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1236

RECOMBINANT DNA ADVISORY COMMITTEE  
WORKING GROUP ON TOXINS  
MINUTES OF MEETING<sup>1</sup>

August 16, 1985

The Working Group on Toxins of the Recombinant DNA Advisory Committee was convened at 9:00 a.m. on August 16, 1985, at the National Institutes of Health, Building 31A, Conference Room 4, 9000 Rockville Pike, Bethesda, Maryland 20892. The meeting was open to the public. Dr. Susan Gottesman was the Chair. The following people were present for all or part of the meeting:

Working Group Members:

John Collier	Dennis Kopecko
Sam Formal	Myron Levine
Michael Gill	Elizabeth Milewski
Susan Gottesman	(Executive Secretary)
William Habig	

A working group roster is attached (Attachment I).

Other National Institutes of Health Staff:

Carol Anonsen, OD  
William Gartland, NIAID  
Malcolm Martin, NIAID  
Stanley Nagle, NIAID  
Gilbert Ogonji, OD

Others:

Tim Beardsley, Nature Magazine  
Bill Bishai, Harvard Medical School  
Jessie Boyd, University Hospital at Boston University Medical Center  
Jeff Christy, Blue Sheet, FTC Reports, Inc.  
Jack Murphy, University Hospital at Boston University Medical Center  
John Newland, Uniformed Services University of the Health Sciences  
Alison O'Brien, Uniformed Services University of the Health Sciences  
John Williams, Beth Israel Hospital

<sup>1</sup>The working group is advisory to the RAC, and its recommendations should not be considered as final or accepted.

Dr. Gottesman, Chair, called the meeting of the Recombinant DNA Advisory Committee (RAC) Working Group on Toxins to order at 9:00 a.m. on August 16, 1985.

Dr. Gottesman said the current National Institutes of Health (NIH) Guidelines for Research Involving Recombinant DNA Molecules require specific RAC review and NIH and Institutional Biosafety Committee (IBC) approval of certain experiments before initiation. Among these experiments are those involving deliberate formation of recombinant DNAs containing genes for the biosynthesis of toxic molecules lethal for vertebrates at an LD<sub>50</sub> of less than 100 nanograms per kilogram body weight. A specific appendix referring to the cloning of toxins was constructed in 1980 by the Working Group on Toxins. That appendix, entitled "Containment Conditions for Cloning of Genes Coding for the Biosynthesis of Molecules Toxic for Vertebrates," specifies the containment to be used for the deliberate cloning of genes coding for the biosynthesis of molecules toxic for vertebrates. The appendix was based on a consideration of the pharmacological toxicity of the toxin and specifies that experiments involving cloning of the genes for toxins such as botulinum toxins, tetanus toxin, diphtheria toxin, and Shigella dysenteriae neurotoxin must be reviewed by the RAC and have NIH and IBC approval before initiation.

Dr. Gottesman said three proposals involving two of these toxins are on the working group agenda for the August 16, 1985, meeting: (1) a request for permission to clone Shiga-like toxin (SLT) structural genes from bacterial species classified in the families Enterobacteriaceae or Vibrionaceae according to Bergey's Manual of Systematic Bacteriology; (2) a proposal to remove from Biosafety Level 4 (BL4) containment K-12 host-vector systems expressing a hybrid gene encoding  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH) and portions of diphtheria toxin; and (3) a request for permission to construct a hybrid molecule in which the gene coding for interleukin-2 (IL-2) is joined to a segment of the gene encoding the A subunit and portions of the B subunit of diphtheria toxin. The hybrid gene would be cloned in E. coli K-12 host-vector systems.

#### Request for Permission to Clone Shiga-like Toxin Genes

Dr. Gottesman said Dr. Alison O'Brien of the Uniformed Services University of the Health Sciences (USHS) was requesting permission to clone SLT structural genes (defined either by nucleotide sequence homology with SLT gene probes from E. coli or by antigenic cross-reactivity of their gene products with purified E. coli SLT) from bacterial species classified in the families Enterobacteriaceae or Vibrionaceae according to Bergey's Manual of Systematic Bacteriology into E. coli K-12 under the conditions specified in Federal Register Volume 49, Number 179. Dr. O'Brien had previously sought and had obtained permission from the NIH to clone SLT from E. coli in E. coli K-12 host-vector systems. The language of that permission appeared in Federal Register 49, Number 179, and was incorporated into the NIH Guidelines as Appendix F-IV-H.

Dr. Gottesman said Appendix F-IV-H of the NIH Guidelines currently reads:

"The intact structural gene(s) of the Shiga-like toxin from E. coli may be cloned in E. coli K-12 under BL3 + EK1 containment conditions.

"E. coli host vector systems expressing the Shiga-like toxin gene may be moved from BL3 to BL2 containment conditions provided that: (1) the amount of toxin produced by the modified host-vector systems is no greater than that produced by the positive control strain 933 E. coli 0157:H7, grown and measured under optimal conditions; and (2) the cloning vehicle is to be an EK1 vector preferably belonging to the class of poorly mobilizable plasmids such as pBR322, pBR328, and pBR325.

"Nontoxinogenic fragments of the Shiga-like toxin structural gene(s) may be moved from BL3 + FK1 to BL2 + FK1 containment conditions or such nontoxinogenic fragments may be directly cloned in E. coli K-12 under BL2 + EK1 conditions provided that the E. coli host-vector systems containing the fragments do not contain overlapping fragments which together would encompass the Shiga-like toxin structural gene(s)."

Drs. Habig and Levine said they might have conflict of interest considerations in dealing with the proposal from Dr. O'Brien as they both have collaborative associations with her. Dr. Gottesman suggested they participate in the discussion and vote on motions. Dr. Milewski would note their votes and the potential conflict of interest in the minutes of the meeting.

Dr. O'Brien said she and her group had been investigating organisms for the presence of SLT. SLT is defined as being cytotoxic to cells and antigenically cross-reactive with purified E. coli SLT. SLT has been identified in members of the families Enterobacteriaceae and the Vibrionaceae, and she was now requesting permission to clone SLT genes from the Vibrionaceae and the Enterobacteriaceae in E. coli K-12. The major goal of this work is to develop a cholera toxin vaccine.

Dr. O'Brien suggested SLT may be a common property of these bacteria; in organisms such as Shigella dysenteriae 1 SLT may play a role in virulence when a large amount of the protein is produced and other virulence factors are present.

Dr. O'Brien said SLT has also been recently identified in one strain of Campylobacter and two strains of Aeromonas. The Aeromonas are classified as Vibrionaceae. The Campylobacter had at one time been classified by Bergey's Manual as Vibrionaceae but had recently been reclassified as an orphan species.

Dr. O'Brien pointed out that a number of Campylobacter characteristics are common to the Vibrionaceae. She asked whether she might receive permission to clone SLT genes from Campylobacter.

Dr. Milewski said the August 19, 1985, Federal Register announcement (50 FR 33462) published 30 days before the RAC meeting and describing the proposal stated that Dr. O'Brien requested permission to clone the SLT gene from members of the families Enterobacteriaceae and Vibrionaceae as classified in Bergey's Manual. She suggested the working group first vote on the proposal as it

appeared in the Federal Register. The group could subsequently offer a recommendation on experiments involving Campylobacter.

Dr. Levine pointed out that investigators in other parts of the world may perform the types of experiments requested by Dr. O'Brien under much less restrictive conditions. He thought U.S. investigators are unfairly shackled by differences in rules on the cloning of toxin genes.

Dr. Gottesman said there are two approaches to dealing with differences in rules between countries: (1) the working group may suggest the NIH Guidelines be modified; and (2) the investigator may request more generic approvals.

Dr. Gottesman suggested the working group proceed by voting on those portions of the request which were published in the August 19, 1985, Federal Register and subsequently propose and vote on other actions the working group deems reasonable.

Dr. Gottesman asked whether the SLT structural gene is carried on a phage. Dr. O'Brien replied that high toxin production in bacteria usually appears to be associated with the presence of SLT converting phages such as 933J and H19A/J but not always. A low level of toxin production can consistently be detected in the absence of phage, and may be associated with a chromosomal gene. Dr. O'Brien hypothesized that the phage gene may be a variant of the chromosomal gene. She suggested other variants of the gene might be found in other bacterial species. She added that the Shiga toxin gene appears to be a chromosomal gene in Shigella dysenteriae 1 since the phage cannot be induced.

Dr. Gottesman asked what is known about the host range of the SLT converting phages. Dr. John Newland of USUHS said the phages can infect other E. coli strains; other bacterial families have not been studied to determine whether these phages will infect members of these families.

Dr. Gottesman asked how much toxin was produced when the SLT gene under its own regulation on a multicopy plasmid was introduced into the E. coli host-vector system. Dr. O'Brien replied that a seven fold increase in toxin production compared to the standard strain 933 E. coli 0157:H7 occurs when the SLT gene under its own regulation is introduced into the E. coli host-vector system.

Dr. O'Brien said the issue of increased SLT production by the E. coli host-vector system should be kept in perspective. Under optimal laboratory conditions, Shigella dysenteriae 1 strain 60R produces  $10^8$  cytotoxic toxin doses per milligram (mg) protein per cell lysate; the standard strain 933 E. coli 0157:H7 produces  $10^7$  cytotoxic doses (CDs) per mg protein under optimal conditions. The E. coli host-vector strain carrying the SLT gene on a multicopy plasmid produces  $7 \times 10^7$  CDs per mg protein under optimal conditions. This level of toxin production is still less than that produced by the Shigella dysenteriae 1 strain. She added that Shigella flexneri produces approximately  $10^4$  CDs per mg protein under optimal conditions. The Vibrio cholerae vaccine strain produces  $10^4$  CDs per mg protein under optimal conditions.

Dr. Collier pointed out that at this time the specific activity of SLT toxin is not known. Dr. Gottesman said arguments on mechanism of entry into the animal and treatability of symptoms support a recommendation to RAC that this proposal be recommended to the NIH Director. Dr. Formal said E. coli host-vector systems offer an additional safety factor since experience has shown E. coli K-12 will not ordinarily colonize the bowel.

Dr. Collier moved that the working group approve of Dr. O'Brien's request as it appears in the August 19, 1985, Federal Register. Dr. Kopecko seconded the motion.

Dr. Gill said he would like the record to show the working group was taking this action because it judged that E. coli host-vector systems carrying the SLT gene are no more harmful than Shiga toxin expressing strains found in nature.

By a vote of six in favor, none opposed, and no abstentions, the working group recommended RAC approve Dr. O'Brien's request as it appeared in the August 19, 1985, Federal Register. (Drs. Levine and Habig voted in support of the motion.)

Dr. Newland asked whether 933 E. coli 0157:H7 would be the standard against which activity is measured regardless of what strain was used as the source of the SLT gene. Dr. Gill said the strain specified in Appendix F-IV-H of the NIH Guidelines would be the standard strain regardless of the source of the SLT gene.

Dr. O'Brien asked the working group to consider substituting in Appendix F-IV-H a shigella strain which produces more toxin than 933 E. coli 0157:H7 as the standard against which to measure toxin production. Shigella dysenteriae 1 strains produce approximately  $10^8$  CDs per mg protein in cell lysate; standard strain 933 E. coli 0157:H7 produces  $10^7$  CDs per mg protein under optimal conditions.

Dr. Collier said the question is whether there is any reason to believe an E. coli K-12 host-vector strain expressing SLT or Shiga toxin on a high copy number plasmid would be any more toxic than Shigella found in nature. Dr. Levine pointed out that E. coli K-12 does not colonize the gut and a K-12 host-vector system carrying the SLT gene would not be more pathogenic than Shigella dysenteriae. He thought  $10^8$  CDs per mg protein in cell lysates would be an appropriate upper limit for permitting a clone to be removed from BL3 containment.

Dr. Gill said an extra margin of safety is provided by the specification in Appendix F-IV-H of  $10^7$  CDs per mg protein in cell lysates as the upper limit for removal of SLT clones from BL3 containment. He would prefer strain 933 E. coli 0157:H7 which produces  $10^7$  CDs under optimal containment be the standard strain for determining whether a particular host-vector strain could be removed from BL3 containment. If cloning of the Shiga toxin gene on a high copy number plasmid in E. coli K-12 resulted in a 10 fold increase in toxin production, a very high level of toxin,  $10^9$  CDs, would be produced.

Dr. Kopecko said it was important to clone the Shiga gene from Shigella dysenteriae 1 in order to understand how toxin gene expression is controlled since Shigella dysenteriae 1, an epidemic strain, produces more toxin than other bacteria. He thought  $10^8$  CDs per mg protein is equivalent to the highest level of Shiga toxin expression found in nature; therefore, the working group should recommend E. coli K-12 host-vector systems expressing  $10^8$  CDs per mg protein be permitted to be moved from BL3 to lower containment. K-12 strains producing more than  $10^8$  CDs per mg protein will not be permitted to leave BL3 containment under the proposed language.

Dr. Gottesman said the issue is whether E. coli K-12 producing high levels of Shiga toxin would be hazardous to animals.

Dr. Habig said he did not see why E. coli K-12 host-vector systems carrying the Shiga toxin gene would pose a greater danger than Shigella dysenteriae 1. Dr. Kopecko pointed out that clinical laboratories routinely work with Shigella dysenteriae 1 at BL2 conditions. He did not think E. coli K-12 host-vector systems would be more dangerous than Shigella dysenteriae 1. Dr. Gottesman said she felt comfortable with permitting E. coli host-vector systems expressing  $10^8$  CDs of SLT to be used at BL2 containment since E. coli K-12 does not colonize the gut.

Dr. Levine suggested a number such as  $10^8$  CDs be chosen as the upper limit of toxin a host-vector system might express if it is to be removed from BL3 to a lower containment level.

Dr. Levine said E. coli K-12 is not an invasive pathogenic organism. Several factors are necessary for pathogenicity, and even for Shigella more is required than simply expression of the toxin gene. An attenuated Shiga bacillus vaccine strain which produces as much Shiga toxin as the parent pathogenic strain but lacks invasiveness factors did not cause disease when fed to human volunteers. In a the single case in which the strain reverted to invasive, the human volunteer became ill.

Dr. Formal said he had constructed a strain by introducing half of the Shigella flexneri chromosome and an SLT converting phage into E. coli K-12. The introduced Shigella flexneri genes were thought to include all of the genes necessary for virulence. The constructed strain had no effect on monkeys in feeding experiments although it produced  $10^7$  CDs per mg protein of SLT under optimal laboratory conditions.

Dr. Gill asked whether concern exists about transfer to other organisms of a high copy number plasmid carrying the Shiga toxin gene and its control elements.

Dr. Newland said the probability of transfer of the SLT or Shiga toxin gene by phage in nature is higher than the probability of transfer from K-12 by poorly mobilizable plasmid vectors.

Dr. Gottesman asked what laboratory conditions are necessary to obtain optimal expression of the SLT gene in the E. coli K-12 host-vector system. Dr. Newland replied that antibiotic pressure is necessary. In addition, several other procedures such as using specially treated medium are also required. If an

SLT clone is simply cultured in the conditions usually used to culture the host-vector system, toxin production is generally a factor of  $10^2$  lower than the highest level of expression obtained under optimal conditions with that clone.

Dr. Newland said he had observed that the recombinant plasmid carrying the SLT gene appears to be rapidly lost from the E. coli host-vector system in the absence of selective antibiotic pressure. The plasmid without the SLT gene is not so rapidly lost in the absence of selective pressure. He and Dr. O'Brien hypothesized that high levels of toxin may be toxic to bacteria. The rapid loss of the recombinant plasmid in the absence of selective pressure may be an additional safety factor.

Dr. Gottesman asked whether the working group would consider modifying Appendix F-IV-H by raising the upper level of toxin expression to  $10^8$  CDs per mg protein in cell lysates in exchange for an assurance that the plasmid will be rapidly lost in the absence of antibiotic pressure. Alternatively, an upper level of expression need not be fixed if the plasmid will be rapidly lost since rapid plasmid loss will provide a measure of safety.

Dr. Levine said one problem with Dr. Gottesman's proposal is that language generic for experiments involving E. coli may not apply to experiments involving Vibrio or Shigella. A second concern is the variability of the SLT assay; without an internal standard it is difficult to know how much toxin is produced. He asked Dr. O'Brien how great a difference is observed using the same procedure from experiment to experiment. Dr. O'Brien replied that a 100 fold difference in the amount of CDs produced may be observed from experiment to experiment.

Dr. Gill moved that Appendix F-IV-H be amended to read in part:

"E. coli host-vector systems expressing the Shiga-like toxin gene product may be moved from BL3 + EK1 to BL2 + EK1 containment conditions provided that: (1) the amount of toxin produced by the modified host vector systems be no greater than that produced by the positive control strain 933 E. coli O157:H7, grown and measured under optimal conditions, or ten times this level if the maintenance of the plasmid carrying the gene is dependent upon growth in the presence of an antibiotic...."

Dr. Levine suggested the motion be amended to read in part:

"E. coli host-vector systems expressing the Shiga-like toxin gene product may be moved from BL3 + EK1 to BL2 + EK1 containment conditions provided that: (1) the amount of toxin produced by the modified host vector strain be no greater than  $10^8$  CDs per mg protein...."

Dr. Formal suggested the motion should name a toxin producing strain as the standard rather than citing a specific number. He suggested Shigella dysenteriae 1 strain 60R be used for this purpose. Shigella dysenteriae 1 strain 60R, a rough strain not capable of colonizing the human gut, produces about ten

times more toxin than 933 E. coli 0157:H7. Language specifying a specific strain would provide an internal standard and permit comparison from laboratory to laboratory. Dr. Kopecko agreed.

Dr. Levine amended his motion to require use of Shigella dysenteriae strain 60R as the standard strain. Dr. Formal seconded this motion.

Dr. Gottesman seconded Dr. Gill's original motion in order to permit a vote on this motion. By a vote of two in favor, four opposed, and no abstentions, Dr. Gill's motion was refused by the working group. (Dr. Levine opposed this motion. Dr. Habig supported it.)

The working group then voted on Dr. Levine's motion. By a vote of five in favor, one opposed, and no abstentions, the working group accepted this motion. (Drs. Habig and Levine supported this motion.)

Dr. Kopecko then offered the following motion:

"Campylobacter species have long been recognized as members of the family Vibrionaceae. Recently Campylobacter species have been separated taxonomically into an orphan genus. Because of the similarities between Campylobacter and Vibrionaceae, the Working Group on Toxins recommends that for purposes of cloning SLT Campylobacter can be considered as members of the Vibrionaceae."

Dr. Levine seconded this motion.

Dr. Gill said the most important consideration in evaluating a proposal is not the species from which the gene was originally isolated but the characteristics of the gene's product and the host-vector system. He said he would support Dr. O'Brien's request to permit cloning of the SLT gene from Campylobacter. He thought Dr. Kopecko's motion did not convey the idea that the gene, and the characteristics of its product are the most important considerations. He suggested a substitute motion which would not refer to the source of the gene be developed.

Dr. Formal said although he agreed with Dr. Gill, at the present time it would be difficult to precisely define the characteristics of SLT toxin. A substitute motion would have to contain such a definition.

Dr. Milewski said Dr. Kopecko's motion was preferable because of the way the August 19, 1985, Federal Register announcement had been written. She said the minutes of the working group meeting would show the group strongly supports the concept that the gene is the most important consideration.

Dr. Levine called the vote on Dr. Kopecko's motion. By a vote of six in favor, none opposed, and no abstentions, the working group approved of the motion. (Drs. Levine and Habig supported the motion.)

Request to Remove Clones Carrying the  $\alpha$ -MSH-Diphtheria Toxin Gene From High Containment

Dr. Gottesman said Dr. John Murphy, then of Harvard Medical School and now with the University Hospital of the Boston University Medical Center, in a letter dated October 5, 1982, requested permission to construct a hybrid molecule in which the gene coding for  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH) is joined to a segment of the gene encoding diphtheria toxin. The gene segment would encode the A subunit and portions of the B subunit of the diphtheria toxin gene. The segment would not contain the diphtheria toxin binding domain. The  $\alpha$ -MSH gene would be a synthetic oligonucleotide. The  $\alpha$ -MSH-diphtheria toxin hybrid gene would be introduced into poorly mobilizable plasmids such as pBR322, PUC9, or PUC8 and cloned in E. coli EKI host-vector systems. The goal of these experiments is to construct a family of chimeric toxin genes whose products have the potency of diphtheria toxin and the receptor binding specificity of  $\alpha$ -MSH.

This request was discussed by the RAC at the October 25, 1982, meeting; RAC recommended these experiments be permitted at BL4 containment at the Frederick Cancer Research Facility. The RAC also recommended that data on the characteristics of the recombinant organisms expressing the  $\alpha$ -MSH-diphtheria toxin hybrid gene should be evaluated before the clones are permitted to leave the BL4 facility. The RAC charged the Working Group on Toxins with reviewing the data on the E. coli strains carrying the hybrid toxin; the group also was charged with offering a recommendation to the Director, NIH, on whether the strains may be removed from BL4 containment. The recommendation of the Working Group on Toxins could be acted upon by the NIH Director without RAC review of the data. A report of the Working Group on Toxins would, however, be sent to the RAC.

Dr. Gottesman said Dr. Murphy made the following three separate requests concerning removal from the BL4 facility of the E. coli K-12 host-vector systems:

- (1) that fragments of the diphtheria toxin structural gene (expressed from either the tox or lambda phage  $P_{\lambda}$  promoter) which include fragment A and fragment B up to, but not beyond, the SphI site be classified at BL1;
- (2) that the diphtheria toxin structural gene modified by the introduction of a C-terminal cysteine residue up to, but not beyond, the SphI site (pARC508) be classified at BL1;
- (3) that the diphtheria toxin-related  $\alpha$ -MSH fusion genes up to, but not beyond, the SphI (pABM508, or pABM1508) site be classified at BL1.

Dr. Gottesman said Dr. Murphy had supplied data in the form of two tables. Table 1 contains data on intra-peritoneal injection challenge of guinea pigs with viable and boiled Escherichia coli host-vector systems and E. coli pABM1508 carrying the  $\alpha$ -MSH diphtheria toxin fusion gene. Table 2 contains data on guinea pig survival after injection with partially purified conjugate protein at graded doses up to 1000 minimal lethal dose equivalents (based on 160 nanograms diphtheria toxin per kg body weight as minimal lethal dose.)

Dr. Murphy said these data suggest E. coli host-vector systems carrying the hybrid gene are not harmful to guinea pigs. Table 2 data suggest that up to 1000 equivalent diphtheria toxin doses of conjugate protein challenges to guinea pigs cause no effect. He said one animal died during the course of these experiments; autopsy showed no evidence of the pathology of the adrenal glands characteristic of diphtheria toxin intoxication.

Dr. Gill said the crucial question in review is whether lack of human toxicity can be predicted from the data submitted by Dr. Murphy. He said one question is whether the  $\alpha$ -MSH-diphtheria toxin hybrid protein could be lethal to humans or whether an exposed individual might become albino. He said it is not known whether destroying cells possessing  $\alpha$ -MSH receptors would be lethal to a human.

Dr. Habig asked whether human  $\alpha$ -MSH would be recognized by guinea pig  $\alpha$ -MSH receptors. Dr. Murphy said human  $\alpha$ -MSH is recognized by guinea pig melanocytes.

Dr. Gottesman asked Dr. Murphy what cells having  $\alpha$ -MSH receptors would have been encountered by the toxin during the guinea pig tests. Dr. Murphy said skin melanocytes in the G2 phase of the division cycle are sensitive to diphtheria toxin. Cells in the brain also have MSH receptors but would not be exposed in these tests because of the blood-brain barrier.

Dr. Kopecko asked whether the guinea pig has  $\alpha$ -MSH receptor-possessing cells in the peritoneum. Dr. Murphy said guinea pigs are very sensitive to intra-peritoneally introduced diphtheria toxin.

Dr. Gill asked Dr. Murphy if he knew whether guinea pig melanocytes were destroyed during the test. Dr. Murphy said he did not know if melanocytes had been destroyed.

Dr. Levine asked how long the animals had been observed during these tests. Dr. Murphy said the animals had been observed for at least 150 hours. This is approximately 24 hours beyond the observation period employed when animals are challenged with one minimal lethal dose equivalent of diphtheria toxin.

Dr. Murphy said diphtheria toxin is only fatal to sensitive cells in the G2 portion of the cycle since sensitive cells only express  $\alpha$ -MSH receptor in the G2 phase of division. Normal melanocytes have a very low basal rate of protein synthesis and replicate about once a year. Therefore, only a very small fraction of the melanocyte population would be expressing the  $\alpha$ -MSH receptor at a given time. In order to envisage fatality, either a long colonization period by toxin producing bacteria or a long serum half life of the  $\alpha$ -MSH-diphtheria toxin hybrid molecule would have to be hypothesized.

Dr. Habig asked whether the  $\alpha$ -MSH-diphtheria toxin hybrid protein could enter brain cells through hydrophobic insertion if administered intracerebrally or intraneurally. Dr. Murphy said the conjugate toxin probably is as potent as the CRM 45 fragment when injected intracerebrally or intraneurally.

Dr. Habig said diphtheria toxins with an intact A chain possess a fair amount of residual enzymatic activity. He asked Dr. Murphy if he had any data on the

toxicity of the SphI fragment. Is the SphI fragment an active toxin if the  $\alpha$ -MSH moiety is cleaved off? Is the level of toxicity of the SphI fragment similar to that of the CRM 45 fragment? He noted that the CRM 45 fragment is smaller than SphI but is toxic injected intracerebrally or intraneurally.

Dr. Gill pointed out that the CRM 45 fragment is 100 times less toxic than diphtheria-toxin. If the SphI fragment is 100 times less toxic than diphtheria toxin, experiments involving the cloning of the fragment would be classified in the BL1 category.

Dr. Collier asked Dr. Murphy to compare the SphI fragment to the CRM fragments in terms of size. Dr. Murphy said the SphI fragment lacks 67 amino acids of the mature diphtheria toxin. It is larger than the largest known CRM fragment.

Dr. Gill asked if any data exist on the toxicity of a diphtheria toxin fragment of that size. Dr. Murphy said no data exist but felt the SphI fragment would be as toxic as the CRM 45 fragment.

Dr. Levine asked whether the challenged guinea pigs mounted an antibody response against the  $\alpha$ -MSH-diphtheria toxin hybrid protein. Dr. Murphy said he had not tested for an antibody response but assumed antibodies would be produced.

Dr. Habig asked whether the conjugate protein could cause an antibody response against  $\alpha$ -MSH itself. Dr. Levine said another question is whether the conjugate would be effective in humans if more than one course of therapy was necessary since most humans would mount an antibody response against the  $\alpha$ -MSH-diphtheria toxin hybrid molecule.

Dr. Habig said 80 per cent of the immunized population have some antibodies to the A chain portion of the diphtheria molecule. He questioned whether the  $\alpha$ -MSH-diphtheria toxin molecule would be therapeutically effective in immunized populations. Dr. Murphy hypothesized that it would. He said only those monoclonal antibodies that block toxin binding to the receptor neutralize the toxin; monoclonal antibodies that bind to the toxin but do not block receptor binding do not neutralize the toxin. He hoped that only antitoxin antibodies capable of blocking receptor binding would be able to neutralize the  $\alpha$ -MSH-diphtheria toxin molecule in vivo.

Dr. Habig said the  $\alpha$ -MSH-diphtheria toxin hybrid might be neutralized in the animal by the formation of polyvalent complexes. Tetanus toxin can be neutralized by lattice formation even if the receptor binding site is not blocked by antibodies.

Dr. Gottesman asked whether fragment SphI has an internal stop codon. Would fusion proteins result if "read-through" occurred? Dr. Murphy said the SphI fragment has no internal stop codon. A fusion protein results when the SphI gene fragment is linked directly to the vector DNA.

Dr. Gottesman asked Dr. Murphy whether any toxicity data exist for random SphI fusion molecules. Dr. Murphy said he had no toxicity data on SphI fusion

proteins. He felt the fusion proteins would be unstable and rapidly degraded in the bacteria and in crude lysate preparations.

Dr. Gill said this later possibility could be tested by spiking the preparation with whole purified diphtheria toxin. This would give a limited answer, however, since fusion proteins may be degraded at different rates than the whole toxin.

Dr. Habig said some toxicity questions could be answered by permitting the  $\alpha$ -MSH-diphtheria toxin hybrid protein to be purified and tested in clinical trials.

Dr. Gottesman said the working group must deal with two questions. The first is whether fusing the SphI fragment with the  $\alpha$ -MSH gene would confer on the hybrid molecule properties quite different from the properties of diphtheria toxin. The second is the toxicity of the SphI fragment and any randomly generated fusion proteins.

Dr. Formal said the crux of the matter is whether the host-vector system carrying the hybrid molecule is safe. One important question is whether diphtheria toxin could be produced by E. coli K-12 in the lumen of the bowel. Dr. Formal said there is no evidence K-12 produces diphtheria toxin in the bowel.

Dr. Malcolm Martin of the National Institute of Allergy and Infectious Diseases (NIAID) said the working group should focus on an evaluation of the E. coli host-vector system carrying the hybrid gene rather than on the toxicity of the hybrid toxin.

Dr. Gottesman said Appendix F of the NIH Guidelines is based on the pharmacological activity of toxins. The working group, therefore, would consider toxin toxicity. Dr. Gill agreed.

Dr. Gottesman said if the  $\alpha$ -MSH-SphI conjugate protein is not as toxic as diphtheria toxin, then containment lower than BL4 is indicated. She asked whether the working group is willing to lower containment to the BL1 level for procedures involving the  $\alpha$ -MSH-SphI conjugate protein.

Dr. Collier said a judgment call was made when diphtheria toxin was classified in Appendix F of the NIH Guidelines in 1980. On the basis of diphtheria toxin toxicity, this toxin might have been classified as requiring either BL3 or BL4 containment. He thought the BL4 containment assigned at that time was inappropriately high. He pointed out that Appendix F permits the cloning at BL1 + FK1 of Pseudomonas aeruginosa exotoxin A which is only ten to fifty times less toxic than diphtheria toxin.

Dr. Habig said the pathogen elaborating diphtheria toxin, Corynebacterium diphtheriae, is grown at BL2 containment in clinical laboratories and large culture production. The NIH Guidelines also require BL2 containment for work with Corynebacterium diphtheriae.

Dr. Kopecko said E. coli K-12 host-vector systems are not pathogens. He thought the worst case scenario would involve the potential transfer of the recombinant DNA plasmid to another organism. Dr. Levine said even if the E. coli K-12 host-vector system could colonize the gut the rate of transfer of plasmids in the gut is extremely low under normal conditions.

Dr. Murphy said all of the conjugate protein produced by the E. coli host-vector system is exported to the periplasmic compartment. Dr. Habig asked if this was the case even in the stationary phase of bacterial growth. Dr. Murphy said he did not know but reminded the group that experiments involving intra-peritoneal injection of viable and heat killed organisms showed no different response to organisms carrying the hybrid gene and the host-vector system without the hybrid gene.

Dr. Levine said he was not sure the observation the protein was not secreted is an argument for safety. Bacterial cells are not immortal and will lyse and secrete the fusion protein into the immediate environment.

Dr. Murphy thought a large number of cells would have to lyse to produce protein levels lethal to the animal.

Dr. Gill questioned whether the toxicity data presented by Dr. Murphy might have been generated using degraded conjugate protein.

Dr. Habig asked Dr. Murphy whether he had ascertained that the  $\alpha$ -MSH-diphtheria toxin hybrid protein was isolated intact. Dr. Murphy said he had not looked at the C terminal sequences to determine whether the hybrid protein had been degraded. The active fraction was homogeneous on gels and was close to the deduced molecular weight of 50,000, however.

Dr. Collier asked Dr. Murphy how he knew the fraction injected into the guinea pigs contained active  $\alpha$ -MSH-diphtheria toxin hybrid protein. Dr. Murphy replied that the protein fraction was active against sensitive cells in culture.

Dr. Gottesman asked how sensitive these tissue culture cells are to diphtheria toxin. Dr. Murphy replied that the control cells, African green monkey CV-1 cells, are sensitive to  $10^{-10}$  or  $10^{-11}$  molar diphtheria toxin.

Dr. Gill said the important considerations are the toxicity of the SphI fragments and of the conjugate protein. He thought the toxicity of the SphI fragment is very likely to be very low; however, it is not possible in BL4 conditions to obtain sufficient purified material to determine toxicity.

Dr. Gill suggested Dr. Murphy be given permission to work in BL3 conditions to purify enough material for toxicity testing and to quantitatively assay the hybrid protein in more species of animals over a longer period of time. Drs. Gottesman and Collier agreed that Dr. Murphy should be permitted to remove the clones from BL4 containment for the purpose of obtaining additional data.

Dr. Levine said he would prefer Dr. Murphy be permitted to use BL2 containment. He moved that BL2 be recommended as the containment level for Dr. Murphy's three requests, and that this language be recommended for approval by the working group. This motion would permit Dr. Murphy to produce enough material to test toxicity. Dr. Forman seconded this motion.

Dr. Collier re-emphasized that the toxicity of the SphI fragment is not known. He offered a friendly amendment to Dr. Levine's motion; the modified motion would specify BL3 containment conditions for Dr. Murphy's three proposals. Dr. Levine accepted this modification.

Dr. Martin suggested the working group specify BL2 containment plus BL3 practices for Dr. Murphy's proposals. A negatively pressurized room is the only difference between BL3 containment and BL2 containment plus BL3 procedures. He did not think a negatively pressurized room offered any additional safety factor for Dr. Murphy's proposed experiments.

Dr. Levine modified his motion to require BL2 containment plus BL3 practices for Dr. Murphy's three requests.

Dr. Gill suggested additional language be added to the motion to indicate the working group wished to permit Dr. Murphy the opportunity to acquire better toxicity data on the purified protein with the implication containment could be further lowered on the basis of these data.

Dr. Levine accepted Dr. Gill's proposed amendment.

By a vote of six in favor, none opposed, and no abstentions, the working group approved of Dr. Levine's motion.

#### Proposal to Construct a IL-2-Diphtheria Toxin Hybrid Protein

Dr. Gottesman said in this proposal, Dr. John Murphy of the University Hospital of the Boston University Medical Center requests permission to construct a hybrid gene composed of the gene coding for interleukin-2 (IL-2) and the SphI segment of the diphtheria toxin gene. The hybrid gene would be cloned in E. coli K-12 host-vector systems.

The long-term goal of this research is to develop novel IL-2 receptor-targeted cytotoxic agents to combat organ rejection and to create a state of graft "tolerance" in organ transplantation. Some of the products may have therapeutic potential for treating leukemia and lymphoma.

Dr. Gottesman said the primary difference between the  $\alpha$ -MSH-diphtheria toxin hybrid molecule proposal and the IL-2-diphtheria toxin hybrid molecule proposal is the type of cell postulated to be sensitive to the conjugate protein.

Dr. John Williams of Beth Israel Hospital said IL-2 receptors are found on proliferating, antigen-activated T cells and to a lesser extent on activated B cells. These cells would be the targets of the IL-2-diphtheria toxin hybrid

protein. Resting or memory lymphocytes do not express IL-2 receptors. There is no evidence of IL-2 receptors on tissues outside of the lymphoid compartment.

Dr. Williams said the goal of this project is to selectively remove activated T cells and activated B cells during a well controlled time period following organ transplantation. It is hoped the IL-2-diphtheria toxin molecule would be more selective than the immunosuppressive techniques currently in use. Experiments in mice using anti-IL-2 receptor-monoclonal antibody conjugates to suppress cells which cause rejection in cardiac transplants support this hypothesis. In these experiments, the life of the graft in mice was extended from 10-14 days to three months or more in 80 percent of the animals.

Dr. Gill said the "worst case" scenario he could conjecture would be that an animal's T cells and B cells would be eliminated by this hybrid protein. A long observation of test animals might be necessary before such an effect would be detected. Dr. Habig said a long observation period might also be necessary for animals treated with the  $\alpha$ -MSH-diphtheria hybrid protein.

Dr. Levine said the IL-2-diphtheria toxin hybrid protein could be an important therapeutic agent, but it would be a new toxin and could be harmful. He suggested containment be initially set at the BL4 level.

Dr. Martin said the largest BL4 facility at the Frederick Cancer Research Facility is run by NIAID. Any request by Dr. Murphy for use of the facility will need to be considered by NIAID in relation to other competing requests.

Dr. Levine said since little is known about the potential toxicity of the proposed hybrid protein, the working group approach should be conservative. If BL4 containment is not available, the experiment should be permitted under RL3 containment with a recognition that concern exists about the nature of the proposed hybrid protein.

Dr. Gottesman suggested containment could be set at BL3 with a requirement for the use of EK2 host-vector systems. Dr. Levine said he would accept the suggestion to specify EK2 vectors; he would not accept the suggestion to specify EK2 hosts.

Dr. Gottesman asked whether any type of risk assessment experiment could be performed which would answer basic questions about the nature of the protein.

Dr. Gill said risk assessment experiments with this hybrid protein would differ from previous risk assessment protocols because acute lethality is not a likely outcome. Tests would have to be devised to look for chronic impairment of the immune system. This type of data and the protocols necessary to generate it are different from that generally required for toxins.

Dr. Gill offered a motion to recommend permission to proceed under BL3 conditions; EK2 vectors would be used. Dr. Levine said he would prefer the language of the motion specified the use of "poorly mobilizable plasmid vectors such as the EK2 certified plasmids." Dr. Gill agreed to this modification. Dr. Levine seconded the motion.



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