

DEPARTMENT OF HEALTH AND HUMAN SERVICES  
PUBLIC HEALTH SERVICE  
NATIONAL INSTITUTES OF HEALTH

RECOMBINANT DNA ADVISORY COMMITTEE

MINUTES OF MEETING

OCTOBER 25, 1982

A. Proposal to Clone an E. coli Toxin Gene

Dr. Gill said Drs. Alison O'Brien and Randall Holmes of the Uniformed Services University of the Health Sciences request (tab 1083) permission to clone the structural gene of the Shiga-like toxin of Escherichia coli. The E. coli Shiga-like toxin has biological activity similar to the activity of Shigella dysenteriae neurotoxin. The Shiga-like toxin gene would be cloned in E. coli EKI host-vector systems using plasmid, cosmid, or lambda cloning vectors.

Drs. O'Brien and Holmes argued that these experiments pose minimal risks for the following reasons:

- (1) Clinical isolates of E. coli have already been demonstrated to elaborate large amounts of a toxin indistinguishable from that produced by Shigella dysenteriae 1 (Shiga toxin). Therefore, the genes for Shiga-like toxin production are present in the E. coli gene pool in nature.
- (2) Human volunteers fed large numbers of Shigella dysenteriae 1 organisms that produce Shiga toxin but do not colonize the bowel, did not become ill. Therefore, any accidental ingestion of the organism to be manufactured, a toxin-producing E. coli K-12 strain that cannot colonize the human intestinal tract, should pose little hazard to man.
- (3) Purifications of Shiga toxin and E. coli Shiga-like toxin has not identified any excessive risk from the toxin aerosolization that probably occurs during the purification process. In fact, in one laboratory, toxin was isolated from 500 liters of culture under P1 physical containment conditions.
- (4) Shiga toxin is a potent cytotoxin for a HeLa cell subline (a human cervical carcinoma tissue culture cell line), but the toxin has no effect on many other human, monkey, and rodent tissue culture cells. Therefore, the toxin is quite cell-type specific, and this limited spectrum of activity suggests that it would be non-toxic for most cells in the human body.
- (5) Contrary to the old literature, Shiga toxin is not a neurotoxin. By 1955, it was established that the paralysis observed in rabbits and mice (but not monkeys, guinea pigs, hamsters, or rats) when toxin is administered intravenously is a reflection of the effect of toxin on the endothelium of small blood vessels, not a direct effect on nerve cells.

Dr. Gill said Shiga toxin has three known properties: (1) it is cytotoxic to certain epithelial cells in culture, (2) it is enterotoxic in that it causes fluid release from the jejunum, and (3) it causes

paralysis and death apparently by damaging the endothelium of blood vessels in the nervous system. Shiga toxin inhibits protein synthesis and is, to man, one of the most potent toxic proteins.

Dr. Gill said the Guidelines that apply to experiments involving Shiga toxin should be applied to the Shiga-like toxin which has been discovered in E. coli. Dr. Gill said under the current Guidelines human toxicity data are to be of paramount importance in setting containment levels. If no human toxicity data are available, toxicity data from primates are next most important. If neither human or other primate toxicity data are available, containment levels shall be determined from the LD<sub>50</sub> of the most sensitive of three small animal species (mice, guinea pigs, and rabbits). The available data show Shiga toxin to be highly toxic to primates. These data, however, are a little soft. Data generated using rabbits show the toxin to be extremely toxic. In contrast, Shiga toxin is not very toxic to mice. He said that Shiga toxin has the same toxicity range in the most sensitive animals as tetanus toxin and the botulinum toxins.

Dr. Gill said in vitro gene expression of Shiga toxin can differ by factors of 10,000. How this correlates to in situ (in the gut) expression is not known.

Dr. Kaper said, taxonomically, Shigella, and Escherichia are so similar that in the future they may be classified with the same name. He added that the Shiga toxin gene has undoubtedly been cloned many times in the shotgun cloning of the E. coli genome.

Dr. Kaper said that studies in human volunteers have shown that invasiveness in the most important virulence determinant: nontoxicogenic, invasive Shigella strains cause disease; toxicogenic, noninvasive Shigella strains do not cause disease in humans.

Dr. King Holmes said certain aspects of the Shigellosis syndrome such as disseminated intravascular coagulation, the hemolytic uremic syndrome, and central nervous system symptoms may be related to toxins such as Shiga toxin. He asked if the E. coli Shiga-like toxin is encoded by a chromosomal gene. Upon receiving an affirmative answer, he questioned whether cloning the gene on a high copy number plasmid might not result in increased virulence. For this reason, he argued in favor of containment higher than P1.

Dr. King Holmes said the investigators wish to prepare a DNA probe with the Shiga-like toxin gene in order to screen E. coli isolated from patients with diarrhea. Dr. Gottesman suggested a probe need not consist of the entire toxin gene sequence; a non-toxic fragment of the gene would suffice. The research to generate the probe might be performed under higher containment; containment for experiments involving the probe could then be lowered through a multistep process. Dr. Gill endorsed Dr. Gottesman's suggestion of generating a partial or a defective gene probe.

Dr. Randall Holmes said the approach of generating a defective gene, without using recombinant DNA technology, is currently not feasible; there are no E. coli strains known to lack the genes; there are no probes available to screen for the genes; there is no good selection method to isolate mutants. He added that generating a probe is only one goal of the project. He said characterizing the structural genes and the mechanisms involved in regulating expression in E. coli are also objectives.

Dr. Gottesman asked if it would be possible to perform these experiments using classical genetic methods. Dr. Randall Holmes replied that some experiments could be performed by classical genetic techniques, others, however, can only be performed using recombinant DNA technology.

Dr. Randall Holmes said additional information has become available since the proposal was originally submitted. He said two more high producers of Shiga-like toxin have been selected from strains isolated by the Centers for Disease Control (CDC). These E. coli strains are associated with human diarrheal disease, usually mild. Four independent isolates which produce as much Shiga-like toxin as Shigella dysenteriae are now available. The role the Shiga-like toxin plays in the pathogenesis of enteric disease is unknown.

Dr. Randall Holmes said approximately 13 E. coli strains have been tested to determine if they produce Shiga-like toxin. All produce some Shiga-like toxin. Therefore at this time, the possibility exists that all E. coli strains produce Shiga-like toxin. He added that Shiga-like toxin activity can be detected in E. coli K-12 lacking detectable plasmids; therefore, functional chromosomal copies of these genes must be present in E. coli K-12.

Dr. Randall Holmes said evidence suggests that in E. coli infections, the toxin, if it has any relevance to pathogenesis, is an enterotoxin which functions as a cytotoxin. Injected intravenously the toxin would probably be highly toxic to monkeys. No data suggest that it is highly toxic by the aerosol route of exposure. The route of administration is, therefore, important. Dr. Gill said that in an incident in which Shiga toxin was accidentally administered to monkeys' tracheas, the monkeys did suffer damage.

Dr. Maas moved that the experiments be permitted at the P3 + EK1 level of containment. The motion was seconded. Dr. Gill pointed out that the motion would permit Shiga-like toxin to be handled at lower containment than diphtheria toxin, yet Shiga-like toxin is probably 100 times more potent than diphtheria toxin.

Dr. Martin asked if it was possible to study regulation of expression by in vitro mutagenesis of a fragment probe. Dr. Randall Holmes replied that in cases where such an approach was successfully employed, the structural gene was known. The structural gene for the Shiga-like toxin is not known. Dr. Martin suggested in vitro complementation

with the protein product could be employed. Dr. Randall Holmes said the amounts of toxin available for biomedical studies are minuscule. Studies of the biological functions associated with the toxin are not at the same level of sophistication as those for cholera or diphtheria toxin.

Dr. Fedoroff suggested mutagenesis might be employed in conjunction with immuno-identification to isolate an inactivated gene. Dr. Randall Holmes replied that approach might be reasonable but pointed out that Shiga-like toxin may have an essential function as it appears to be expressed by all E. coli strains. Dr. Fedoroff asked if any Shigella strains are Shiga toxin minus. Dr. Randall Holmes said no Shigella strains are truly Shiga toxin minus; those termed toxin minus actually produce small amounts of the toxin.

Dr. King Holmes said whether high amounts of toxin are produced by bacteria in the gut is a critical question. Dr. Gill added that the question of whether the toxin, if produced, crosses the gut into the blood stream is equally important. Dr. Randall Holmes said Shigellosis caused by strains producing low levels of shiga toxin induces a systemic antitoxin response.

Dr. Kaper suggested a low copy number plasmid might be employed. Dr. Gottesman said more bacteria would then have to be grown to obtain sufficient DNA for the studies. She suggested that the most reasonable approach is to set high containment for the initial experiments to produce a defective probe. She suggested P4 + EKI containment. If considerations of inherent toxin toxicity are not of primary importance and issues of invasiveness are given more weight, P3 containment might be reasonable.

Dr. King Holmes asked if risk assessment studies would be required before the gene could be sequenced or gene regulation and expression studied. Dr. Randall Holmes replied that would depend on whether the fully functional wild type gene would be studied or whether methods for working with a mutant toxin could be developed.

Dr. Maas called the question on his motion (i.e., to allow the experiments at the P3 + EKI level of containment). Dr. Fedoroff asked if Dr. Maas would accept an amendment to raise containment to P4. Dr. Maas refused. By a vote of fourteen in favor, none opposed, and no abstentions, the question was called. By a vote of five in favor, seven opposed, and one abstention, the motion was denied.

Dr. King Holmes moved to permit the experiment at P4 containment in a P4 facility. Dr. McKinney said the P4 facility at Frederick Cancer Research Facility (FCRF) is fully equipped for P4 conditions. He asked how experiments would be scheduled. Dr. Talbot said scheduling could be arranged to permit the experiments on Shiga-like toxin.

Dr. Gill said many investigators would like to study a mutated Shiga toxin gene. He asked if the Shiga-like toxin gene would be available to other researchers if it were made in a P4 facility. Dr. Randall Holmes said his laboratory would make the strains available as soon as the data are published, assuming the strains can be removed from the facility.

Dr. Berns called the vote on the motion. By a vote of twelve in favor, none opposed, and one abstention, the motion to permit the experiments at the P4 containment level was carried.

Dr. Landy offered a motion to permit the experiments using P3 laboratory practices and containment equipment in a P4 facility. Dr. McKinney said that when P3 practices are followed in a P4 facility, additional protection beyond P3 is provided as the facility has a completely separate ventilation system; all liquid waste from the facility goes to a waste treatment plant for sterilization prior to discharge, and all materials in the facility are doubly sterilized. When work is performed under full P4 conditions, a Class III glove box is used.

Dr. Landy called the question on his motion. By a vote of nine in favor, one opposed, and one abstention, the question was called. By a vote of five in favor, seven opposed, and one abstention, the motion was defeated.

B. Request to Reevaluate Conditions Under Which the Shiga Toxin Gene may be Cloned

Dr. Berns introduced the letter (tab 1085) of Dr. K. N. Timmis of the Universite de Geneve for discussion. Dr. Timmis requested that the NIH reevaluate the conditions under which the Shiga toxin gene may be cloned in Escherichia coli host-vector systems. He argued that Shigella dysenteriae and E. coli are closely related organisms and that the degree of uncertainty inherent in "shotgun" cloning is related to the evolutionary distance between the two DNAs being combined. He contended that the joining of genetic segments from closely related species known to exchange genetic information with one another by natural processes, involves predictable hazards that do not exceed the sum of the hazards exhibited by each of the contributing organisms. He felt the overriding principle of the Guidelines should be the relatedness of the DNA species being combined, rather than the specific toxicity of the toxin molecules. He thought use of the later criteria introduces an illogical inconsistency into the Guidelines.

Dr. Gill said no inconsistency exists in the Guidelines; Section III-A takes precedence over other sections of the Guidelines. He emphasized that the fact the organisms exchange genetic information does not imply the absence of risk. Dr. Gottesman reaffirmed that the Guidelines are internally consistent. She recommended that ORDA, in responding to Dr. Timmis' letter, relate the RAC discussion and RAC's action regarding the Shiga-like toxin gene.