did not attempt to analyze the technical expertise of potential enemies or investigate their ongoing military research.

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SUMMARY

In 1972 the United States unilaterally destroyed all of its Biological Weapons (BW). Since then, it has neither performed nor contracted any Research and Development for offensive or retaliatory biological weapons. The official U.S. government policy is to conduct BW research for defensive purposes only and to limit studies to those that would protect the soldier from the effects of BW agents delivered by hostile forces. This document is the first in a series of reports to assess and reevaluate the future threats from biological weapons as a result of new developments in biotechnology and related scientific disciplines. The expressed opinions and conclusions do not represent official U.S. Army Chemical Research, Development and ngineering Center; U.S. Army Materiel Command; or Department of the Army positions or policy.

For many years, military commanders were not overly concerned about the threat of biological weapons because more immediate and pressing problems took precedence over the somewhat nebulous threat from bacteria, viruses, etc. The incubation period or length of time between exposure to the biological agent and the resulting effect on the infected troops reduced the tactical value. Also, the impossibility of controlling the spread of the organisms following release discouraged their use. New scientific advances might permit the development of new or modified biological weapons with greater effectiveness and reliability. Other modifications could lead to agents that were difficult to detect with current technology. Changes in the tactics of terrorist groups, who do not consider the long-term consequences of their actions, could lead to a deliberate attack with a biological agent.

Although it is unlikely that any clandestine organization could conduct a major research effort to develop a sophisticated weapon, there is so much ongoing basic and applied research in the medical, academic, and industrial communities that the essential components will eventually become available without any military involvement.

Biological weapons, which were previously considered threats, should still be considered threats until there is sufficient scientific or intelligence data to permit a reevaluation. This report does not assess the risk or threat of any biological agent. It addresses the question of what could be done with present and developing biotechnology to alter biological materials and increase their potential as weapons.

Recent advances in molecular biology catalyzed enormous research and created a new industry in biotechnology. The central dogma of molecular biology is that genetic information flows from deoxyribonucleic acid (DNA) to ribonucleic acid (RNA) to protein. It is now possible and reasonably simple to remove genes (sections of DNA) from one organism and introduce them into a second so that the original protein is produced by a different organism.

By proper manipulation of the system, thc quantity of protein produced is much greater in the recombinant organism than in the original.

Cell-fusion techniques, which provide the basis of monoclonal antibodies, permit the production of large quantities of proteins without isolating and cloning the corresponding gene. Site-specific mutagenesis alters a gene in a prescribed manner so that the resulting protein is changed in a defined way.

Toxins can be divided into two large classes. The proteinaceous toxins are proteins that are synthesized biologically by polymerizing amino acids in the order prescribed by the corresponding genes. In contrast, the nonproteinaceous toxins are smaller molecules that are synthesized biochemically by a series of enzyme catalyzed reactions. Although cloning techniques are directly applicable to the manipulation of proteinaceous toxins, they would be less useful for nonproteinaceous toxins because the corresponding gene for each enzyme must be cloned and expressed in a coordinated manner.

Effective biological warfare agents must (1) exert a detrimental effect on some physiological process, (2) be produced in sufficient quantity, and (3) enter the body and reach the site of action at an effective concentration. Biotechnology could be applied to developments in all three areas.

There are so many toxins and pathogenic organisms that could be selected for development by potential enemies that any list not based on extensive intelligence would be either conjecture or too lengthly to be useful. The approach in this report is to select a few examples and describe representative modifications.

In the near future, biotechnology could probably be used most effectively to produce large quantities of nonproteinaceous toxins by culturing organisms in fermentors and isolating the products. Saxitoxin or some of the related toxins from the marine unicellular dinoflagellates are reasonable choices.

Anatoxin A from blue-green algae is extremely toxic. Because it has a relatively simple structure, it could be synthesized in considerable quantity by traditional chemical methods. Biotechnology does not appear to offer any significant advantage for development of Anatoxin A at this time.

In the mid term, bacteria could be modified by recombinant DNA techniques to produce different proteinaceous toxins or modified into hardier or more virulent organisms.

Viruses might become the long-term or ultimate threat. These intracellular parasites have efficient mechanisms for invading or infecting cells. Because viruses can control cellular metabolism, large quantities of viral protein are produced and then released when the cell lyses. Recombinant DNA techniques could be used to introduce genes for proteinaceous toxins or, more likely, for small neuropeptides like enkaphalins. Then, infection by a few virus particles would result in the replication of the virus and production of lethal amounts of the toxin *in vivo.*

PREFACE

The work described in this report was authorized under Project 1L162706A553A, Chemistry and Effects of Threat Agents. This work was started in October 1985 and completed in March 1987.

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CONTENTS

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POSSIBLE APPLICATION OF BIOTECHNOLOGY TO THE DEVELOPMENT OF BIOLOGICAL AGENTS BY POTENTIAL ENEMIES

1. OBJECTIVES

This project assessed how biotechnology could be used by potential adversaries to enhance the threat of biological agents. For example, toxins that are available only in research quantities might soon be produced in large amounts. Consequently, agents that currently pose little threat (except perhaps for assassination) could become major threats in the future. What are the problems associated with particular agents that limit their effectiveness? How might biotechnology overcome them? Is the required technology currently available? If not, what are the critical steps that must be achieved before the technology will become feasible? Are such developments likely to be reported in the open literature? What are the indicators that potential adversaries are pursuing this research?

The author did not attempt to evaluate the technological competence of potential enemies, and this report does not include evidence of ongoing research unless it has been published in the open literature. Also, the report does not include classified data relating to the terminated biological warfare (BW) program involving pathogenic organisms. In order for a particular biological material to pose a direct threat to military forces, it must be toxic, must be produced in sufficient quantity, and must be delivered into the soldier. Biotechnology is developing the capability of modifying each of these parameters. Rather than examining all the complex relations between toxins, production, and delivery, this report focuses on selected toxins and other biological material and how they could be altered, produced, and delivered. The principles and approaches illustrated by the particular examples would usually be applicable to other biological systems.

Brief summaries of some of the important areas of biotechnology such as molecular biology and cell fusion are included so that individuals who are unfamiliar with recent discoveries in the field can interpret and evaluate the results. The broad area commonly called biotechnology is advancing so rapidly that no one can anticipate all of the major advances. What is currently possible may be surmised with reasonable accuracy; however, what will be possible in the future is only conjecture. If the technology moves as suggested, the proposed developments may be possible. If new breakthroughs occur (as they will), other approaches will become feasible. By including the information leading to the speculations, it will be possible to revise continually the estimates when new developments occur.

2. INTRODUCTION

Biological agents, both vegetative organisms and toxins, have posed a considerable threat to the military for many years. New scientific developments, as well as changing political and ethical climates, have increased the number of possibilities for development, deployment, and use of these weapons by overt military forces and terrorist groups.

Recombinant DNA techniques permit the transfer and subsequent expression of genetic information from one species to another in a planned and prescribed manner. Cell-fusion techniques, in which the genetic information from two different cells is combined to produce a hybrid cell, permit the production of large quantities of particular proteins by eukaryotic cells. Thus far, this technology has been used principally to produce monoclonal antibodies; however, hybridomas that secrete other proteins such as enzymes have been developed [1].

Rapid progress in fermentation technology has been fueled by industry in anticipation of the need when recombinant DNA products reach the production stage. In the past, fermentation was restricted to the food industry (e.g., wine, beer, cheese) or the pharmaceutical manufacturers for antibiotic production [2]. New advances in bacterial culture increased cell densities from 10-20 g/liter to over 200 g/liter . This progress will permit greater production in smaller reactors, which should reduce capital cost and also increase the difficulty of discovering covert production facilities. The higher yields should reduce the cost of nutrients for the organisms. Lower production costs are important for commercial operations in competitive markets but may be secondary for small-scale military applications. New fermentors are also being developed that generate less mechanical shear. Bacteria and yeast have rigid cell walls composed of lipopolysaccharides and other macromolecules, whereas the cells from higher organisms have only a membrane composed of a lipid bilayer containing a few proteins that do not add to the stability. As cell density increases in a fermentor, oxygen usually becomes the limiting factor. Frequently cells shift to anaerobic metabolism in spite of attempts to maximize the introduction of oxygen. In the past, bacteria and yeast were the principal organisms grown in fermentors. Consequently, they were designed to maximize dissolved oxygen by vigorous mixing. Today with the interest in culturing mammalian cells, new low-shear fermentors are reaching the market that provide high oxygen availability.

Analytical techniques are also advancing in an attempt to keep pace with biological technology. High Performance Liquid Chromatography (HPLC) offers the possibility of separating and purifying substantial quantities of biomolecules quickly without the need for exhaustive research to develop purification schemes for the particular polymer.

Gas-phase protein sequencers permit the sequencing of picomole quantities of material. The ability to sequence with less material permits protein purification by analytical techniques rather than preparative methods and thereby reduces considerably the time required for sample preparation. DNA sequencers are also becoming automated using fluorescent adducts.

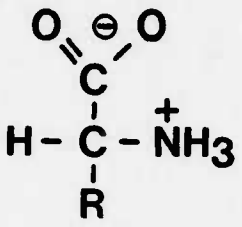
3. TECHNOLOGIES

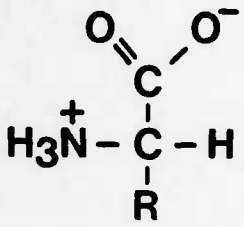
3.1 *Gene Cloning.*

3.1.1 *Molecular Biology.*

All living things are composed of cells [3]. Bacteria (prokaryotic organisms) consist of cytoplasm surrounded by a cellular membrane that is encased by a cell wall. The genetic material or DNA is distributed through the cytoplasm. In all other organisms (eukaryotic), the DNA is contained in discrete bodies or organelles. Most of the DNA is chromosomal and located in the nucleus; however, smaller amounts are found in mitochondria and chloroplasts.

Proteins constitute the largest portion of biological macromolecules. Each protein molecule is a polymer of individual amino acids that have been coupled by peptide bonds. The carboxyl moiety on one amino acid residue is connected to the amino group on the adjacent amino acid (Figure 1). Although there are only 20 common amino acids, they can be arranged in an infinite array to produce a wide variety of proteins.





D - AMINO ACID **L - AMINO ACID**

R = ALKYL, AROMATIC, HYDROXYL, ACIDIC, BASIC, SULPHUR

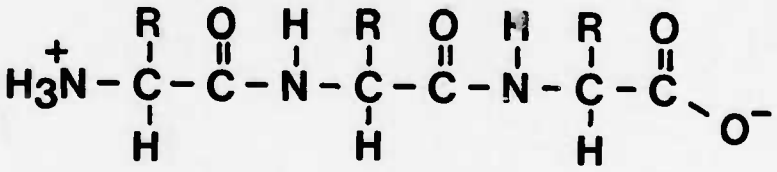


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Figure 1. **Amino Acids and Proteins**

Whereas proteins have different functions based on their individual structures, the only known function of DNA is the maintenance and expression of genetic information. Deoxyribonucleic acid (DNA) consists of a copolymer of sugar molecules (deoxyribose) connected by phosphates (Figure 2). A heterocyclic base (adenine, guanine, cytosine, or

thymine) is connected to the deoxyribose at the one position. Adenine and guanine (purines) are larger than cytosine and thymine (pyrimidines).

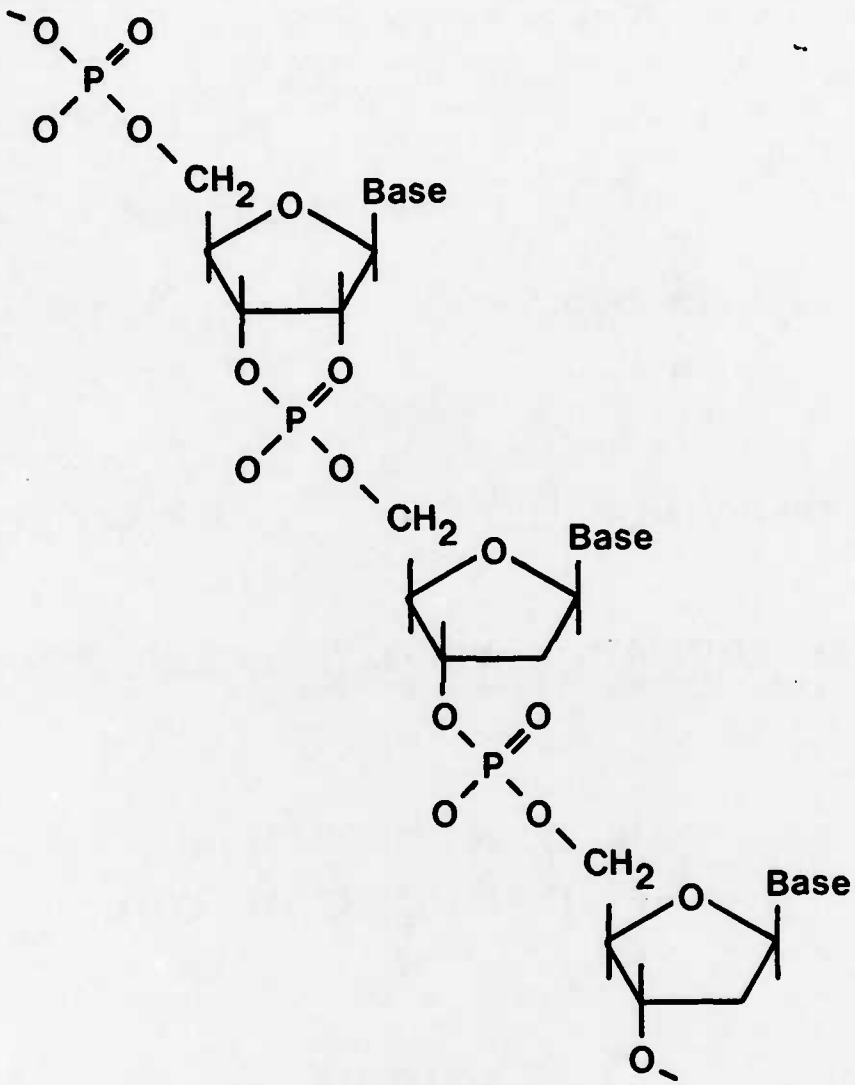

Figure 2. Nucleic Acids

The most common conformation for DNA is the double helix that was described by Watson and Crick in 1953 [4]. The two polydeoxyribose phosphate chains are wound into a double helix and are oriented in opposite directions so that the "head" of one is opposite the "tail" of the other. The bases are positioned inside the helices and perpendicular to the long axis of the molecule. There is only room for one purine and one pyrimidine inside the helix. Because of structural parameters and hydrogen bonding interactions, adenine pairs with thymine and guanine pairs with cytosine. These interactions provide the basis for life. During

DNA replication, the two strands separate as new strands form. Thus, after cell division, a DNA molecule consists of one original strand and one newly synthesized or nascent strand.

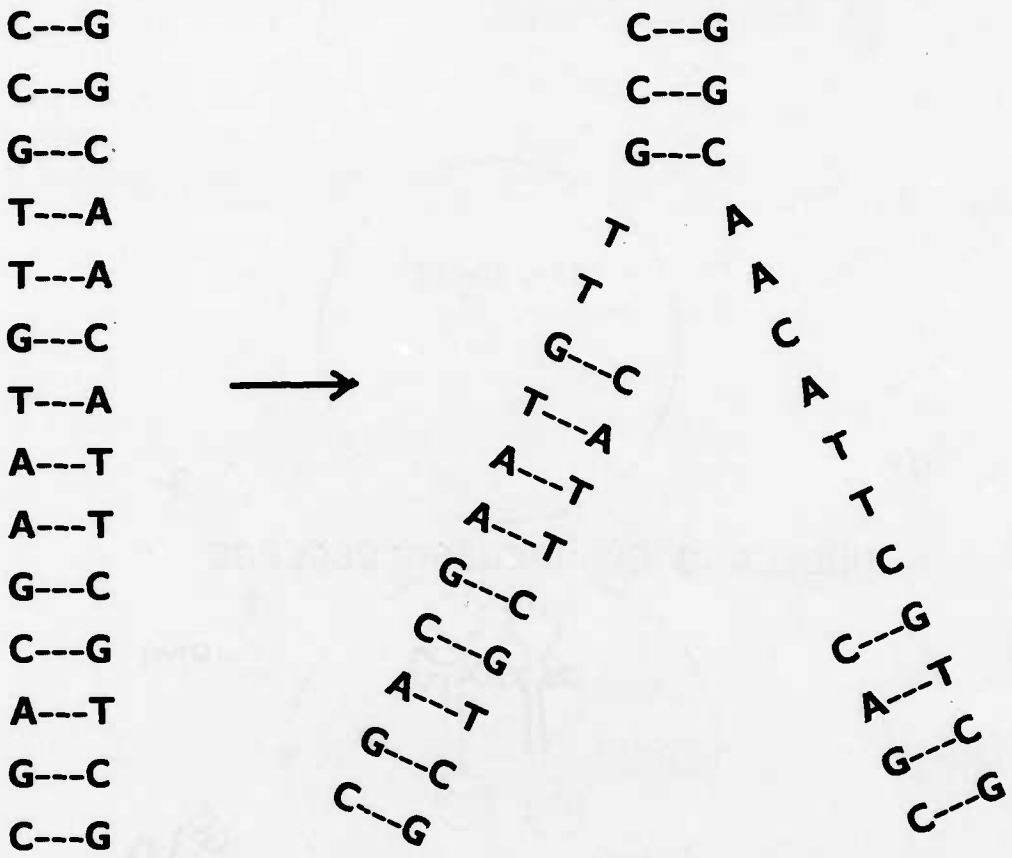

Figure 3. **DNA Replication**

Ribonucleic acid (RNA) is another class of biopolymer that is very similar to DNA. The principal difference is the presence of an oxygen or hydroxyl at the two position of ribose and the absence at the two position of deoxyribose (hence the name). The additional hydroxyl makes the phosphodiester bond more labile so that the half-life of some RNAs is considerably less than that for DNA. Also RNA contains the pyrimidine uracil (U) rather than thymine (T), which is 5-methyl uracil.

The central dogma of molecular biology is that information flows from DNA to RNA to protein. The only known exceptions occur in a few RNA viruses that initially produce DNA from RNA. There are no known examples of information flowing from protein to RNA or DNA. The sequence of bases in DNA contains the information that is eventually translated into protein. In order to provide better regulation of protein synthesis and minimize DNA damage (in some cells DNA must last a lifetime), the information is first transcribed into messenger RNA (mRNA) in a manner somewhat similar to DNA replication. Following transcription, the mRNA is translated into the correct protein sequence at the ribosome. (Figure 4.) A three base sequence on the mRNA or codon is required for each amino acid. The 64 combinations (4 to the third power) provide at least one codon for each of the 20 amino acids plus punctuation for starting and stopping protein synthesis at the correct site. For example,

UCA (uridine-cytodine-adenosine) codes for serine while AAG codes for lysine and UAG terminates protein synthesis. The more common amino acids have several codons, whereas the rare ones have only one.

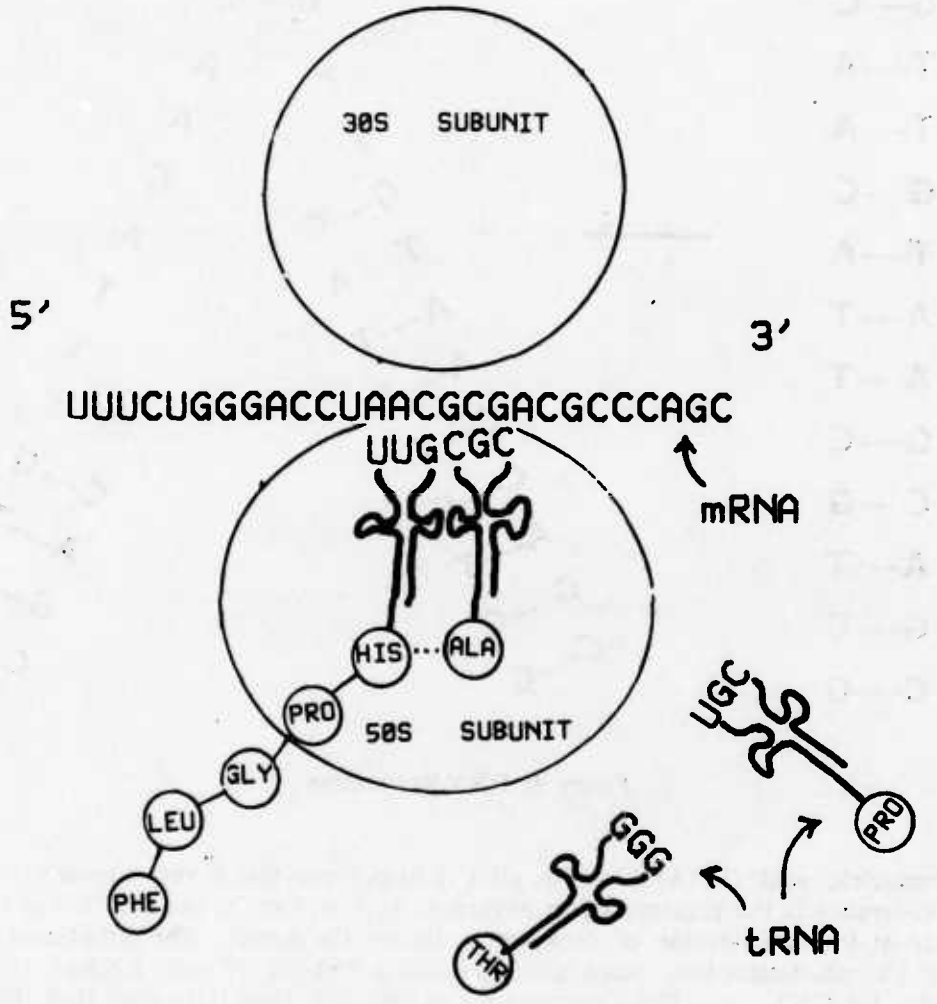

Figure 4. **Protein Synthesis**

Although all cells of an organism contain the same genetic information, not all cells of the organism are alike because different cells express their DNA differently. The control or regulation of gene expression is a major area of research today. The reasons why only blood

cells make hemoglobin, why neurons release neurotransmitters, and why skin cells produce melanin are not well understood. This research will have an impact on military applications if foreign genes are inserted into new biological hosts to produce a new or improved biological weapon.

3.1.2 *Introns and Exons.*

Until DNA sequencing techniques emerged, scientists generally believed that genes were located in discrete units along the chromosome. They believed that genes contained a beginning and an end, and everything between was part of the genetic information. This concept still appears to be true for prokaryotes, which are bacteria, and the few other organisms that do not contain a nucleus. In contrast, the eukaryotic (organisms containing chromosomal DNA inside the nucleus) genes consist of coding regions called exons separated by noncoding regions called introns. The entire sequence, from the initiation site to the termination site, is transcribed into mRNA precursor. Inside the nucleus, the intron regions are removed (by a procedure not well understood) to form processed mRNA, which is then transported outside the nucleus into the cytoplasm where protein synthesis occurs. In bacteria, there is no nuclear membrane to separate transcription from protein synthesis; therefore, it would not be possible to remove noncoding segments from precursor mRNA. Consequently, prokaryotic organisms do not have introns nor the machinery for removing them. The inability of bacteria to process mRNA has hampered the production of eukaryotic proteins by recombinant DNA techniques. DNA removed from eukaryotic organisms and inserted directly into bacterial hosts seldom produces a useful protein. There are examples of eukaryotic genes without introns; however, they are the minority.

The limitation imposed by introns can be overcome by inserting cDNA into bacterial vectors. The usual procedure is to isolate all of the mRNAs from the cytoplasm of a cell and then use them as a template to produce a synthetic DNA. The enzyme reverse transcriptase (RNA dependent DNA polymerase), which is obtained from retroviruses, is the only enzyme known that can produce a DNA chain from RNA. The synthetic gene, which contains no introns because it was derived from a processed mRNA, can be inserted into an appropriate vector and then introduced into a bacterium for amplification and subsequent expression. It is usually easier to produce an entire cDNA library, containing all the functioning genes, and select the desired recombinant rather than isolate the desired mRNA first in order to produce the corresponding DNA molecule.

3.1.3 *Restriction Endonucleases.*

Restriction endonucleases are used to remove a region of DNA from one locus and insert it at another site. The discovery and subsequent commercial availability of restriction endonucleases made recombinant DNA research feasible. Bacterial DNA contains about 4 million base pairs, whereas eukaryotic DNA contains about 2 billion. The enzymes (DNAses) that were discovered years ago digest DNA essentially randomly and produce so many fragments in such low concentrations that can not be used in further studies. In contrast, restriction endonucleases are very specific and, therefore, afford identical polynucleotides in reasonable concentrations. For example, EcoR1 (isolated from an *E.coli* bacterium) recognizes the sequence GAATTC. A prescribed sequence of six nucleotides would mathematically occur once in every 4096 base pairs (4 to the sixth power). Of greater significance is the location of the cleavage. EcoR1 cleaves or cuts the polynucleotide between the G and the A. Because the

sequence has an inverted symmetry (as do most sequences recognized by restriction enzymes), the complementary strand will also be cut between the G and A.

G*A-A-T-T-C
C-T-T-A-A*G

Thus, following restriction the two strands will have unpaired or "sticky ends" that can be joined to or recombined with other polynucleotides that have the complementary sticky ends. Therefore it is possible to cut a large DNA molecule into a few smaller units and then insert the fragments in a prescribed manner into other DNA molecules. Many restriction endonucleases with different recognition sites are available commercially. Collectively, these provide considerable flexibility for recombinant DNA experiments.

3.1.4 *Site-Specific Mutagenesis.*

Although many scientists are studying protein structure, the total design of a protein with predetermined structure and function is beyond current technology. A major objective of academic research is the discovery of basic principles that lead to a thorough understanding of a particular phenomenon. The development of an artificial protein with a predetermined function that resulted from a planned structure is an example of a thorough understanding of molecular interactions. Those in industry would like to use "designer" proteins, particularly enzymes, for commercial operations because their stereospecific and regiospecific catalysis permits the manufacture of a variety of unique products. Because of the theoretical, as well as practical aspects, work will continued with funding from many sources so that the design of proteins will eventually become feasible.

The difficulty is in designing the proteins -- not in producing them. Because the genetic code is well understood, genes can be conceived that would produce the protein. Polynucleotides can be synthesized with automated equipment with sufficient fidelity to permit introduction into vectors for production. For long polynucleotides, it is usually easier to use a block approach. A portion of the gene is synthesized and introduced into a plasmid to increase the concentration. Then the polynucleotide is removed, and additional nucleotides are added sequentially. This stepwise approach reduces contamination that results from incomplete reactions.

The primary sequence of a protein ultimately determines its secondary, tertiary, and quarternary structures. The primary structure is the amino acid sequence. The secondary structure refers to the conformation of the peptide backbone and may differ in different regions of the protein. The protein may exist in helices, sheets, or random coils. The tertiary structure refers to the overall shape of the entire protein. It may be globular, linear, or bent in some other manner. The quarternary structure refers to the aggregation of individual peptides into a larger unit. Occasionally, enzymes aggregate into large complexes. Some of the subunits are catalytic and bind substrates, whereas others bind regulatory molecules. Upon synthesis, a protein bends and folds in order to achieve the lowest energy state (i.e., the most stable conformation). The amide hydrogens form hydrogen bonds with neighboring acyl oxygens. The hydrophobic residues may be clustered in the center of the molecule, while the charged and hydrophilic ones are located on the outside where they can hydrogen bond to water molecules. There are also bulk steric requirements of the larger amino acids as well as stacking interactions between aromatic amino acids. For even a small protein containing 75 amino acids -- each with 15 atoms, the complexity of calculating the interactions is too great for current computers.

Design and synthesis of short peptides with unique functions are possible. Valinomycin is a cyclic dodecapeptide that facilitates transport of potassium ions across biological membranes and lipid bilayers. The molecule exists as a helix with a cavity of about 2.6 Angstroms, which

is optimal for binding a potassium ion when coordinated with six acyl oxygen moieties. By understanding the hydrogen bonding and other interactions that resulted in the appropriately sized cavity, it was possible to design an analog whose cavity would be optimal for binding calcium. The peptide was prepared by solid-phase synthesis, its conformation determined by spectroscopy, and its affinity for calcium confirmed [5].

The synthesis of large molecules with relatively simple structures has also been successful. The synthetic protein, betabellin, was designed to provide a stable protein backbone upon which active elements could be superimposed. Betabellin, containing 66 amino acid residues, is designed to fold into antiparallel beta pleated sheets of four beta strands that are connected by beta turns. Several different Betabellins, containing slightly different amino acid sequences, were synthesized to provide samples for spectroscopic studies that confirmed that the structure is similar to the intended [6].

Although it is not currently feasible to design entire enzymes, it is possible to modify them. Originally, modification involved treating the protein with a chemical reagent that altered some of the amino acids. Usually, this approach resulted in loss of activity. Random mutagenesis of the gene, which codes for the protein, affords a permanent change so that the modified protein can be continually produced by the microorganism. A bacterium can be subjected to UV radiation, dimethyl sulfate, or other mutagens to produce a heterogeneous population that can be screened for the desired trait. If this trait is critical, an enrichment will lead to growth by the desired bacterium only. If there is no exploitable property, then isolating the desired mutant from the others is virtually impossible.

The availability of numerous restriction endonucleases and improvements in chemical synthesis of polynucleotides makes site-specific mutagenesis feasible. The cassette or module approach is among the more common (Figure 5). After the sequence of the gene has been delineated, it is possible to determine which bases must be replaced in order to effect the desired change. The size of the cassette is determined by the location of unique restriction sites at each end, which are needed for removal of the original sequence and substitution of the new. A polynucleotide (DNA) is synthesized with a sequence identical to the native except for the intended substitution. Then the segment is removed from the native gene and replaced with the synthetic. The sticky ends are allowed to hybridize, and the ends of the phosphodiester backbone are joined by a ligase (an enzyme that connects phosphates to sugars without adding additional nucleotides). The result is the creation of a specific mutation at a prescribed locus.

For example, bacterial alkaline phosphatase catalyses the hydrolysis of several phosphate monoesters. Hydrolysis is initiated by attack of the serine 102 on the phosphate to form a phosphoralated enzyme. Subsequent hydrolysis regenerates the free enzyme. Replacement of the serine 102 with cysteine (OH replaced by SH) results in a modified enzyme that is still active but has a different substrate specificity [7].

Mutations can also be used to increase enzyme stability. Subtilisin is a proteolytic enzyme from *Bacillus subtilis* that is used in laundry detergents to remove blood stains and other proteins like chlorophyll. Methionine 222 is the primary site of inactivation because of oxidation. Resistance to oxidation would improve the enzyme compatibility with other laundry constituents like bleach. DNA cassettes that would replace methionine 222 with the 19 other amino acids were introduced into the genome. Activity ranged from 138% for cysteine to 0.3% for lysine. The cysteine analog was more resistant to oxidation than the wild type containing methionine; however, the cysteine analog was still sensitive to 1M hydrogen peroxide. Those analogs containing alanine, serine, and leucine (activity = 55%, 35%, and 12%, respectively) were

resistant to peroxide [8]. Similar modifications would improve the compatibility of agent degrading enzymes with oxidizing components of military decontamination solutions.

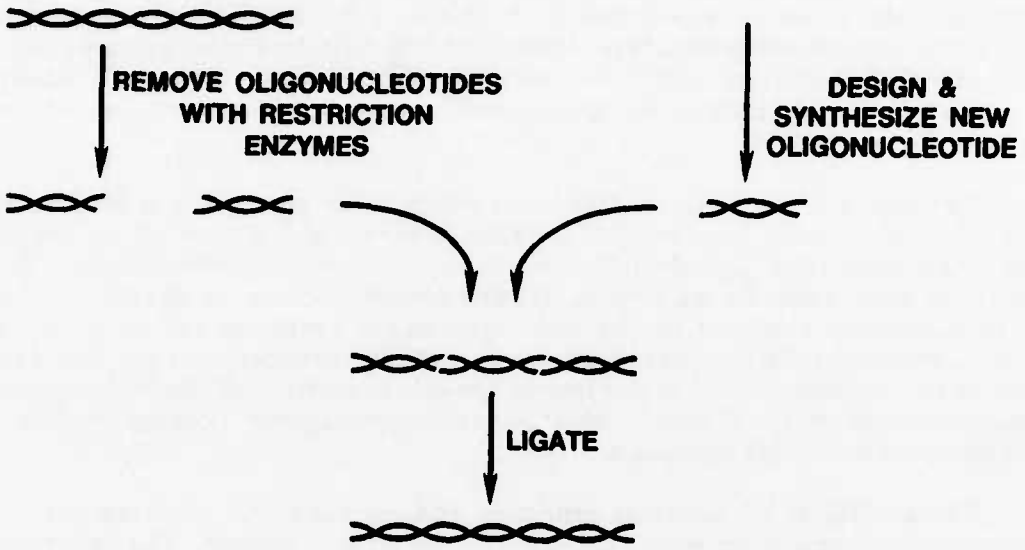

Figure 5. **Site-Specific Mutagenesis**

Thus, it is possible to alter the properties of proteins by introducing changes in the protein sequence. Because the results are not always predictable, it is necessary to make several substitutions and select the best candidate. Most mutations result in a protein with less activity and poorer binding parameters; however, one or more mutations may have properties that surpass the naturally occurring protein.

3.2 *Cell Fusion and Hybridomas.*

Cell fusion offers the possibility of manipulating cells to produce large quantities of proteinaceous and possibly nonproteinaceous products without identifying, isolating, and cloning the essential genes.

For many years, isolation of individual cells from multicellular organisms and subsequent culture in vitro were very difficult. With improvements in biological hoods to

reduce bacterial and fungal contamination, the development of new antibiotics, and the commercial availability of high quality serum, the technology for initiating and maintaining cultures increased dramatically so that today the techniques are reasonably routine. Most of the developments in cell culture have been with mammalian cells because of the interest of the medical community; however, there doesn't appear to be any major physiological impediments for the exploitation for military purposes of nonmammalian cell culture.

One of the major difficulties of mammalian cell culture is the apparent mortality or programming of cells to die. Normal human cells will only divide about 50 times before the cultures stop growing. The genetic or physiological basis for this barrier is not understood. Occasionally, some cells transform into immortal cell lines and are able to divide continually. Frequently, these immortal cells possess unusual karyotypes that result from lost or additional chromosomes.

In contrast, some cells don't divide at all. In a human, the total number of neurons is present at birth or soon after. It is possible to dissect brain tissue and isolate the neurons that will survive in culture for some time. However, the population is eventually overrun by glial cells whose concentration continually increases while the neuron population remains the same or slowly decreases. Neuroblastoma cells are cancer cells that grow in an unregulated manner. Because they have neural origin, neuroblastoma cells possess many functions of neurons. They can be isolated and introduced into tissue culture flasks where they continue to grow and divide and thereby provide a large supply of identical cells that can be frozen indefinitely for comparing future experiments with current. Also they can be distributed to other laboratories so that experiments in different regions can be compared. Unfortunately, many of the neuroblastoma cell lines no longer express the desired neural properties.

Cell fusion was used to recover some of the repressed or lost neural functions. Neither the mouse neuroblastoma cell line (N18TG-2) nor the rat glioma line (C6Bu-1) possessed any morphine receptors in their cell membranes. Hybridization was induced by Sendai virus exposure. The resulting hybridomas contained about 300,000 morphine receptors per cell. In addition, they also possessed choline acetyltransferase, intracellular acetylcholine, nicotinic acetylcholine receptors, and electrically excitable membranes. Thus, hybridization produced a valuable tool for neurophysiology experimentation by permitting the expression of properties not found in either parent [9].

The greatest use of hybridomas has been for the development and production of monoclonal antibodies (MAb). Lymphocyte stem cells in the bone marrow produce lymphocytes that respond to foreign biopolymers by engineering genes that will eventually produce antibodies against the foreign material. The antibodies identify and "tag" the intruders so that the complement system (an enzyme cascade) can destroy them.

The lymphocyte stem cells grow and divide; however, the lymphocytes lose the ability to divide as they mature and develop the capability of producing and secreting antibodies. The cells that are actively secreting antibody are commonly called plasma cells. Each plasma cell produces many copies of a single antibody. Myeloma cells, a type of lymphocytic cancer, secrete large quantities of antibodies. These cells have the ability to grow and divide in an unregulated manner. Because all the myeloma cells from a particular patient derive from one original cancer cell, all of the antibodies are identical. Many of the early studies on the structure of antibodies were performed with myeloma antibodies because of the ease of isolation of large quantities of identical proteins.

A group of cells drived from a single cell in which all the daughter cells are genetically identical is commonly called a clone. The total lymphocyte population in a mammal come from many stem cells; and because different populations are stimulated by differ antigens, a wide

range of antibodies is produced even though each plasma cell produces only one antibody (although many identical copies). Even antibodies to the same antigen are different because the antigen may have several antigenic determinants (sites on the molecule that actually stimulate the immune response and bind to the antibody). Also, binding affinities to the same antigenic determinant may be different because of different protein sequences at the binding site of the antibodies.

In contrast to the usual polyclonal antibodies, which are a mixture of different antibodies, monoclonal antibodies come from the same lymphocyte; therefore, all the antibodies are identical. The hybridomas that produce them can be frozen and stored indefinitely so that current and future production runs will produce the same antibodies. Synthesis of polyclonals stops when the immunized animal dies. To continue production, new animals must be immunized and slightly different antibodies will be produced.

To produce MAb's, animals (usually mice) are injected with an antigen. If the molecule is small, it must be conjugated with a large biomolecule (usually a protein) in order to elicit an immune response. Frequently, additional immunizations are required. Several days later, the spleen is removed and homogenized. The spleen cells are cultured with myeloma cells under appropriate conditions to produce hybrid cells.

There have been several improvements in cell fusion since the original report by Kohler and Milstein in 1975 [10]. They fused equal numbers of cells from each parental line with inactivated Sendai virus. The selected cells had a karyotype slightly less than the sum of the parent's after 5 months in culture. Replacement of the Sendai virus with a solution of polyethylene glycol produced a much more predictable level of cell fusion. In the original procedure, consistency was difficult to maintain because many or no fusions would occur in a single well containing several cells. Development of an electrofusion technique increased the frequency of cell fusions and, thereby, reduced the requirement for myeloma cells with exploitable biochemical markers such as resistance to antibiotics. These markers were essential for selecting hybrid cells from the normal excess of spleen cells.

A very promising new technique is isoselective cell-cell fusion. The strong affinity of avidin for biotin is exploited to increase the surface contact between the lymphocytes and the myeloma cells. Biotin is covalently attached to the myeloma cells. Avidin is covalently attached to surface antigens for the immunoglobins on the surface of the lymphocytes. When the two cells and the linkers are mixed, a strong interaction (lymphocyte-immunoglobin-antigen-avidin-biotin-myeloma cell) occurs. Then, a strong electric field is used to fuse the cells to form a hybrid [11].

Success of this technique relies on the presence of antibodies at the surface of the lymphocyte. This procedure is not directly applicable to cell fusions of other types of cells (e.g., liver). It may be possible to produce antibodies to some marker on the surface of a nontransformed cell that could be used to link it with a myeloma or other cell. Normally, antibodies contain two identical binding sites. Consequently, the antibody would connect two identical cells rather than different cells. Bispecific antibodies in which the two binding sites have affinity for different haptens could possibly be used to connect different cells. Bispecific antibodies have been developed but have not been used to facilitate cell fusions.

Although monoclonal antibodies are the principal product from hybridoma research today, many other products will be developed and marketed in the future. Enhanced production of certain nonproteinaceous toxins is one possibility with military significance.

Research is moving rapidly in many directions to provide a better understanding of the biology and chemistry of life. Although most of the results will lead to new and improved

products for prevention of diseases and therapy, there are numerous possibilities for developing new weapons that could pose a considerable threat to the United States and its allies. Recognizing this potential is the first step in discovering the intentions of potential enemies and in developing new equipment and doctrine for detection, protection, and decontamination. Some of the possible applications are described below.

4. PROTEINACEOUS TOXINS

Usually, proteinaceous toxins are produced by prokaryotic organisms, whereas nonproteinaceous toxins come from eukaryotic species. Ricin, from caster beans, and abrin, from the tropical legume *Abrus precatorius*, are obvious exceptions. Also, venoms from snakes, spiders, etc. contain a mixture of proteinaceous as well as low molecular weight toxins.

Frequently, the proteinaceous toxins consist of two or more protein chains that are held together by disulfide bridges. One of the peptide chains may be involved with binding to a cellular receptor and/or transport through the cell membrane while the other chain produces the toxic effect -- often by an enzyme catalyzed process. The toxin is usually synthesized as a single chain protoxin that folds into a low energy (more stable) conformation, which is held intact by disulfide bonds. Activation of the toxin occurs by proteolytic cleavage of the single chain into two peptides. Enzymes found in the digestive system, such as trypsin, can activate botulinum toxin.

The following table lists some of the proteinaceous toxins that have been reasonably well characterized.

Table 1. Proteinaceous Toxins

NAME	STRUCTURE	SOURCE	ACTION
Abrin	65,000	*Abrus precatorius* (tropical legume)	Inhibits protein synthesis inactivates ribosome by ADP ribosylation; different target from diphtheria toxin
Anthrax	80,000 protective antigen edema factor lethal factor	*Bacillus anthracis*	Unknown
Botulinum	150,000	*Clostridium botulinum*	Inhibits acetylcholine release

β-Bungarotoxin	21,000 9,000 subunit 11,400 subunit	*Bungarus multicinctus*	Blocks release of acetylcholine from nerve terminals and phospholipase activity
Cardiotoxic cobra toxin	low MW	*Naja naja atra*	Membrane effects (?)
Neurotoxic cobra toxin	low MW	*Naja naja atra*	Nicotinic receptor (curarimimetic)
Crotoxin	A subunit 9,000 MW	*Crotalus durissus* (South American *terrificus* rattlesnake)	Chaperon for B subunit with other membranes) (prevents interactions
	B subunit 14,000 MW		Phospholipase that attacks the presynaptic membrane
α-Conotoxin	14-15 amino acids	*Conus magnus* *Conus geographus* (marine cone snail)	Blocks acetylcholine receptor
μ-Conotoxin	22 amino acids	*C. geographus* *C. magnus*	Blocks muscle sodium channels
ω-Conotoxin	25 amino acids	*C. geographus* *C. magnus*	Blocks presynaptic calcium channels
Diphtheria	63,000 2 subunits	*Corynebacterium diphtheria*	Inhibits protein synthesis by ADP ribosylation of elongation factor 2
Dysentery	65,000 1 alpha chain 6-7 beta chains	*Shigella dysenteriae type 1* plus other	Inhibits 60S ribosome in eukaryotes

E. coli enterotoxin (heat labile)	2 subunits	*Escherichia coli*	Inhibits cAMP levels by ADP ribosylation, similar to cholera toxin
E. coli enterotoxin (heat stable)	2,000	*Escherichia coli*	Stimulates guanylate cyclase
Erabutoxin A	62 amino acids asparagine 26	*Laticauda semifasciata* sea-snake	Nondepolarizing block at cholinergic receptors
Erabutoxin B	62 amino acids histidine 26	*Laticauda semifasciata* sea-snake	Nondepolarizing block at cholinergic receptors
Exotoxin A	66,000	*Pseudomonas aeruginosa*	Inhibits protein synthesis
α-Latrotoxin	~130,000	*Latrodectus mactans tredecimguttatus* (black widow spider)	Stimulates release of acetylcholine and possibly other neurotransmitters
Microcystin	cyclic peptide 12 amino acids	*Microcystis aeruginosa* (freshwater algae)	--
Noxiustoxin	39 amino acids	*Centruroides noxius* (Mexican scorpion)	Blocks voltage-dependent potassium channels
Pertussis toxin	A fragment 28,000	*Bordetella pertussis*	ADP ribosylation (different from cholera)
Plague	120,000	*Yersinia pestis*	Inhibits electron transport in mitochondria by blocking NADH-CoQ reductase

Ricin	65,000	*Ricinus communis* (Caster Bean)	Similar to abrin
Staphyloccal alpha toxin	26,000 to 39,000	*Staphyloccus aureus*	Haemolysis
Staphyloccal gamma toxin	2 subunits 26,000 29,000	*Staphyloccus aureus*	Heamolysis
Staphyloccal delta toxin	15,000	*Staphyloccus aureus*	Surfactant detergent action
Streptolysin-O Streptolysin-S plus others	--	*Streptoccus pyogenes*	Heamolysis
Tetanus	150,000 2 subunits	*Clostridium tetani*	Inhibits acetylcholine release
Tityustoxin	--	*Tityus serrulatus* (Brazilian yellow scorpion)	Inhibits closing of sodium channels
At least 12 toxins		*Clostridium perfringens*	Necrotizing hemolytic phospholipase

4.1 *Botulinum Toxin.*

Botulinum is a proteinaceous toxin produced by an anaerobic spore-forming bacteria belonging to the genus Clostridium [12]. Because *C. botulinum* is not able to grow in the presence of oxygen, the toxin is usually encountered in canned foods that were improperly preserved. The bacteria grow and produce toxin while the food sits on the shelf. These bacteria do not possess the cytochromes necessary for electron transport to molecular oxygen. They have flavoenzymes that reduce molecular oxygen to toxic intermediates like peroxide and superoxide but lack the necessary catalases, peroxidases, or superoxide dismutases to degrade them. In an aerobic environment, these toxic intermediates accumulate and kill the bacterium. Botulism is manifested as a flaccid paralysis that results from action on the peripheral rather than the central nervous system. It is currently believed that botulinum toxin does not cross the blood brain barrier [12].

The *Clostridium botulinum* spores, which are widely dispersed in the soil, are not sensitive to oxygen because they are not growing. Unlike *Clostridium tetani*, which is responsible for tetanus, *Clostridium botulinum* rarely infects humans directly. Occasionally, the intestinal track of infants will become infected; however, the usual bacterial flora prevents colonization in adults [13].

Seven types of toxin (A-G), which are classified serologically according to their reaction to specific antibodies, have been identified. Although the spores are resistant to heat, the toxin itself is almost totally inactivated by heating for 10 min at 100°C. Botulinum toxins are the most toxic compounds known with an LD50 for humans of 0.00001 mg/kg. In comparison, the LD50 for strychnine is 2 mg/kg [14]. The seven types of toxin are quite similar. They consist of a large or heavy chain of approximately 100,000 MW that is covalently attached by a disulfide bond to a light chain of 50,000 MW. There is also another internal disulfide bridge in the heavy chain [15]. Apparently, a single protein is synthesized, which is subsequently activated by proteolytic cleavage. The protoxin is relatively nontoxic. Unlike insulin, in which a large segment of proinsulin is removed, only a single nick is introduced (probably at an arginine residue) between the disulfide bridges so that no amino acids are removed [16]. Research to discover the protein sequence continues, but only a portion has been reported [17].

The genes for the toxin are carried on a plasmid that can be cured or removed by culturing the bacterium in the presence of ethidium bromide or by irradiating with UV light [18]. Culturing with acridine orange also resulted in a loss of toxicity (type C1) that could not be restored by further culturing in the absence of the dye [19]. Similar results were obtained with a type D culture. When a cured D culture was incubated with lysates from a C culture, the resulting cultures produced type C toxin. These results demonstrate that the nature of the toxin was due to the particular plasmid and not to a subpopulation of Clostridium [20].

Because botulinum toxin is a large protein, introduction through the skin is unlikely unless the skin is broken; however, introduction through the respiratory tract is feasible. Although it is possible to administer botulinum toxin in such a massive amount that toxicity occurs, other agents are available that easily penetrate the skin. Recombinant DNA techniques could be used to produce large quantities of toxin by fermentation, but the principal application of biotechnology may be the development of new hardier bacteria that produce the toxin in situ in infected troops.

Because of the inverse correlation between bacterial growth and toxin production, the fermentation system must be closely monitored [21]. After the toxin is produced, it should be partially purified to remove various enzymes that would reduce shelf-life. Affinity chromatography using a galactose analog was used in a one-step procedure to obtain toxin of 99% purity [22]. Immunoprecipitation was also used with type E toxin to produce a stable product [23].

Clostridium botulinum is not very virulent. About 2×10^7 spores introduced into the wound of an experimental animal are needed to produce symptoms [12]. In contrast, human infants occasionally develop symptoms from bacteria that have infected the gut. In these cases, the Clostridia are able to grow rapidly and secrete toxin molecules that are absorbed through the lining into the blood stream because the normal microbial flora have not become established. Because *E. coli* is a normal constituent of the colon, introduction of the toxin genes into this bacterium and subsequent infection could produce serious effects. Secretion of the protoxin would lead to activation by trypsin, a digestive enzyme normally found in the intestines and colon. Because different strains of *E. coli* are endemic to different geographic regions, it might be possible to introduce the toxin genes into specific *E. coli* so that only Western troops would be adversely affected.

To accomplish this task, the gene must be isolated, identified, cloned, and introduced into the appropriate *E. coli* where it is expressed. Because the genes for the toxins are located on plasmids, identifying them would be faster than if they were chromosomally coded. Only a few DNA fragments result from digestion of plasmids by restriction endonucleases. The partial sequence for both chains of type A neurotoxin has been reported. The protein sequence data permits construction of a DNA probe for locating the gene on the plasmid. The selection of *E. coli* as the host also facilitates subsequent expression of the toxin gene because the requisite promoters and ribosomal binding sites are known for *E. coli* and are available as integral parts of expression vectors. Introducing the gene into a less well characterized system would be more difficult because the proteinaceous toxin may not be expressed in the new bacterium if the ribosomal binding sites were incorrect, which would probably be the case.

The critical feature is secretion of the toxin by *E. coli*. Unlike many Bacillus and yeast strains, *E. coli* do not normally transport their proteins through the cell wall and release them into the extra-cellular medium. Researchers throughout the world are investigating methods for promoting protein secretion in Gram-negative bacteria in order to manufacture industrial enzymes and hormones more efficiently.

Modification of *Clostridium botulinum* so that it could grow in air might permit infection by an aerosol that enters and colonizes the respiratory tract. Vegetative organisms that would be resistant to air could also be used.

As indicated earlier, the inability of Clostridia to grow in air results from an accumulation of toxic metabolites such as peroxide and superoxide during respiration. These intermediates are not produced in the absence of oxygen. Introduction of genes for superoxide dismutase or other enzymes on appropriate plasmids would probably eliminate this defect. The toxin genes are carried on plasmids; therefore, one might introduce the nascent genes directly into native plasmid. Because the bacteria carrying the oxygen$^+$ genes would have a selective advantage over the native oxygen$^-$ organisms, the toxin genes would be maintained due to their location on the same plasmid. Unless their introduction caused the plasmid to become unstable because of increased size, the plasmid would be replicated. Very little work has been reported on the Clostridium plasmids. The ribosomal binding sites are currently unknown; therefore, the protein might not be expressed even if the gene is present. By inserting the oxygen$^+$ gene between other structural genes, the gene might be expressed as part of a polycistronic messenger. This manipulation would require identification or construction of a restriction site at the proper location.

4.2 *Diphtheria Toxin.*

Diphtheria toxin is a protein that is secreted by the rod-shaped Gram-positive bacterium, *Corynebacterium diphtheria* [24]. Because the toxin is a catalytic enzyme that eliminates a vital function (protein synthesis), one toxin molecule is sufficient to kill a cell. *C. diphtheria* is an obligate aerobe that colonizes such highly oxygenated organs as the upper respiratory tract.

The toxin is synthesized as an inactive protoxin of 68,000 MW. Activation occurs upon cleavage of the protein into two fragments and reduction of disulfide bridges [25]. Proteolysis can be accomplished *in vitro* with trypsin; however, the *in vivo* activating enzyme is not currently known. Fragment A, containing 193 amino acid residues, is an enzyme that inactivates protein synthesis. Fragment B, containing 342 residues, facilitates transport of the toxin into the cell [26,27]. Although the A fragment is catalytic *in vitro*, it is not toxic because it can't enter the cell [28]. Apparently the toxin binds to a receptor on the cellular membrane and is subsequently transported into the cell [29,30]. Hybrid proteins containing fragment A coupled to concanavalin A enter the cell by passing through the membrane and subsequently inhibit

protein synthesis [31]. The purpose for these receptors on the cellular surface is unknown and varies considerably. HeLa cells contain about 4000 sites, whereas the highly sensitive Vero cells may contain 100,000 to 200,000 sites per cell [32]. Because mice cells have no cellular receptors, they are quite resistant to diphtheria toxin even though it is very effective in inhibiting protein synthesis in a cell-free system derived from mice.

Protein synthesis occurs at ribosomes, which are small organelles composed of proteins and nucleic acids. At the ribosome, tRNA's containing their correct amino acids are arranged in proper order according to the mRNA sequence. Diphtheria toxin catalyzes the inactivation of a particular protein called elongation factor 2 by the addition of ADP ribose. Elongation factor 2 normally moves the tRNA from the amino site to the peptide site so that a new tRNA may bind. Because diphtheria toxin is an enzyme that functions catalytically, one toxin molecule can inactivate all elongation factors in a cell and thereby kill it by stopping protein synthesis [33].

- *Limitation of Diphtheria Toxin*

Most people in Western cultures were immunized against Diphtheria as children and, consequently, have immunity against the classical disease. The toxoid used to elicit the immunological response is prepared from the toxin itself rather than from some surface antigen on the bacterium, *Corynebacterium diphtheria.* Consequently, the antibodies circulating in the blood should provide some protection against diphtheria toxins although the residual titer could be overwhelmed by a massive dose of toxin.

Like many of the proteinaceous toxins, diphtheria toxin contains three domains. One is the active enzyme, another aids in transport of the protein across the cellular membrane, and the third binds to cellular receptors. The active toxin consists of two peptides that are covalently linked by disulfide bridges. The toxin precursor is synthesized as a single peptide, which is activated by proteolytic cleavage. One of the chains contains the enzyme, whereas the other facilitates passage through cellular membranes. Just as hybrid proteins can transport the enzyme fragment A into a cell, the transport peptide B could possibly carry other unrelated toxins into the cell.

- *How might biotechnology be used*?

Gene fusion is receiving considerable attention now although few results have been attained because the technique is very new. The objective is to isolate two genes and join them together in the proper sequence with correct punctuation so that they produce one large protein with properties of the two respective proteins. This technology may make it possible to clone a toxin gene with a gene for the transport region and thereby produce a new toxin with more mobile characteristics. This new toxin could be produced industrially and disseminated in bulk, or it could be produced *in vivo* through some microbial vector. In this case the transport protein might facilitate transport of the toxin from the site of synthesis to the ultimate target. This feature could be important if the toxin was synthesized in cells different from the target because proteins normally have difficulty getting out of cells just as they have difficulty entering.

In contrast, *in vivo* synthesis of native diphtheria toxins is probably not feasible unless the inactive protoxin is excreted because the active toxin would inactivate protein synthesis and stop toxin production. The *C. diphtherium* does not kill itself because the structure of the target protein in eukaryotic organisms is different from the corresponding protein in bacteria. Consequently, the toxin inhibits eukaryotic protein synthesis but does not inhibit bacterial.

- *What would have to be done*?

First, the gene for diphtheria must be cloned and the sequence determined. This has already been accomplished for the nontoxic variants but not for the toxic protein nor the toxin precursor. Then the region that codes for the transport peptide must be excised with a suitable restriction enzyme. If an industrial production is planned, the gene is inserted into an appropriate vector and introduced into a production host. The peptide is produced in large quantities, isolated and purified, and then attached chemically to a toxic moiety. Proteinaceous toxins could be joined by disulfide bonds. Small molecular weight toxins like tetrodotoxin, batrachotoxin, etc., could also be attached. It is important to remember that attaching these to a protein may destroy or reduce the toxic properties. Also, those toxins that reach the target easily may not be aided.

The second approach requires considerable expertise in many areas as well as extensive effort at the basic level. In the next few years, it should be easy to isolate two genes and join them so that one predictable protein is produced. Diphtheria toxin, as well as several other proteinaceous toxins, are inactive as single chains. Therefore, one would predict that a hybrid toxin should have two chains. It seems extremely unlikely that two proteins could be produced inside a cell and then join spontaneously to form an active toxin. The only reasonable approach follows the natural one. A single protein is produced that adopts an appropriate conformation based on its total amino acid sequence. Disulfide bonds form by spontaneous oxidation of sulfhydryls and only then is the chain nicked to produce the two peptides.

The toxin precursor must have the correct structure to form the disulfide bonds and present only the correct sequence to the activating enzymes. This approach would then require the synthesis of appropriate genes and introduction into hosts for production. This aspect of the project would not be difficult.

- *Outlook*?

The total design of a toxin is beyond current technology. It may be possible to link two genes together and immediately produce a new toxin; however, such an approach would seem fortuitous. The other approach for producing hybrids by chemically joining two independently prepared peptides is feasible. This is the approach currently employed by Eli Lilly to produce human insulin. Producing the correct disulfide bridges requires experimentation in order to discover the optimal conditions. In the distant future it will probably be possible to design one protein that can be nicked by proteolytic enzymes to produce a predicted toxin or other protein.

4.3 *Staphylococcal Toxins.*

Staphylococcous aureus is the principal agent for Staph infections [34]. Boils and interconnected abscesses called carbuncles frequently result. *S. aureus* is a Gram-positive, nonmotile faculative anaerobe that usually grows faster under aerobic than anaerobic conditions and sometimes prefers an increased carbon dioxide pressure. Staphylococci are among the hardiest of the nonspore forming bacteria. They remain viable for months on agar plates and may be cultured from dried pus that is many weeks old. Some strains can tolerate heating to 60°C for 30 min. They are also more resistant to disinfectants than most bacteria. In fact, Staphylococcal strains can be isolated from others by culturing in media containing 7-10% sodium chloride because most bacteria cannot grow under these conditions.

When penicillin was discovered, most Staphylococcal strains were sensitive; however, 60-90% are resistant today because of the presence of a plasmid gene that codes for

penicillinase (beta-lactamase). Other plasmids carry resistance to heavy metals, erythromycin, chloramphenicol, tetracyclines, neomycin, and kanamycin. Unlike the enterobacteria, the Staphylococci can not conjugate (sexual exchange) so that plasmids must be transferred by transduction.

The family of skin diseases collectively termed the Staphylococcal scalded skin syndrome is caused by an *S. aureus* toxin. One of the genes, Exfoliative Toxin B gene, was located on the chromosome and cloned into plasmid pDH5060. The gene was subsequently removed from this plasmid and transferred to expression vector M5248 where it was expressed in *E. coli*. This particular toxin doesn't appear to pose much of a military threat [35].

Four hemolytic toxins have been discovered in *S. aureus*. All of the proteins cause beta-hemolysis of red blood cells; however, their species specificity and mechanisms of action differ. Some strains may secrete more than one toxin. Alpha-hemolysin (alpha-toxin) is the principal toxin found in strains that infect humans. It lyses rabbit blood cells as well as those from humans. The beta-hemolysin is commonly found in animal strains. The 30,000 MW enzyme catalyses the hydrolysis of sphingomyelin to N-Acylsphingosine and phosphorylcholine. The gamma-hemolysin consists of two proteins that lyse rabbit, human, and sheep blood cells but not those from horse and fowl. Because the action of gamma-hemolysin is inhibited by agar and other sulfated polymers, it may not be detected by the usual blood-agar assay. The delta-toxin is a mixture of subunits of 5,000 MW. In addition to red blood cells, delta-toxin also lyses white cells, cultured mammalian cells, and bacterial protoplasts. The mechanism probably involves some surfactant action [34].

The Staphylococcal enterotoxins are responsible for more than 40% of the food poisonings in the United States. Vomiting and diarrhea usually begin within 6 hr after eating contaminated food. Poisoning is usually from ingestion of the toxin rather than from ingestion of the bacteria followed by toxin synthesis in the gut. Generally, the disease is of short duration and rarely fatal. These enterotoxins are different from cholera toxin and those from *E. coli* because they do not act directly on intestinal cells. The same clinical response is obtained in monkeys following intravenous injection of the toxin or oral introduction. When the toxin activates receptors in the abdomen, the stimulus reaches the vomiting center via the vagus nerve. Thus, the Staphylococcal enterotoxins can be considered neurotoxins. The diarrhea effect is not well understood. There is some evidence that the toxin might function like cholera toxin by affecting adenylate cyclase and, thereby, increasing the rate of sodium and chloride efflux. Other contradictory evidence indicates that the increased fluid flow does not result from enhanced levels of cyclic AMP [36]. In support of this hypothesis, enterotoxin A (SEA) does catalyze the hydrolysis of NAD to nicotinamide and ADP-ribose by first-order kinetics [37].

The Staphylococcal enterotoxins are a group of globular proteins with molecular weights ranging from 27,000 to 35,000. At least eight (A,B,C1,C2,C3,D,E,&F) serologically distinct (bind to different antibodies) types have been discovered. This heterogeneity may result from post-translational modifications. The complete amino acid sequence is known for the B & C1 toxins. The C1 toxin consists of 239 residues with a molecular weight of 27,500. This protein is similar to the B toxin especially in the carboxy terminus; however, the region spanned by the disulfide bond is three residues shorter [38].

The toxic shock syndrome is a much more serious consequence of Staphylococcal enterotoxins that can result in death. Thus far, the A,B,C,&F toxins have been implicated. Those who develop the syndrome appear to have difficulty developing or maintaining sufficient antibody titers to the toxin [39].

Currently, there are few good models in which to study the toxicity of the enterotoxins. Monkeys show many of the symptoms of toxic shock syndrome; however, cost and other

considerations limit the research that can be performed on nonhuman primates. The development of monoclonal antibodies coupled with enzymed linked immunosorbent assays (ELISA) has facilitated the detection of the enterotoxins in food [40-41].

- *What is the potential for Staphylococcal toxins as weapons*?

The vomiting, diarrhea, and painful cramps associated with the Staphylococcal toxins could make them effective incapacitating agents. The effects would be more persistent (about a day) than many potential chemical incapacitants. Also, it would be difficult to prove conclusively that biological agents had been used because food poisoning is always a possibility during military conflicts. The fact that an entire combat unit became ill could readily be attributed to food poisoning. The toxins normally enter the body through the intestinal mucosa so they are not readily degraded by enzymes that digest other proteins. Also, their ability to induce symptoms when injected intravenously suggests that they could be introduced via the respiratory system. Use of purified toxin would probably result in simultaneous incapacitation of all the troops, whereas use of the bacteria would produce a longer effect with some troops recovering as others became ill.

- *What are the limitations*?

The toxins are quite stable to thermal denaturation. They retain activity after 30 min at 100°C. Production, isolation and purification, and developing the delivery system seem to be the major limitations. The Soviet's have shown some interest in these toxins. A recent Russian article reports a method for producing the type D toxin [42].

The gene for the A toxin has been cloned. One paper reported the cloning into the plasmid pBR322 and subsequent expression by *E. coli* in which the protein was secreted into the periplasmic space. The gene is located on the chromosome between the purine and isoleucine-valine markers in most species. Those strains that do not produce toxin A usually lack this entire genetic region [43]. The gene is also contained in a bacteriophage that is capable of transmitting the gene to other strains. The phage integrates into the host chromosome by circularization and reciprocal crossover; the entire entA gene is located near the phage attachment site [44].

This phage could be used to introduce mutant genes into various Staphylococcus strains that would code for toxin analogs with different properties. It is unlikely that any more "troublesome" enterotoxins will be developed by genetic engineering soon. There seem to be more efficient avenues. For example, the toxin was modified by chemical methods. Carboxymethylation of either five or six histidine residues reduced the toxicity and the antibody specificity. The loss of activity was due to modification of a specific amino acid residue and not to a general change in conformation of the protein [45].

Post-translational modification is one of the anticipated problems associated with cloning, gene amplification, and production in a host. If the foreign host can not modify the protein into its active state, toxicity will be lost or reduced. The gene for *S. aureus* enterotoxin B (entB) was cloned into pBR322, but no toxin was produced until the gene was inserted into an expression vector with a strong lamda promoter. The mature toxin, not the protoxin, was found in the cytoplasm while the protoxin was associated with the cell membrane. Thus, *E. coli* can modify SEB into an active species [46].

Apparently, post-transitional modification would not be a problem to large-scale production of the enterotoxin by recombinant DNA techniques.

4.4 *Ricin.*

Lectins are plant proteins that bind and agglutinate animal cells. Ricin is perhaps the best known of the toxic lectins. Ricin should not be confused with the nonproteinaceous toxin ricinine, a pyridine analog with molecular weight of 164 that is also found in the castor bean, *Ricinus communis*. Ricin is a 65,000 MW toxin consisting of A and B chains that are held together by disulfide bonds. Individual chains are not toxic. The mechanism of toxicity is similar to diphtheria toxin and involves the inhibition of protein synthesis by inactivation of the 60S ribosomal subunit. A single toxin molecule in the cytoplasm is sufficient to kill a cell.

The A chain is the toxic enzyme, whereas the B chain aids in translocation through the cellular membrane. The B chain binds to the galactose containing glycoproteins or glycolipids on the cell surface. When different proteins (hormones, growth factors, or antibodies) are substituted for the B chain, the binding sites on the membrane change, but the toxicity remains. One application of immunotherapy is based on attaching toxins to antibodies that are specific for neoplastic cells so that only cancer cells will be killed.

Ricin immunotoxins have been prepared by reducing the interchain disulfide bonds and isolating the A chains by chromatography. The A chains are usually coupled to antibodies by a chemical crosslinking agent such as N-succinimidyl 3-(2-pyridithio)propionate. This approach was used effectively *in vitro* to kill BCL cells, a mouse tumor line that is lethal in BALB/c mice at a dose of 1-10 cells. The immunotoxins were used in combination with other therapies *in vivo*. Mice with a tumor burden of 10 E10 tumor cells were first treated with total lymphoid irradiation or spleenectomy to reduce the tumor burden and then treated with immunotoxins to kill the remaining cells. Animals that received no immunotoxins or control immunotoxins (the antibody was directed to other cells) were dead within 7 days, whereas those receiving the experimental immunotoxins were alive and apparently tumor free after 18 weeks [47].

This approach has also been attempted for human colorectal cancer using diphtheria toxin. Generally, diphtheria toxin is not as effective because of the prevalence of antibodies directed against diphtheria toxin in the general population.

- *What is likely to be developed*?

Immunotoxins hold great promise for cancer therapy because they offer the possibility of directing the agent to the cancer cells even when the cancer cells have metastasized to other organs. Also, normal cells should not be damaged because the toxins employed are not hazardous outside the cell. Much research will probably be directed toward immunotherapy by the medical community. These studies may result in gene clonings and in smaller or modified toxins that penetrate the cells easily. The success of the therapy depends on producing sufficient quantities of antibodies directed toward the patient's tumor cells quickly so that treatment can begin before the patient dies [48].

Knowledge gained by medical research in targeting toxins to specific cells could have military implications. Lethality from chemical agents usually results from cessation of a particular vital function. When an agent binds to a variety of receptors, its effective concentration is diluted by noncritical interactions. By targeting toxins to specific tissues or classes of cells, the effective dose and thereby the toxicity could be increased considerably. Exploitation by potential enemies will not be possible for many years; however, the advantages for medical therapy are so great that the medical community will continue to support this research.

- *What are the possibilities for producing an active protein with recombinant DNA methods?*

There is a recent report of crystallizing the ricin A chain that was obtained from a cloned gene expressed in *E. coli.* Unfortunately, no data was presented regarding the nature or activity of the protein [49]. Presumably more information will be published soon.

Ricin is produced by eukaryotic organisms so the corresponding gene probably contains several introns. The toxin is produced in reasonable quantities by the plant so there should be sufficient mRNA for preparation of a cDNA that contains the desired gene. The main problem would be the post-translational modifications. Ricin is a glycoprotein with sugar moieties added enzymatically after the protein chain is synthesized. Frequently, elimination of the sugar molecules results in loss of biological activity. It is possible that the function of the sugars is to aid in binding the toxin to the cell membrane and has nothing to do with inactivation of the ribosome. If this is true, then cloning a gene for the A chain would produce an active toxin if it could be transported inside the cell. Based on results from immunotoxin research, the A chain itself will fold and refold into its active conformation. This is not the case with certain other proteins (i.e., insulin). The proinsulin molecule folds into its most stable conformation as it is synthesized. After the structure has been "locked" with disulfide bonds that connect cysteine residues, 33 residues are removed enzymatically to produce two chains. If the disulfide bonds are reduced so that the A and B chains separate, it is difficult to recombine them into an active molecule.

Large-scale production of ricin by recombinant DNA techniques will probably be an option for weaponry in the future. At this time, the easiest approach for ricin production would be to grow the castor bean plants and isolate the toxin.

4.5 *Bacillus anthracis.*

Bacillus anthracis is frequently considered the quintessential biological agent because of its toxicity and stability in the environment. Gruinard Island off the coast of Scotland remains uninhabitable today in spite of numerous attempts at decontamination because of the persistence of *Bacillus anthracis* spores that were disseminated during Biological Warfare agent tests [50].

Concern about the continued development of anthrax as a biological weapon by the Soviet Union has increased as a result of numerous cases of pulmonary anthrax at Sverdlovsk in 1979. Western officials attribute the outbreak to an aerosol discharge of *B. anthracis* spores from the biological weapons research facility, whereas the Soviets blame contaminated meat from infected grain. Western sources point out the extensive decontamination efforts, which the Soviets justify by claims that the anthrax was widely dispersed when undisciplined workers disposed of the contaminated meat in open garbage containers [51].

B. anthracis is a large Gram-positive organism that grows best under aerobic conditions but can shift to anaerobic when oxygen is limited. Spores form readily when conditions for further growth become unfavorable. Aerobic conditions are required for sporolation but not for germination. Under natural conditions, anthrax is principally a disease of domestic and wild animals; however, virulence varies considerably among species. Mice are the most susceptible with an LD_{50} of about 5 spores whereas that for dogs, cats, and rats is over a million. Under natural conditions, humans contract the disease only by direct contact with diseased animals or animal products such as hides and wool. Pulmonary anthrax results from inhalation of spores that are subsequently phagocytized by alveolar macrophages and transported to the lymph nodes where they germinate and multiply [52].

Anthrax toxin consists of three proteins each with a molecular weight of about 85,000; however, none of the proteins is toxic by itself. The edema factor plus the protective antigen leads to skin edema while the lethal factor plus protective antigen leads to lethality. The exact physiological effect and the critical target responsible for death are currently unknown. There is some speculation that the edema and lethal factors compete for the same receptor because addition of edema factor to a mixture of protective antigen and lethal factor did extend the life of experimental animals [53]. Current evidence indicates that the edema factor is an adenylate cyclase (catalyzes the formation of cyclic AMP from ATP). The enzymatic activity is dependent on a cellular cofactor, probably calmodulin [54].

In order for a strain of *B. anthracis* to be virulent, it must produce a capsule containing poly-D-glutamic acid and must produce the toxin. Much of the anthrax toxin used for research and vaccines is produced by strains that lack capsids and, therefore, are not pathogenic. Vaccines composed of precipitated protective antigen have replaced the attenuated bacteria that were developed by Pasteur; however, the current vaccines will probably be replaced by products of recombinant DNA research that were developed by the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID).

Virulent strains of *B. anthracis* contain a plasmid, designated pX02, that is involved in the synthesis of the capsules, whereas avirulent strains like the Pasteur strains lack pX102. Transfer of this plasmid into *B. cereus* and *B. anthracis* strains that were noncapsulated resulted in the production of capsules [55].

Cloning of the gene for the protective antigen has been completed. A library of restriction fragments from plasmid pBA1 was constructed in plasmid vector pBR322 and introduced into *E. coli.* Two clones producing protective antigen were identified by enzyme-linked immunosorbent assay (ELISA). A Western blot indicated that the entire protein had been produced and a cell elongation assay demonstrated biological activity [56].

The successful cloning of one of the toxin genes and expression in *E. coli* suggests that cloning of the other genes is probable. The transfer of these genes into other plasmids and other bacteria would increase the difficulty of detecting the disease when using methods that rely on detection of surface markers or metabolic reactions of *B. anthracis* per se.

The persistence of the spores, despite extensive efforts at decontamination reduces the value of anthrax as a tactical weapon because the infected area would not be usable without protective equipment. Undoubtedly, there are scenarios for the use of anthrax as a strategic weapon or terrorist tool when the long-term effects are not considered important by the user.

A tactical weapon might result from engineering properties into the *B. anthracis* spores that would make them less persistent - either more sensitive to environmental factors like sunlight or more susceptible to chemical disinfectants. Irradiation of living cells with ultraviolet radiation or sunlight damages the DNA by producing thymidine dimers as well as other defects. Even though DNA is continually damaged by environmental insults, most of the defects are quickly repaired so that no permanent effect is manifested. Occasionally, the defects may not be repaired, and a neoplasia or cancer may result [57]. The enzymes and corresponding genes for excision repair (the most common and generic type of DNA repair) are well characterized for many bacteria. The techniques for developing bacteria that are sensitive to sunlight are well documented in the literature.

In summary, the principal applications of biotechnology with respect to anthrax would be the attempts to conceal the toxin genes inside another organism or to decrease the stability of the spores in the environment.

5. NONPROTEINACEOUS TOXINS

Nonproteinaceous toxins are normally produced by eukaryotic organisms. Most are neurotoxins; however, the large class of trichothecene mycotoxins inhibit protein synthesis. A major limitation in military exploitation of these toxins would be the difficulty in producing sufficient quantities to pose a serious threat. Because they are not proteins, nonproteinaceous toxins are not coded by individual genes. Instead, they are synthesized metabolically by a series of enzymatic reactions. The essential enzymes are coded by genes, however.

Table 2 is a somewhat arbitrary list of nonproteinaceous toxins. The usual definition of a toxin is a toxic substance produced by a living organism. Nicotine, certain biological nitrogenous wastes, and possibly ethanol would be considered toxins according to this definition; however, they are not usually considered as toxins although picrotoxin, which is considerably less toxic than nicotine, is usually included.

Table 2. Nonproteinaceous Toxins

NAME	MW	STRUCTURE	SOURCE	ACTION
Anatoxin A	165	bicyclic	*Anabaena flos-aquae* (blue green alga)	cholinergic receptor agonist
Aconitine	633	polycyclic	*Aconitum napellus*	persistent activation of sodium channels
Batrachotoxin	538	polycyclic	*Phyllobates aurotacnia* and *P. terribilis* (South American frog)	causes persistenent activation of sodium channels
Brevetoxin	900	cyclic polyether	*Ptychodiscus brevis* (dinoflagellate)	neuron depolarization
Caulerpin	398	pentacyclic	*Caulerpa racemosa* and other C. spp (marine alga)	--
Ciguatoxin	--	oxygenated polyether	*Gambierdiscus toxicus* *Ostreopis lenticularis* plus other dinoflagellates	membrane depolarization
Debromoapysiatoxin	--	polyester	*Lyngbya gracillis* (seaweed)	--

Gephyrotoxin	289	trycyclic	*Phyllobates terribilis* and other	inhibits ion passage through channels
Grayanotoxin	398	tetracyclic	*Rhododendron ericaceae*	causes persistent activation of sodium channels
Histrionicotoxin	285	bicyclic	*Phyllobates terribilis* and other including *Dendrobates histrionicus*	blocks outward potassium movement through channels blocks acetylcholine evoked ionic conductance
Maitotoxin	--	--	*Gambierdiscus toxicus* (dinoflagellate)	increases calcium influx through voltage channels
Palytoxin	2677	cyclic	*Palythoa toxia* and other species	depolarization of myelinated nerve fibers
Picrotoxin	602	$C_{30}H_{34}O_{13}$	*Anamirta cocculus* (fish berries)	inhibits chloride channel - indirect GABA inhibitor
Pumiliotoxin B	324	bicyclic	*P. terribilis* and other	stimulates calcium release; inhibits calcium storage
Pumiliotoxin C	195	bicyclic	*P. terribilis* and other	inhibits ion passage through channels
Saxitoxin	286	tricyclic	*Gonyaulax tamarwnsis* *G. excavata* (dinoflagellates)	inhibits opening of sodium voltage channels
Strychnine	310	polycyclic	*Strychnos nux vomica*	central nervous system
Tetrodotoxin	320	tricyclic	*Takifugu poecilonotuss* (dinoflagellates)	inhibits opening of sodium voltage channels
Trichothecene toxins	~560	polycyclic	Fusarium fungi (several species)	inhibit protein synthesis
Veratridine	673	polycyclic	*Veratrum album* *Schoenocaulon officinale*	causes persistent activation of sodium channels

5.1 *Batrachotoxin.*

Batrachotoxin is a three-ring compound that binds to voltage channels in neurons. These channels conduct neural impulses by opening briefly to allow sodium ions to enter the cell and then closing quickly [58]. Batrachotoxin inhibits closure of the channels so that sodium continues to enter the neuron until it is completely depolarized. The neuron is unable to transmit a signal until the ion gradient is reestablished. Batrachotoxin is isolated from the skin of several brightly colored frogs that are native to South America [59]. South American Indians cover the points of their hunting darts with a mixture of toxins secreted by these frogs. The toxins are stored and probably synthesized in glands located in the skin. When the frog is traumatized, the toxins pass through the epidermis via ducts and cover the external surface with a milky film. Dart tips are then rolled through the film and dried. The toxin remains active for at least a year. It seems unlikely that enough frogs could be grown in captivity to permit production of the toxin in threat quantities.

Very little is known about the biochemical pathways for synthesizing batrachotoxin. Currently, there is no major effort to elucidate them because the principal interest in batrachotoxin is its role in studying neurophysiology. Undoubtedly, many enzymes are involved. A recombinant DNA approach would require the isolation of the essential enzymes and subsequent cloning of respective genes. Sometimes all the genes that code for enzymes involved in synthesis of a compound are located at adjacent regions on a chromosome with a single regulatory site. A single large mRNA, called a polycistronic messenger, is transcribed that provides the code for sequential synthesis of all the proteins. This arrangement assures the availability of essential enzymes when they are needed. If such a batrachotoxin operon exists, it could be inserted into an rDNA vector; however, it is extremely unlikely that the regulatory parameters for the eukaryotic system would be compatible with the bacterial machinery. A eukaryotic host such as yeast would be more likely to succeed, but the knowledge gaps are too great to permit production by recombinant DNA techniques. Moreover, it is very unlikely that a batrachotoxin operon exists. Although operons are common in prokaryotes, none have been reported for eukaryotes. The difficulties imposed by mRNA processing and transport of the final messenger from the nucleus probably preclude polycistronic messengers. The closest example in eukaryotes involves protein processing rather than genetic control. Certain RNA viruses, during the early part of the cell cycle, translate the entire RNA sequence into a single long polypeptide that is subsequently cleaved into several functional proteins.

For some time, it has been possible to remove cells from an organism and grow them in culture. Such an approach could lead to production of batrachotoxin because only the skin cells that synthesize it would be grown. As a general rule, toxin production occurs in highly differentiated cells; however, the cells that typically grow best in culture are the less differentiated. It is possible that little toxin would be produced even though the cells grew. More likely, the cells would survive in culture but would not divide. The result would be limited production.

With today's knowledge and technology, the best approach for large-scale production of batrachotoxin would be to develop a hybrid cell by fusing the toxin producing cells with an immortal cancer cell line. This technique has been very effective for production of certain proteins from mammalian cells but has not been used to produce nonproteinaceous products. Also, the number of frog cell lines with which to fuse the desired cells is limited. Because cell fusion is a low probability event, efficient assays would have to be developed to screen a large number of clones for the desired product. This could probably be accomplished with an assay that measures the passage of sodium-22 through voltage channels. Cells containing the highest radioactivity would have been exposed to the highest concentration of batrachotoxin.

Although it is possible to develop a research plan that, if successful, could lead to large-scale production of batrachotoxin, there are so many unknowns and major hurdles that it would probably be more prudent to select a toxin from another source that would be more amenable to scale-up. Because so many other toxins exist with similar toxicities, there doesn't appear to be any compelling reason to select batrachotoxin.

5.2 *Tetrodotoxin.*

Whereas batrachotoxin keeps the voltage channels open, tetrodotoxin keeps them closed so that no sodium ions pass through the membrane upon stimulation; and the neural impulse is lost [60]. Tetrodotoxin is a three-ring compound that is produced in the visceral organs of puffer fish. Called "fugu" in the Orient, puffers are a delicacy especially valued by the Japanese. The aura of fugu probably results from the narcotic effect caused by ingestion of low levels of tetrodotoxin.

Puffer fish can be maintained in aquariums; however, they are difficult to catch in quantity and are not known to breed in captivity. The principal difficulties for large-scale production of tetrodotoxin would be the same as for batrachotoxin.

5.3 *Toxins from Dinoflagellates.*

Plankton are small, usually unicellular plants and animals that float freely in the ocean. The plants or phyloplankton belong to the algae phylum because they contain chlorophyll and produce food via photosynthesis. They are the primary sources of marine organic matter and constitute the principal food source for shellfish and many finfish. Although diatoms are rigid because of silica in their external cell walls, dinoflagellates are flexible and therefore more susceptible to physical damage, especially in culture. The dinoflagellates are capable of limited motion with their two flagella. When the water temperature, nutrients, salinity, and sunlight are optimal, the dinoflagellates can grow very rapidly and form a bloom or red tide that results in a brown, amber, or yellow-green discoloration of the ocean. These pigments probably aid in photosynthesis by expanding the usable region of the visible spectrum [61].

It seems that an inordinately large number of toxins are produced by these marine unicellular organisms. Toxin synthesis is not constant but rises to a maximum toward the end of logarithmic growth and continues briefly into stationary phase [62]. For blue-green algae and dinoflagellate toxins, there is no simple relation between the concentrations of cells and of toxin because optimum conditions for cell growth and toxin synthesis are different. Also, many of the toxins are endotoxins that are released into the environment only upon cell lysis, which occurs most often near the end of the growth phase [63].

A few years ago, E.S. Silva hypothesized that the paralytic shellfish poisons (PSP) were produced by bacteria associated with the dinoflagellates or as a result of a symbiotic relation in which some intracellular bacterium stimulated the production of the toxin. Silva supported this hypothesis with data reporting the presence of a bacterium in the cytoplasm of *P. tamarensis* and in the nucleus of *G. instriatum.* Today it is apparent that dinoflagellates influence bacterial growth by releasing nutrients and possibly bacterial inhibitors. Sometimes different bacteria dominate different isolates of the same species. In one study, no bacteria could be detected by electron microscopy in a very toxic strain of *P. tamarensis.* In another study, no PSP toxins could be detected in bacterial isolates. Thus far, it has not been possible to transfer toxicity into nontoxic strains by incubating with bacteria from toxic strains. Current evidence does not disprove Silva's hypothesis, but evidence against it is increasing faster than the evidence supporting it [64].

There is some evidence that bacteria may modify toxins. Both a Pseudomonas species and a Vibrio species isolated from the viscera of a crab and a turban shell were able to convert gonyautoxins to saxitoxin by reductive elimination of the C-11 hydroxylsulfate and the N-1 hydroxyl moieties. This finding partially explains why these mollusks contain saxitoxin and neosaxitoxin while their principal food alga, *Jania sp.*, produces only gonyautoxin I, II, and III [65].

5.3.1 *Saxitoxin.*

Saxitoxin is one of the causative agents of paralytic shellfish poisoning. It binds to the voltage channels along the axon of neurons and, like tetrodotoxin, maintains an active state so that sodium continuously enters until the neuron is depolarized. There is no direct effect on the outward diffusion of potassium ions. Clinical effects are numbness of the lips, tongue, and fingertips soon after ingestion that spreads to the legs, arms, and neck. A general muscular incoordination leads finally to respiratory distress and muscular paralysis. Classical therapy involves palliative treatment of the symptoms [66].

Saxitoxin was first isolated from the Alaskan butter clam, *Saxidomas giganteus*, but further studies indicated that it was produced by dinoflagellates, particularly those of the genus Gonyaulax including *G. tamarensis*. Shellfish consume the dinoflagellates and thereby become contaminated with the toxin. Recently, saxitoxin was discovered in the livers, ovaries, and digestive tracts of puffer fish. Presumably, the puffers obtained the toxin from bivalves that had previously ingested the dinoflagellate *Protogonyaulax tamarensis*. If this is prevalent, then some of the toxicity associated with puffer fish may be due to saxitoxin [67]. Although saxitoxin is usually considered a marine toxin, it was identified in the freshwater blue-green alga *Aphanizomenon flos-aquae* [68].

The metabolic pathway for synthesis of saxitoxin was studied, but only limited knowledge is available concerning the origin of the tricyclic ring system because the photosynthetic dinoflagellates resist utilization of exogenous organic tracer compounds. Feeding [13C]-glycine resulted in enrichment of all the ring carbon atoms. Use of [13C]-acetate resulted in preferential enrichment of C-5 and C-6, and, coupled with previous data, led to the conclusion that arginine and acetate condense to form an intermediate that subsequently cyclizes to the saxitoxin nucleus [69].

An improved procedure for the total synthesis of racemic saxitoxin was recently reported. This approach is reasonably efficient (0.5-1.0 g was produced in the laboratory) and requires no chromatographic purifications of the intermediates. The sequence requires several reactions -- some may be difficult to adapt to industrial processes [70].

- *How could saxitoxin be produced more efficiently?*

Dinoflagellates are among the more difficult phytoplankton to culture. Because their cell wall is more fragile than bacteria and yeast, vigorous aeration is frequently detrimental. As a general rule dinoflagellates are sensitive to metal ions, particularly copper. Light is also a critical factor. Intensity and spectral quality should be regulated to correspond to the natural light where the isolates were obtained. Optimal lighting may be quite different from whole sunlight if the dinoflagellates were isolated several feet below the surface. Several excellent media have been developed that permit the initiation and maintenance of dinoflagellate cultures in the laboratory when good techniques are followed [71].

- *Could saxitoxin be modified to facilitate synthesis or increase toxicity?*

Studies on saxitoxin analogs suggested that the 7,8,9 guanidinum region and the C-9 and C-10 hydroxyls are possibly the essential moieties for binding to the receptor and for toxicity. These are the same as the active groups on tetrodotoxin. The carbamyl group in saxitoxin contributes to binding but is not essential; however, removal reduces the toxicity considerably. A new model proposes that saxitoxin and tetrodotoxin bind to a receptor located on the outside surface of the membrane near the actual sodium channel [72-73].

- *Outlook.*

It is unlikely that new saxitoxin analogs will have much greater toxicity than the parent compound. Current research directed toward elucidating the binding parameters may lead to the successful design of smaller molecules that can be synthesized chemically with less effort. These compounds may not possess the binding specificity of saxitoxin; therefore, their toxicity may be reduced by nonspecific binding to noncritical targets. Design of new compounds that penetrate the skin and other barriers could lead to a faster acting toxin. Although chemical synthesis of saxitoxin is feasible, the cost is probably prohibitive on any production scale.

Production of the toxin by culturing dinoflagellates will probably be feasible in the future. New fermentors should reduce mechanical damage to the organisms. Optimizing culture conditions should increase yields.

5.3.2 *Brevetoxin.*

The brevetoxins are a series of macrocyclic polyethers that are produced by the dinoflagellate *Ptychodiscus brevis* (formerly *Gymnodinium breve*) [74]. The Red Tides caused by these organisms are responsible for fish kills due to their neurotoxic properties. The mechanism of action is associated with activation of the sodium channels [75]. Axons on both adrenergic and cholinergic fibers are affected. Calcium flux is also affected, but this may be a secondary effect [76].

The principal limitation of the use of brevetoxins as a weapons system is the lack of material. They are fairly large for nonproteinaceous toxins with numerous asymmetric centers so that a total chemical synthesis on a large scale is not feasible. There is no single gene that codes for a brevetoxin molecule because it is not proteinaceous. The metabolic pathway for biological synthesis is unknown but undoubtedly requires several enzymes. Theoretically, it would be possible to identify, isolate, and clone the genes for these enzymes after the synthetic path had been elucidated, but the effort required would far exceed the value of the toxin as a weapon.

Currently, the toxin is obtained by extraction of cultures of the dinoflagellate *Ptychodisus brevis*. In a typical isolation, 90 mg of crude toxin was obtained from 50 liters of artificial sea water [77]. The feed stocks are not particularly expensive so that scale-up is feasible. The toxin is soluble in ether and other solvents so that separation of the toxin from protein and other cellular material is relatively easy.

Although it doesn't seem feasible today to insert all the genes into bacteria, it may be feasible to amplify production in the dinoflagellate through experiments with gene regulation. The yield of penicillin has been increased enormously by developing and selecting better strains.

It should be possible to produce large quantities of brevetoxins by culture of appropriate dinoflagellates; however, the brevetoxins are considerably less toxic [78] than other nonproteinaceous toxins so the effort and resources required to optimize conditions would probably be more profitable for another toxin unless some significant breakthrough occurred quickly.

5.3.3 *Ciguatoxin.*

Ciguatera, the most common poisoning resulting from eating fish, has been known since the 15th century. Ciguatoxin, the causative agent, is produced by the dinoflagellate, *Gambierdiscus toxicus*, which is loosly attached to algae growing on coral reefs. Ciguatoxin has been reported in Australia, Japan, Hawaii, and the Atlantic. Small herbivorous fish consume the contaminated algae and thereby enter the toxin into the food chain [85]. Although the toxin is harmless to fish, ingestion by humans leads to nausa, vomiting, and abdominal pain. Most cases are mild; however, occasionally the poisoning is severe or fatal. Currently, there is no effective therapy. Atropine, EDTA, steroids, and electrolytes were tried without benefit [86].

Ciguatoxin appears to be a cyclic polyether with a molecular weight about 1100. Although the toxin has been isolated in different forms, some if not all are interchangeable on alumina chromatography. The toxin has been crystalized, and the proton nuclear magnetic resonance (NMR) spectrum recorded [87]. This progress suggests that the complete structure will be reported soon.

The problems in the fish industry caused by contaminated fish led to the development of numerous qualitative and quantitative tests for ciguatoxin. One biological assay relies on sensitivity of mosquitoes to the toxin in fish preparations extracted with organic solvents [88]. A radioimmunoassay (RIA) [89] and an enzyme-immunoassay [90] use sheep anticiguatoxin serum. A new enzyme immunoassay stick test uses a special coating to adsorb ciguatoxin and related polyethers onto the solid phase [91]. This assay simplifies detection considerably and permits monitoring suspected fish outside the laboratory. These tests are much faster than the traditional mouse bioassay that was used to determine an LD_{50} of 87 mg/kg [92].

Currently, ciguatoxin is believed to interact and modify sodium channels. At concentrations of 0.25-1.25 x 10^{-3} μg/ml, ciguatoxin produced spontaneous action potentials in isolated frog nerve fibers that were reversed when the toxin was removed [93]. Similar effects occur in the atrial and papillary muscle of the guinea pig heart. Ciguatoxin stimulates channel opening but has little effect on channel closing. There is some indication that electrical stimulation enhances toxin binding. The effects of ciguatoxin could be reversed by tetrodotoxin, a toxin that inhibits channel opening [94].

There have not been any reports of simplified procedures that would lead to large-scale production of the toxin. Most approaches rely on extraction of the toxin from fish followed by purification by chromatography [95]. "Major advances in treatment for ciguatera and detection of ciguatoxin await the means of producing additional ciguatoxin" [96]. The most reasonable approach for scale-up would be culture of the dinoflagellates. If *G. toxicus* cannot be cultured alone in fermentors, it might be necessary to grow them on the surface of algae. This modification would require a light source for photosynthesis but could reduce the cost of nutrients. Because of the large size of ciguatoxin and its apparent complexity, chemical synthesis is probably not a viable approach for producing significant quantities of toxin.

5.3.4 *Maitotoxin.*

Maitotoxin, a potent marine toxin, is a principal cause of seafood poisoning that is isolated from the dinoflagellate *Gambierdiscus toxicus*. Because it was discovered recently and has not been purified to homogeneity, the published data should be considered preliminary. Both maitotoxin and ciguatoxin have been found in the same organisms [97]. Although maitotoxin's structure has not been elucidated, it is probably nonproteinaceous. Maitotoxin is currently believed to be the most potent marine toxin with toxicity about 50 times that of tetrodotoxin.

In the past, purification was achieved with silicic acid, DEAE cellulose and Sephadex chromatography, whereas newer methods rely on reverse-phase high-performance liquid chromatography (HPLC) with methanol as the mobile phase [98].

The toxin is usually manifested by cardiotoxicity resulting from increased calcium influx into cells. Stimulated calcium influx may result in secondary effects such as modifying the release of dopamine [99], GABA [100], arachidonate, and prolactin [101]. In cell culture with myocardial cells, 0.1-10 ng/ml maitotoxin caused an increase in contraction and arrhythmogenic action that was eliminated by calcium-free medium [102].

Because the structure is not known, it is not possible to be too specific about applications of biotechnology. As indicated above, it is probably feasible to grow quantities of the dinoflagellate and then to extract and purify the resulting toxin. If the toxin is proteinaceous (which seems unlikely), it should be possible to clone the respective gene and express it in a bacterial or eukaryotic system.

5.4 *Anatoxin.*

Anatoxin is a bicyclic alkaloid produced by the freshwater blue-green alga *Anabaena flos-aquae*. The toxic algal blooms have been responsible for the deaths of fish, livestock, and birds. Anatoxin A was originally called "very fast death factor" because of lethality resulting from respiratory paralysis. Death in mice occurs in 2-5 min. Subsequent work has shown that this toxin binds to the nicotinic cholinergic receptor and stimulates the neuron in a manner similar to the natural neurotransmitter, acetylcholine. Because anatoxin is not an ester, it is not hydrolysed by acetylcholinesterase so that stimulation of the nerve terminal continues until the neuron is depolarized [79]. There is also evidence that anatoxin A inhibits acetylcholinesterase. At concentrations between 0.35 and 0.60 μg/kg, anatoxin A completely inhibited rat serum cholinesterase; however, inhibition of cholinesterase is not the principal mechanism for toxicity because the LD_{50} (0.05 μg/kg for mice given intraperitoneally) is considerably lower [80]. The magnitude of the threat from anatoxin A depends on its toxicity. If the toxicity for humans is equal to the value listed above, there could be a serious threat. If the human toxicity is closer to that for ducks (LD_{90} = 50 mg/kg given intraperitoneally) [81], the threat is considerably less.

The chemical synthesis of anatoxin A has received considerable attention. Since the identification of its structure in 1977 [82], at least seven publications have reported improvements in the synthetic approaches. One approach begins with D or L glutamic acid and produces anatoxin A with high optical purity [83]. A more recent approach could probably be scaled-up to produce large quantities of toxin in about 60% yield with a four-step procedure [84].

Anatoxin A can also be produced by culturing the algae. Significant quantities are produced biologically when the cultures reach maturity in about 14 days. Because much of the toxin is found in the supernatant (either excreted by the algae or released during cell lysis), it is usually isolated from the entire culture by extraction or by ion exchange chromatography.

The relatively simple structure of anatoxin A provides several chemical approaches for large-scale production and possible binary reaction. Although the toxin could probably be obtained from microbiological culture as effectively as other toxins, it seems more probable that anatoxin could be produced more efficiently by chemical methods unless some new strains are developed. It also doesn't seem realistic to produce the toxin by another organism - either at a manufacturing site or inside a human by some pathogenic organism.

6. SHORT PEPTIDES

Most classical neurotransmitters are small molecules that are released at the nerve terminal, diffuse across the synaptic junction, and stimulate a response (neural, muscular, etc.) on the postsynaptic receptor. Examples include acetylcholine, gamma aminobutyric acid, dopamine, epinephrine, glutamate, glycine, histamine, norepinephrine, and serotonin. More recently it has become evident that small peptides also act as neurotransmitters and/or modify neural responses. Examples of some of these neuropeptides are listed in Table 3.

Table 3. Neuropeptides [103]

Angiotensin 1	Motilin
Angiotensin 2	Neurophysin
Angiotensin 3	Neuropeptide Y
Bombesin	Oxytocin
Bradykinin	Pancreatic polypeptides
Calcitonin	Physalaemin
Carnosine	Neurotension
Cholecystokinin	Pituitary peptides
Corticotropin-releasing factor	Proctolin
Ependymin	Prolactin
beta-Endorphin	Secretin
Gastrin	Somatomedin
Glucagon	Somatostatin
Gastrointestinal polypeptide	Substance P
Insulin	Thyrotropin
[Met]enkephalin	Lysine vasopressin
Melatonin	Vasotocin
Melanocyte-stimulating hormones	Vasointestinal polypeptide

Among these neuropeptides, the enkephalins seem to offer the greatest potential for military weaponry by potential aggressors. Their small size (about 5 amino acids) permits large-scale synthesis and facilitates distribution through tissues. Like the opioids, the enkephalins exert two principal effects on nerve function. They inhibit the rate of firing and they reduce the amount of neurotransmitter released by the neurons. [Met]enkephalin has the structure tyr-gly-gly-phe-met, whereas [leu]enkephalin consists of tyr-gly-gly-phe-leu.

It is feasible to produce large quantities of enkephalins by chemical synthesis. Solid-phase protein synthesis was developed several years ago by Bruce Merrifield. The carboxyl terminal amino acid is covalently attached to a resin, and the additional amino acids are attached chemically one at a time. Because the product is attached to a solid phase, the excess reagents and solvents can be removed easily by filtration with minimal loss of product. Modern equipment is automated and controlled by microprocessors so that the technician programs the amino acid sequence and adds the chemicals while the protein synthesizer runs through the addition, washing, and unblocking steps.

Because chemical reactions never go to 100% completion, substantial errors can occur that alter protein function when long peptides are attempted. Production of short peptides like enkephalins should not be a problem. Whereas solid phase protein synthesis is ideal for single preparations, genetic approaches offer advantages for long-term, large-scale manufacturing operations. A theoretically superior method would be to design and synthesize a gene that would code for the desired peptide and introduce the synthetic gene into an appropriate host for biological synthesis.

It is probable that neuropeptides will become available in the near future as a result of research in the medical community. Natural peptides, as well as new analogs, will be produced and marketed by pharmaceutical companies.

Even though these peptides are small, they have very low volatility and have difficulty passing through natural barriers like skin. Chemical analogs that are designed without peptide bonds could reduce some of these limitations. The greatest threat from these biologically active peptides could come from *in vivo* synthesis inside a human following infection with a new biological agent.

7. VIRUSES

7.1 *Properties.*

Viruses are small, obligatory intracellular parasites because they do not have sufficient cellular machinery to perform the necessary functions independently. Consequently, the host must supply the required enzymes, metabolic intermediates, and energy sources. Some viruses contain their genetic material as RNA whereas others have DNA. Both single-stranded and double-stranded viruses have been identified. The small RNA phage (virus with bacterial host) contains only three genes that code for an A protein, a capsid protein that forms the external shell of the virion, and a replicase that produces additional copies of the viral RNA chain. In contrast, about 140 genes have been discovered in T4, the bacteriophage that infects *E. coli*.

The life cycle of viruses differs considerably from that of bacteria. In bacteria, after DNA replicates; the cell divides to produce two identical daughter cells. Soon after a virion enters a cell, it can no longer be identified as an intact entity. The coat has dissolved or disappeared, and the genetic material is distributed through the cell. Eventually, new virions start to assemble by formation of a capsid around the viral genome. There are only a few different proteins in the capsid, but the numerous copies aggregate (usually in a regular pattern) to form a coat. The resulting virion may be icosahedral, helical, or complex. Following assembly, the cell is lysed and the virions are dispersed. The expression of viral genes is rigidly controlled. As a general rule, the genes that are expressed early code for enzymes that replicate the genome and molecules that inhibit the host's metabolism. Those that are expressed late are involved with formation of the capsid and with cell lysis.

During infection of bacteria, the coat attaches to the cell surface before the viral genome is injected directly into the bacterium. Usually the capsid does not enter the bacterium - perhaps because of the rigid cell wall. In contrast, animal cells normally phagocytize the entire virion.

7.2 *Applications.*

In order for a gene to produce a toxic protein inside an organism, the DNA comprising the gene must first enter a cell where the necessary biological machinery is located. In the laboratory under controlled conditions, DNA precipitated by addition of calcium salts can be taken up by mammalian cells. A few of the DNA fragments are eventually incorporated into chromosomal DNA. Sophisticated screening techniques employing biochemical markers are required to select the transfected cells from the large number of native cells that do not contain the new DNA inserts. It is also possible to inject DNA into the nucleus of a cell with a micropipette having a diameter of 0.1 to 0.5 microns. Experienced investigators can inject 500-1000 cells per hour. About half of these cells will stably integrate the DNA into its genome. Although these techniques are very useful in the laboratory for experimental and developmental studies, they would not be applicable to a weapons system.

Several factors make viruses ideal vectors for introducing genes that code for specific toxins. A toxin must enter the body in order to exert any physiological action. For small hydrophobic molecules with high vapor pressures or which cross biological membranes readily this is not a problem; however, proteins are usually restricted to a few paths of entry. Viruses possess natural methods for infecting their hosts. Because the host range is usually narrow, only a few viruses would be candidates. Viruses could be selected so that indigenous livestock, wild animals, and plants would not be affected. Second, the viruses have extraordinary power to regulate cellular functions. During a severe infection, most of a cell's energy is directed toward making viral products. If one of the products is a toxin (either natural or engineered), the *in situ* yield could be enormous. Third, the viral growth cycle normally ends with cell lysis so that the toxins and newly produced virions would be discharged into the lymphatic and circulatory systems for distribution through the body. Fourth, the viral genome is small so that it is easy to manipulate. The small number of restriction sites would facilitate the insertion of new genetic information at predetermined sites. Fifth, viruses would provide a complete package to protect the gene. Although some viruses are unstable and persist only a short time outside the body (i.e., some of the viruses that cause venereal disease), others are quite stable. A stable virus would extend the shelf life of the weapon. It would probably be necessary to store the viruses away from strong light (particularly ultraviolet), ionizing radiation, and other mutagens. As a general rule, viruses are very susceptible to mutagens because they do not possess the proofreading enzymes that repair damage to the genome before it is "set" during replication. These agents would reduce their effectiveness as weapons because random mutations usually result in a reduction in virulence.

The development of new biological weapons by inserting genes into viruses would require a long-term effort. The level of knowledge of certain bacterial viruses, particularly lamda viruses, is sufficient that additional genes can be added easily and subsequently expressed. Such is not the case with animal viruses, particularly those of humans. Many in the scientific community, particularly those in academic research, are interested in studying basic fundamental principles. These can be learned with less risk using nonhuman viruses so the development of human viral vectors is likely to be slow. Evidence of research programs for the development of human viral vectors could be suggestive of a biological weapons program. The development of a viral biological weapons program would require research with human viral vectors; however, there are many other applications for this type of research. The medical

community hopes to use nonpathogenic viruses to introduce new genes into patients suffering from certain genetic diseases; however, such therapy, if possible, lies far into the future.

Vaccinia virus was originally used by Edward Jenner to immunize against smallpox. Recently, it was engineered into a vector for the expression of foreign genes [104]. Vaccinia is a large DNA virus with about 185,000 base pairs (bp) whose ends are covalently linked as a hairpin. This hairpin terminus is one type of arrangement that facilitates the replication of linear viral DNA. The virus contains genes for replicating its DNA, a DNA dependent RNA polymerase for producing mRNA, and mRNA processing enzymes so that it can function in the cytoplasm. Some viruses that don't carry all these genes must replicate and assemble into new virions inside the nucleus. To use the virus as a vector, the new DNA must be inserted into the viral genome in such a manner that it is expressed. The virus must also remain virulent because it cannot operate outside a host cell. Frequently, when new DNA is added, the virus becomes less virulent; however, vaccinia seems to be able to accommodate additional DNA without loss of activity -- perhaps because the percentage of increase is small due to its large initial size.

The large size of the vaccinia DNA increases the difficulty of introducing an isolated gene or cDNA directly into the viral genome at a prescribed locus. Instead, a two-step approach is used. The viral DNA at the site of desired insertion is isolated in some manner. The new gene is surrounded by this DNA and then introduced into a plasmid. The host is transfected with the plasmid containing the gene and infected with the virus. By homologous recombination, the plasmid DNA containing the new gene is exchanged for the corresponding DNA on the virus. This recombination occurs rarely (about 0.1%) so some form of selection is needed. One method is to select the insertion site in the middle of the thymidine kinase gene. (Thymidine kinase phosphoralates the deoxynucleoside thymidine prior to incorporation into DNA.) When the thymidine kinase minus recombinants are grown with bromodeoxyuridine (a thymidine analog), the native cells are killed by the incorporated analog while the recombinants, which cannot use the analog, survive.

The expression of the gene, with respect to the amount of protein produced as well as the time of synthesis in the life cycle, depends on the particular promoter. In one experiment about 10^8 surface antigen proteins for hepatitis B were produced by vaccinia virus recombinants. Work is underway in several laboratories tc improve viral vectors for recombinant DNA research.

The gene for human proenkaphalin has been cloned into vaccinia virus. When several mammalian cell lines were infected, all produced and secreted proenkaphalin. The cells from the mouse pituitary line, GH4C1, were able to process the proenkaphalin by proteolytic digestion and secrete mature Met-enkephalin. Although demonstration of secretion of neuropeptides by viral infected cells does not confirm the effect *in vivo*, this study increases the concern about the use of viruses as biological weapons [105]. Because processing of proenkephalins is tissue specific, probably only neural cells would secrete mature enkephalins, whereas the other infected cells would secrete a variety of precursors.

Research with vaccinia virus demonstrated the feasibility of introducing foreign genes into viral vectors and expressing them in human cells; however, vaccinia virus would probably not be an effective component of a biological weapon. When vaccinia virus was used to vaccinate against smallpox, the spread of infection was infrequent and required close physical contact. It might be possible to alter the viral capsid by introducing genes for other coat proteins; however, there is no evidence that this would make the virus more virulent. Moreover, because most people have been immunized with vaccinia virus, the general population has a considerable titer of antibodies to this virus. With the eradication of smallpox, vaccinations have ceased and the population that is immune to vaccinia virus will continue to decline.

7.3 *Prospects for the Use of Viruses.*

The use of human viruses as vectors for dissemination of proteinaceous toxins, whether large proteins or small neuropeptides, may eventually pose the greatest threat of all biological weapons because they provide a mechanism for introducing the biological material into the soldier where the toxic effects will be manifested. Delivering sufficient material inside the target is a major limitation of proteinaceous toxins as biological weapons. The basic principles of gene cloning and expression in viral vectors have been demonstrated. The ability of viruses to invade cells, replicate, and excrete products from lysed cells is a property inherent in the life cycle of viruses. Although it would require considerable time and effort to develop a weapon, most of the basic research has been completed; therefore, a precise program could be delineated. The major limitation today is the lack of appropriate viral vectors. They will probably be developed by the medical community where there is considerable interest in the use of viral vectors as vaccines for preventing the spread of infectious diseases and possibly for curing or controlling certain genetic diseases. Design and modification of candidate viruses is a major area of research in virology [106].

8. RICKETTSIA

Rickettsia are usually classified as bacteria; however, they are smaller and possess fewer enzymes than most bacteria. Rocky Mountain Spotted Fever results from infection by *Rickettsia rickettsi*, whereas the less severe Q fever is caused by *Coxiella burnetii*. All rickettsia are obligate eukaryotic parasites; that is, they cannot grow outside a living cell. Because rickettsia cannot metabolize glucose and probably most other sugars, they must obtain their energy from their host [107]. Preliminary data suggested that the intermediary metabolites, ATP, NAD, and CoA passed freely through leaky membranes, but recent evidence indicates that rickettsia possess unusual as well as traditional transport systems [108].

It might be possible to develop rickettsial organisms that could exist outside cells by inserting one or two genes. Unlike viruses, these organisms have some metabolic capabilities as indicated by their use of pyruvate and other components of the citric acid cycle. These mutant organisms could then be obtained in sufficient quantities for production and would probably vegetate in the environment upon release. Currently, rickettsia are grown in the yolk sac of chicken embryos.

The associated diseases are transmitted to humans via arthropod vectors such as fleas, ticks, lice, and mites. The rickettsia, contained in arthropod feces, enter the human through a minute skin lesion caused by a bite or scratch. Only Q fever, which is seldom fatal, is transported via aerosol-dried arthropod feces and results in respiratory infection. Viability of the organisms decreases rapidly while they are outside the host because of rapid loss of vital metabolites. Insertion of appropriate genes could make the cell wall less permeable and increase survivability. In contrast, one could take advantage of the leaky cell wall and insert genes for additional toxins that might be excreted rapidly.

Genetic modification of rickettsia could increase the difficulty in treating the disease. Current therapy is sufficient for most cases when diagnosed properly because rickettsia are quite sensitive to chloramphenicol and the tetracyclines. Because they are resistant to most other antibiotics, insertion of a few genes coding for antibiotic resistance could make them refractory to most drugs.

There is evidence that rickettsia secrete toxins even though none has been identified. The rapid onset of death when mice are injected with large quantities of rickettsia is consistent with a toxin mechanism. Also, irradiation with ultraviolet light reduces the viability of these organisms but does not reduce the toxicity. There is always the threat from insertion of the gene(s) for this toxin into another host. This could be particularly devastating because the properties of the toxin and how it functions are not known.

9. CONCLUSIONS

The advances in molecular biology and other areas of biotechnology provide new possibilities for modifying existing biological weapons and for developing new ones. Vegetative organisms and toxins that were previously considered to pose minimal threat may present a major risk in the future. The threat or risk of biological warfare (BW) agents that were developed by classical methods is not diminished by the possibility of producing new weapons.

The number of bacteria, viruses, and toxins that could be developed is so numerous that it is not possible to predict the future threats with certainty without extensive intelligence. Nevertheless, some possibilities appear more likely.

Biotechnology could be used in the near term to produce large quantities of nonproteinaceous toxins by large-scale culture of unicellular organisms followed by classical purification techniques. Saxitoxin would be a possible candidate; however, other toxins would be equally effective. Anatoxin A should be considered as a special case because its simple structure permits synthesis by chemical methods as well as isolation from cultured algae.

In the mid term, it could be possible to modify infectious bacteria to make them more virulent or to produce new toxins or other regulatory peptides *in vivo*.

The ultimate threat may come from the development of human viral vectors that code for toxic proteins or other physiologically acting peptides. The virus would provide the mechanism for entry into the body. Once inside the cell, it would replicate, lyse the cell, and distribute the products and new virus particles throughout the body.

The author has examined how potential enemies might apply biotechnology to develop new biological warfare (BW) agents. Many other factors that affect the development of toxic materials into weapons are beyond the scope of this study and must be considered before predicting new threats.

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REPLY TO
ATTENTION OF:

09 APR 2013

RDCB-DPC-RS

MEMORANDUM THRU Technical Director, Edgewood Chemical Biological Center (ECBC) (RDCB-D/Mr. Joseph D. Wienand), 5183 Blackhawk Road, Aberdeen Proving Ground, MD 21010-5424

FOR Office of the Chief Counsel, US Army Research, Development and Engineering Command (RDECOM)(AMSRD-CCF/Ms. Kelly Knapp), 3071 Aberdeen Boulevard, Aberdeen Proving Ground, MD 21005-5424

SUBJECT: Operations Security/Freedom of Information Act (FOIA) Review Request

1. The purpose of this memorandum is to recommend the release of information in regard to RDECOM FOIA Request, FA-13-0027.

2. The ECBC received RDECOM FOIA Request FA-13-0027 from Ms. Kelly Knapp, RDECOM FOIA Officer. The request was for an Operations Security review of documents from the Department of the Army Inspector General's FOIA Office which originated from Paige Tomicelli of the International Center for Technology Assessment.

3. A review of the below requested documents was conducted by an ECBC subject matter expert:

a. ADB-113338 (CB-000027), Possible Application of Biotechnology to the Development of Biological Agents by Potential Enemies, dated June 1987.

b. AB-117238 (CB-00675), Final Report of the AD HOC Sub-group on Army Biological Defense Research Program, dated July 1987.

c. ADA-198966 (CB-001819), Third Annual Conference on Receptor Based Bio-Sensors, dated July 1988.

d. ADA-308957 (CB-030252), Towards a Coherent Strategy for Combating Biological Weapons of Mass Destruction, dated 15 April 1996.

4. Documents 3a, 3c and 3d have been deemed appropriate for release. Document 3a must have the current distribution level changed with Defense Technical Information Center (DTIC) prior to release. ECBC has no objection to the release of document 3b, however, this document requires Headquarters Department of the Army approval prior to release.

5. The point of contact is Mr. Ronald L. Stafford, ECBC Security Specialist, (410) 436-6810 or ronald.l.stafford.civ@mail.mil.

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