

Commentary Integrated into Body of 2/27/76
Proposed Guidelines (Summary of Guidelines used and
also full draft when amplification necessary).

**SUMMARY OF THE PROPOSED GUIDELINES FOR RESEARCH
ON RECOMBINANT DNA MOLECULES**

i. INTRODUCTION pp1-3

1. **Robert Sinsheimer:** In the advent of a new biological research, the research process is changing from one of analysis to one of synthesis. The complications are great. The process is an irreversible one. Vectors will escape and there will be no control. We will not be able to stop production as we could with DDT.
2. **Daniel Callahan:** The underlying question is how to establish a proper policy bias. There is no moral obligation to do this research although it certainly is commendable but there is a moral obligation to do no harm; and given the uncertainty of the hazards, one should incline to caution. Does the burden of proof lie with the scientist to show no harm or with the public to show that there is harm? The benefit of the doubt should be to the "worriers" and not the researchers. This should be the policy bias, and the guidelines should be reviewed accordingly. With this in mind, the guidelines seem to be reasonable and prudent in the conduct of this research.

A. Definition of Experiments Included Within the Guidelines

The proposed guidelines concern experiments in which different segments of DNA chains are joined by biochemical techniques and the resulting recombined DNA molecules are then inserted into living cells in which the molecules can be reproduced. The recipient cells are called the "hosts." In general, one segment of the recombined DNA molecule will be derived from an extra-chromosomal genetic element common to the "host" species. That segment of DNA is referred to as the "vector." The other segment of the recombined DNA is referred to as "foreign" DNA, since it is not normally chemically linked to the vector. "Host" cells will usually be either single-celled microorganisms, such as bacteria, or single cells of higher organisms (plants and animals) grown under special laboratory conditions.

In nature, exchange and recombination of genetic information (that is, DNA segments) ^{are} ~~is~~ a consequence of the sexual mode of reproduction and generally occurs only between individual organisms of the same species. The unique feature of recombinant DNA experiments is that they provide a means for combining genes from diverse species. This unique feature promises revolutionary potential both for the investigation of basic biological processes and for approaches to important practical problems in medicine and agriculture. The same unique feature is the basis for concern over the potential biological hazards that might result from such novel genetic recombinants.

B. Principles Upon Which the Guidelines Are Based

The guidelines are consistent with conclusions formulated at the International Conference on Recombinant DNA Molecules held at Asilomar Conference Center, Pacific Grove, California, in February 1975.

1. Certain experiments are judged to present such serious potential hazard that they should not be attempted at this time.

2. Other experiments can be undertaken provided that they afford new knowledge or benefits not readily obtained by conventional methodology and provided that appropriate safeguards are incorporated into the experimental design. Such safeguards should protect laboratory workers, the community, and the environment from possible infection with potentially hazardous agents. The proposed barriers to dissemination of the agents are twofold: (1) physical containment of organisms containing recombinant DNA; and (2) biological containment, that is the use of "hosts" and "vectors" with limited ability to survive in natural environments. The two types of barriers are complementary and are to be used in combination.

3. Recognizing that present relevant knowledge is limited, ^{we can rank} the various types of experiments ~~can be ranked~~ with regard to expected degree of potential hazard. The level of containment achieved by combined physical and biological barriers should be chosen to match the estimated potential hazard. Containment should be high at the start and should be modified subsequently only if there is a substantiated change in the assessed risks.

4. The guidelines should be reviewed at least annually and modified to reflect new knowledge.

II. METHODS OF CONTAINMENT

1. David Baltimore: He asks whether the proposed guidelines are an appropriate response to the hazard posed by recombinant DNA molecules and whether the process that led to the formulation of guidelines was a responsible one. He notes that recombination of DNA molecules has gone on for aeons; he urges the focus must be on those DNAs that are a potential hazard, not focus on the mere joining of DNA molecules. The hazard is defined as the potentiality of artificially joined DNA molecules escaping from the laboratory and disseminating in the general population. He believes the guidelines are appropriate to meet that hazard. He urges that the guidelines be promulgated as quickly as possible. He cites the scientific interest and the medical justification for these experiments to go on.

2. Robert Sinsheimer: Too little attention has been given to hazards that might result from new combinations of genes that shall be created in this research. Research increases the opportunities for potentially dangerous gene combinations by many, many orders of magnitude that might occur sporadically in nature. In this regard, biological containment may not be adequate. The key element of concern is the irreversibility of the process that will become magnified as more investigators in laboratories pursue research in this area. Rather than going the global approach, the guidelines might be better served by approaching each desired objective in as specific and safe a manner as possible.

1. Peter Barton Hutt: The concept of physical containment is both too imprecise and too subject to the vagaries of human fallibilities. Thus physical containment can only be applied when risk is non-existent. When there is any potential risk, protection must rest with biological containment. Consideration of the P1 through P3 levels should be undertaken. Perhaps P1 and P2 should be combined into a single level of physical containment. Another possibility is to upgrade some of the experiments to a P3 or P4 level that are currently under P1 and P2 levels. The intended requirements for P1 through P3 levels should be spelled out. Perhaps Dr. Barkley who raised several points in his presentation could review this section for additional comments. Description of acceptable practices should be as clear as possible and might well include stated minimum levels of scientific training and experience necessary to conduct the experiments.

1. Philip Handler: What does physical containment mean? Are P1 and P2 levels really physical containment? Should we not consider P3 as the minimum level incorporating P1 and P2? This research must go forward but the pace must be set by Dr. Fredrickson.

Four levels are defined and designated P1, P2, P3, and P4 in order of increasing containment. Each higher level assumes the practices of the lower ones. The specifications reflect existing technology as developed for work with known pathogenic organisms. It is anticipated that technical developments will lead to novel alternate procedures for achieving physical containment.

- P1 (minimal): Strict adherence to the standard microbiological practices widely used in research and clinical laboratories for work with moderately pathogenic organisms. Appropriate training of all personnel is required.
- P2 (low): Access to the laboratory is limited to authorized and informed personnel when potentially hazardous organisms are being used and until after appropriate decontamination. Pipetting by mouth is prohibited, and specific precautions are required for procedures expected to release aerosols containing potentially hazardous material.

- P3 (moderate): Laboratories are separated from areas used for less hazardous experiments and access is limited to those who work therein. Laboratories should have air pressure lower than that of the surrounding areas to limit the flow of airborne organisms into surrounding areas. Exhaust air from laboratories should be appropriately discharged or decontaminated prior to recirculation. Biological safety cabinets should be used for all transfer operations and for all procedures likely to produce aerosols. Gloves are to be worn and all vacuum lines protected by filters.

Alternate procedures, affording at least equivalent physical containment, are suggested for situations in which laboratory air conditions cannot be controlled in the specified manner.

- P4 (high): Special facilities designed to contain highly infectious and hazardous microorganisms are required. Such facilities involve isolation by means of airlocks, negative pressure environments, clothing changes and showers by personnel, biological safety cabinets, and decontamination of all air as well as all liquid and solid wastes. Only a limited number of such facilities exist in the United States.

C. Biological Containment

The nature of these barriers will depend on the particular "host" and "vector" used in each experiment since the barrier is defined by the limited ability of the "host" and its resident recombinant DNA to survive in natural environments. In general, the choice of "host" and "vector" will be determined both by the nature of the experiment and by the required containment. Therefore, experiments are grouped below according to possible "host-vector" systems.

D. Publication

The committee strongly recommends that all publications dealing with recombinant DNA work include a description of the physical and biological containment procedures used.

III. EXPERIMENTAL GUIDELINES

pp 10-12

A. Experiments That Should Not Be Performed at This Time

1. Any experiments in which a portion of the DNA to be joined is derived from highly pathogenic organisms (classes 3, 4, and 5 of the "Classification of Etiologic Agents on the Basis of Hazard," Center for Disease Control, USPHS, Atlanta, Georgia).
 2. Experiments in which a portion of the DNA to be joined contains genes for production of highly toxic agents.
 3. Experiments in which a portion of the DNA to be joined is derived from a plant pathogen if the resulting host may acquire increased virulence or the ability to infect previously unsusceptible species.
 4. Widespread or uncontrolled release into the environment of any organism containing a recombinant DNA molecule unless a series of controlled tests leave no reasonable doubt of safety.
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1. LeRoy Walters: The guidelines are least clear in defining prohibition on experiments where antibiotic resistance may occur. Further clarification is necessary.
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5. Transfer of genes conferring drug resistance to micro-organisms not known to acquire such resistance naturally when such resistance may compromise clinical use of the drug in medicine or agriculture.
 6. Large-scale experiments with recombinant DNAs known to result in the formation of harmful products. Exceptions are permissible for specific experiments of direct societal benefit, provided that they are expressly approved by the NIH Recombinant DNA Molecule Program Advisory Committee.

B. Guidelines for Permissible Experiments

clarify

5. Wallace Rowe: The risks are real. Tests are necessary to determine the nature of these risks including a disturbance in the regulation of the E. coli, the introduction of DNA from E. coli into other cells and the dispersion of toxic products from the E. coli.
6. Roy Curtiss: He outlines possible tests for the risks as the probability of escape, the probability of survival in the outside environment; and the probability of the manifestation of adverse consequences. In this regard we are not only talking about the protection of man but the protection of the entire biosphere.
1. Philip Handler: The NIH should undertake to develop a program of study of the most important hazardous experiments. This should be done at P4 facilities at the NIH or Fort Detrick. These experiments would provide scientific evidence to defuse the debate on the potential hazards. Guidelines can be modified accordingly based on that evidence.

1. Criteria for use of *Escherichia coli*, strain K-12 as "host"

2. Robert Sinsheimer: Experiments should be done with micro-organisms that have no association with man or other animals. These organisms should have a very restricted range of genetic exchange at a very limited biological niche of viability.

1. Paul Berg: Because we are so familiar with the metabolism and genetic systems of *E. coli*, it provides the best opportunity to achieve the benefits in this research. Further, it's the most likely candidate for providing the safest host and vector systems. Developing an entirely new host-vector system will not answer questions of risk. The ominous and catastrophic scenarios raised concerning *E. coli* could equally be raised with any known organism.

3. Philip Leder: He has constructed an EK2 host-vector system and it has been recently announced in the nucleic acid recombinant scientific memoranda. Testing of this system is currently underway. He expects to submit it shortly to the Advisory Committee for certification.

3. Peter Barton Hutt: What would be the cost in not using *E. coli*? Can incentives be created to foster the development of EK2 and EK3 systems. Should there be a 2-year limit on EK1 to force development of EK2 and EK3? If EK1 is to be used, there must be as detailed an explanation as possible of that system and its use.

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developed

The common bacterium *Escherichia coli*, strain K-12, is the "host" of choice at the present time. Well-studied extrachromosomal DNA molecules known to reside and reproduce in this "host" are available for use as "vectors." These include plasmids and bacteriophages. Extensive knowledge of *Escherichia coli* and its plasmids and bacteriophages currently affords the most fruitful approach to the design of containable "host-vector" systems.

Three containment classes for *E. coli* strain K-12 "host-vector" systems are defined. The classes are named EK (for *E. coli* K-12) 1, 2, and 3 in order of decreasing ability to survive in natural environments (that is, increasing containment).

EK1: This class includes most of the currently known and available *E. coli* K-12 "host-vector" systems. Present knowledge suggests a low probability for dissemination of plasmid or bacteriophage "vectors" carrying recombined "foreign" DNA. This conclusion is based in part on the following information: (1) *E. coli* K-12 is not known to colonize the normal bowel; (2) Certain plasmids known to be useful as "vectors" have only limited ability to be transferred to other *E. coli* strains commonly found in animal (including human) bowels and sewerage; (3) Bacteriophages which specifically infect *E. coli*, and which lend themselves well to use in these experiments, are available. Some of these bacteriophages have limited ability either to escape the "host" cell or to reinfect common (not K-12) *E. coli* strains.

1. Biological containment criteria using *E. coli* K-12 host-vectors

EK1 host vectors - These are host-vector systems that can be estimated to already provide a moderate level of containment, and include most of the presently available systems. The host is always *E. coli* K-12, and the vectors include nonconjugative plasmids [e.g., pSC101, ColE1 or derivatives thereof (17-24)] and variants of bacteriophage λ (25-27).

The *E. coli* K-12 nonconjugative plasmid system is taken as an example to illustrate the approximate level of containment referred to here. The available data from experiments involving the feeding of bacteria to humans and calves (28-30) indicate that *E. coli* K-12 did not usually colonize the normal bowel, and exhibited little, if any, multiplication while

variables
passing through the alimentary tract even after feeding high doses (i.e., 10^9 to 10^{10} bacteria per human or calf). However, general extrapolation of these results may not be warranted because the implantation of bacteria into the intestinal tract depends on a number of ~~parameters~~, such as the nature of the intestinal flora present in a given individual and the physiological state of the inoculum. Moreover, since viable *E. coli* K-12 can be found in the feces after humans are fed 10^7 bacteria in broth (28) or 3×10^4 bacteria protected by suspension in milk (29), transductional and conjugational transfer of the plasmid vectors from *E. coli* K-12 to resident bacteria in the fecal matter before and after excretion must also be considered.

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————— Since most conjugative plasmids in nature are repressed for expression of donor fertility, the frequency at which nonconjugative plasmids are mobilized and transferred by this sequence of events in vivo is difficult to estimate. However, in calves fed on an antibiotic-supplemented diet, it has been estimated that such triparental nonconjugative R plasmid transfer occurs at frequencies of no more than 10^{-10} to 10^{-12} per 24 hours per calf (30). In terms of considering other means for plasmid transmission in nature, it should be noted that transduction does operate in vivo for *Staphylococcus aureus* (32) and probably for *E. coli* as well. However, no data are available to indicate the frequencies of plasmid transfer in vivo by either transduction or transformation.

These observations indicate the low probabilities for possible dissemination of such plasmid vectors by accidental ingestion, which would probably involve only a few hundred or thousand bacteria provided that at least the standard practices (Section II-A above) are followed, particularly the avoidance of mouth pipetting. The possibility of colonization and hence of transfer are increased, however, if the normal flora in the bowel is disrupted by, for example, antibiotic therapy (33). For this reason, persons receiving such therapy should not work with DNA recombinants formed with any *E. coli* K-12 host-vector system during the therapy period and for seven days thereafter; similarly, persons who have achlorhydria or who have had surgical removal of part of the stomach or bowel should avoid such work, as should those who require large doses of antacids.

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The observations on the fate of *E. coli* K-12 in the human alimentary tract are also relevant to the containment of recombinant DNA formed with bacteriophage λ variants. Bacteriophage can escape from the laboratory either as mature infectious phage particles or in bacterial host cells in which the phage genome is carried as a plasmid or prophage. The fate of *E. coli* K-12 host cells carrying the phage genome as a plasmid or prophage is similar to that for plasmid-

containing host cells as discussed above. The survival of the λ phage genome when released as infectious particles depends on their stability in nature, their infectivity and on the probability of subsequent encounters with naturally occurring λ -sensitive E. coli strains. Although the probability of survival of λ and its infection of resident intestinal E. coli in animals and humans has not been measured, it is estimated to be small given the high sensitivity of λ to the low pH of the stomach, the insusceptibility to λ infection of smooth E. coli cells (the type that normally resides in the gut), the infrequency of naturally occurring λ -sensitive E. coli (34) and the failure to detect infective λ particles in human feces after ingestion of up to 10^{11} λ particles (35). Moreover, λ particles are very sensitive to dessication.

Establishment of λ as a stable lysogen is a frequent event (10^0 to 10^{-1}) for the att^+ int^+ cI^+ phage so that this mode of escape would be the preponderant laboratory hazard;

While not exact, the estimates for containment afforded by using these host-vectors are at least as accurate as those for physical containment, and are sufficient to indicate that currently employed plasmid and λ vector systems provide a moderate level of biological containment. Other nonconjugative plasmids and bacteriophages that, in association with E. coli K-12 can be estimated to provide the same approximate level of moderate containment are included in the EK1 class.

PP18-25
EK2: This class consists of combinations of modified *E. coli* K-12 "hosts" with modified plasmid or bacteriophage "vectors." The modifications will be achieved by classical genetic manipulation and must be such that the survival rate of the recombined "foreign" DNA fragment, in natural environments, is less than one in 10^8 . Testing procedures for verification of the properties of EK2 systems are described. (EK2 systems are being developed and tested and it is anticipated that they will be available shortly. Responsibility for certification of putative EK2 systems presently lies with the NIH Recombinant DNA Molecule Program Advisory Committee.)

EK2 host-vectors - These are host-vector systems that have been genetically constructed and shown to provide a high level of biological containment as demonstrated by data from suitable tests performed in the laboratory. The genetic modifications of the *E. coli* K-12 host and/or the plasmid or phage vector should not permit survival of the cloned DNA fragment in other than specially designed and carefully regulated laboratory environments at a frequency greater than 10^{-8} . This absolute measure of biological containment has been selected because it is a realistic measurable entity. Indeed, by testing the contributions of preexisting and newly introduced genetic properties of vectors and hosts, individually or in various combinations, it should be possible to substantially indicate, if not prove, that the specially designed host-vector system can provide a margin of biological containment in excess of that required.

For EK2 plasmid vectors, no more than one in 10^8 host cells containing a chimeric plasmid should be able to perpetuate the cloned DNA fragment under nonpermissive conditions designed to represent the natural environment either by survival of the original host or as a consequence of transmission of the cloned DNA by transformation, transduction or conjugation to a host with properties common to those in the natural environment.

In the construction of EK2 plasmid-host systems it is important to use the most stable mutations available, preferably deletions. Obviously, the presence of all mutations contributing to higher degrees of biological containment must be verified periodically by appropriate tests. In testing the level of biological containment afforded by a proposed EK2 plasmid-host system, it is important to design relevant tests to evaluate the survival of the cloned DNA under conditions that are possible in nature and that are also most advantageous for its perpetuation. For example, one might conduct a triparental mating with a primary donor possessing a derepressed F-type or I-type conjugative plasmid, the safer host with $\Delta bioH$ -asd, dapD8, Δgal -chl^r, $\Delta thyA$, deoC, trp and hsdS mutations and a plasmid vector carrying an easily detectable inserted gene such as for ampicillin resistance or trp⁺, and a secondary recipient that is Su⁺ hsdS trp (i.e., permissive for the recombinant plasmid). Such matings would be conducted in a medium lacking diaminopimelic acid and thymine and survival of the Ap^r or trp⁺ marker in any of the three strains followed as a function of time. Survival of the cloned marker by transduction could also be evaluated by introducing a known generalized transducing phage into the system. Similar experiments should also be done using a secondary recipient that is restrictive for the recombinant plasmid as well as with primary donors possessing repressed conjugative plasmids with incompatibility group properties like those commonly found in enteric

microorganisms. Since a common route of escape of plasmid-
host systems in the laboratory might be by accidental
ingestion, it is suggested that the same types of experi-
ments be conducted in suitable animal-model systems. In
addition to these tests on survival of the cloned DNA, it
would be useful to determine the survival of the host strain
under nongrowth conditions such as in water and as a function
of drying time after a culture has been spilled on a lab bench.

For EK2 phage vectors, no more than one in 10^8 recombinant phage particles should be able to perpetuate the cloned DNA fragment under non-permissive conditions designed to represent the natural environment either (a) as a prophage or plasmid in the laboratory host used for phage propagation or (b) by surviving in natural environments and transferring the cloned DNA to a host (or its resident lambdoid prophage) with properties common to those in the natural environment.

The phenotypes and genetic stabilities of the mutations and chromosome alterations included in these λ -host systems indicate that containment well in excess of the required 10^{-8} or lower survival frequency for the cloned DNA fragment should be attained. Obviously the presence of all mutations contributing to this high degree of biological containment must be verified periodically by appropriate tests. Laboratory tests should be performed with the bacterial host to measure all possible routes of escape of cloned DNA such as the frequency of lysogen formation, the frequency of plasmid

formation and the survival of the lysogen or carrier bacterium. Similarly, the potential for perpetuation of the cloned DNA fragment carried by infectious phage particles could be tested by challenging typical wild-type E. coli strains or a λ -sensitive nonpermissive laboratory K-12 strain, especially one lysogenic for a lambdoid phage..

p25


EK3: These are EK2 systems for which the increased containment has been independently confirmed by appropriate tests in animals. EK3 systems are not presently available.

2. Classification of experiments using the E. coli K-12 containment systems

In the following classification of containment criteria for different kinds of recombinant DNAs, the stated levels of physical and biological containment are minimums. It is recommended that higher levels of biological containment (EK3 > EK2 > EK1) be used if they are available and are equally appropriate for the purposes of the experiment.

<a> Shotgun Experiments

These experiments involve the production of recombinant DNAs between the vector and the total DNA or (preferably) any partially purified fraction thereof from the specified cellular source.

2. Donald Brown: The guidelines are too strict and inflexible. Requiring EK2 containment for plant, vertebrate and viral DNAs places in effect an indefinite moratorium on research. Basis for this is that there are at present no EK2 vectors nor a clear mechanism of how EK2 systems will be certified. The NIH needs to conduct shotgun experiments in its P4 facilities. 
1. Robert Sinsheimer: Shotgun experiments with eukaryotic DNA incorporated into E. coli are especially troublesome. A safer and potentially reversible method is to incorporate such DNA into animal viruses where there is already in place a viable defense. One such example that comes to mind is cowpox. Here vaccination is possible and if the hazard were perceived, all infected animals and tissue culture cells could be destroyed.
4. Allen Silverstone: He recommends banning of all shotgun experiments with mammals and birds. For all of the eukaryotes P4 containment is recommended. He believes the possibility of cancer-causing DNA in lower organisms is a potential hazard which requires the highest physical containment. In the case of mammals he also recommends a ban on the use of chemically purified DNA.

(i) Eukaryotic DNA recombinants

P26
Primates - P3 physical containment + an EK3 host-vector, or P4 physical containment + an EK2 host-vector, except for DNA from embryonic tissue or primary tissue cultures therefrom, and germ-line cells for which P3 physical containment + an EK2 host-vector can be used. The basis for the lower estimated hazard in the case of DNA from the latter tissues is their relative freedom from horizontally acquired adventitious viruses.

Other mammals - P3 physical containment + an EK2 host-vector.

Birds - P3 physical containment + an EK2 host-vector.

*distinction
not yet justified
adequately*
Cold-blooded vertebrates - P2 physical containment + an EK2 host-vector except for embryonic or germ-line DNA which require P2 physical containment + an EK1 host-vector.

Other cold-blooded animals and lower eukaryotes - P2 physical containment + an EK1 host-vector. If the eukaryote in this class is a known pathogen (i.e., an agent listed in Class 2 of ref. 5 or a plant pathogen) or carries such an agent, the containment should be increased to P3 + EK2.

Higher plants - P2 physical containment + an EK2 host-vector. If the plant carries a known pathogenic agent or makes a product known to be dangerous to any species, the containment should be raised to P3 physical containment + an EK2 host-vector.

pp27-28 (ii) Prokaryotic DNA recombinants

Prokaryotes that exchange genetic information with *E. coli*² -

The level of physical containment is directly determined by the rule of the most dangerous component (see introduction to Section III).

Thus P1 conditions can be used for DNAs from those bacteria in Class 1 of ref. 5 ("Agents of no or minimal hazard....") which naturally exchange genes with *E. coli*; and P2 conditions should be used for such bacteria if they fall in Class 2 of ref. 5 ("Agents of ordinary potential hazard...."), or are plant pathogens. EKI host-vectors can be used for

² Defined as observable under optimal laboratory conditions by transformation, transduction, phage infection and/or conjugation with transfer of phage, plasmid and/or chromosomal genetic information.

all experiments requiring only P1 physical containment; in fact, experiments in this category can be performed with E. coli K-12 vectors exhibiting a lesser containment (e.g., conjugative plasmids) than EK1 vectors. Experiments with DNA from species requiring P2 physical containment which are of low pathogenicity (for example, enteropathogenic Escherichia coli, Salmonella typhimurium, and Klebsiella pneumoniae) can use EK1 host-vectors, but those of moderate pathogenicity (for example, Salmonella typhi, Shigella dysenteriae type I, and Vibrio cholerae) should use EK2 host-vectors.³

³ The bacteria which constitute Class 2 of ref. 5 ("Agents of ordinary potential hazard....") represent a broad spectrum of etiologic agents which possess different levels of virulence and degrees of communicability. We think it appropriate for our specific purpose to further subdivide the agents of Class 2 into those which we believe to be of relatively low pathogenicity and those which are moderately pathogenic. The several specific examples given may suffice to illustrate the principle. The Committee has asked the Center for Disease Control to help prepare a complete list of Class 2 agents subdivided into low and moderate pathogenicity that could be distributed to interested parties.

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Prokaryotes that do not exchange genetic information with

E. coli - The minimum containment conditions for this class consist of P2 physical containment + an EK1 host-vector, and apply when the risk that the recombinant DNAs will increase the pathogenicity or ecological potential of the host is judged to be minimal. Experiments with DNAs from pathogenic species (Class 2 ref. 5 plus plant pathogens) should use P3 + EK2.

Experiments extending the range of resistance to therapeutically useful drugs and disinfectants should use P2 + EK2 containment or higher depending on the virulence of the donor.

8828 - (iii) Characterized clones of DNA recombinants derived from
shotgun experiments

4. The Environmental Defense Fund: They too urge that E. coli not be used. They also are extremely concerned about use of the terms, "harmful" and "harmless," in describing genes and their products. They point out that accidents will occur and would like as broad a definition for "harmful" as possible to minimize possible accidents. They recommend that EK1 strains never be used except in circumstances where there is no possible risk to humans.

When a cloned DNA recombinant has been rigorously characterized⁴
and there is sufficient evidence that it is free of harmful genes,⁴
then experiments involving this recombinant DNA can be carried out
under P1 + EK1 conditions if the inserted DNA is from a species that
exchanges genes with E. coli, and under P2 + EK1 conditions if not.

⁴The terms "characterized" and "free of harmful genes" are unavoidably vague. But, in this instance, before containment conditions lower than the ones used to clone the DNA can be adopted, the investigator must obtain approval from the granting agency. Such approval would be contingent upon data concerning: (a) the absence of potentially harmful genes (e.g., sequences contained in indigenous tumor viruses or which code for toxic substances), (b) the relation between the recovered and desired segment (e.g., hybridization and restriction endonuclease fragmentation analysis where applicable), and (c) maintenance of the biological properties of the vector.

what about reversion of E

p29

 Purified cellular DNAs other than plasmids, bacteriophages,
and other viruses

1. Donald Brown: The guidelines for vertebrate "shotgun" experiments are too stringent and rigid. They establish a moratorium on gene isolation from all vertebrates. He urged the use of hybridization methods at reduced levels of physical and biological containment. Further, the requirements for chemically purified DNA are not achievable. There can be no guarantees for 99% purity. The Committee did not consider how purified DNA components differ from bulk DNA.

The formation of DNA recombinants from cellular DNAs that have been enriched⁵ by physical and chemical techniques (i.e., not by cloning) and which are free of harmful genes can be carried out under lower containment conditions than used for the corresponding shotgun experiment. In general, the containment can be decreased one step in physical containment (P4 → P3 → P2 → P1) while maintaining the biological containment specified for the shotgun experiment, or one step in biological containment (EK3 → EK2 → EK1) while maintaining the specified physical containment--provided that the new condition is not less than that specified above for characterized clones from shotgun experiments (Section <a>--iii).

⁵ A DNA preparation is defined as enriched if the desired DNA represents at least 99% (w/w) of the total DNA in the preparation. The reason for lowering the containment level when this degree of enrichment has been obtained is based on the fact that the total number of clones that must be examined to obtain the desired clone is markedly reduced. Thus, the probability of cloning a harmful gene could, for example, be reduced by more than 10⁵-fold when a nonrepetitive gene from mammals was being sought. Furthermore, the level of purity specified here makes it easier to establish that the desired DNA does not contain harmful genes.

PP29-32
<c> The following classifications refer to cases where the "foreign" DNA is itself derived from plasmids, bacteriophages, or viruses.

(i) "Foreign" DNA from viruses that infect animals.

P4 and EK2, or P3 and EK3 are to be used. P3 and EK2 may be used after purification by cloning and demonstration that only harmless viral genes are present.

(ii) "Foreign" DNA from viruses that infect plants.

P3 and EK1 or P2 and EK2.

(iii) "Foreign" DNA from purified (99% pure) eukaryotic organelle DNAs.

From primates: P3 and EK1 or P2 and EK2.
Other: P2 and EK1.

(iv) "Foreign" DNA from prokaryotic plasmids or bacteriophages.

If the "foreign" DNA is from a species that exchanges genetic information with *E. coli* and is known not to contain harmful genes, P1 and EK1 may be used. Otherwise, the containment defined in <a> (ii) above is required.

Required by
reference
anyone?

p 32-34 3. Experiments with prokaryotic "host-vector" systems other than *E. coli*

1. Richard Goldstein: As previously noted he recommends *E. coli* be banned as a host-vector within the next two years. He urges that the development of alternative prokaryote systems be developed and suggests one possibility is *B. subtilis*.

Other prokaryotic host-vector systems are at the speculative, planning, or developmental stage, and consequently do not warrant detailed treatment here at this time. However, the containment criteria for different types of DNA recombinants formed with *E. coli* K-12 host-vectors can, with the aid of some general principles given here, serve as a guide for containment conditions with other host-vectors when appropriate adjustment is made for their different habitats and characteristics.

In general, the strain of any prokaryotic species used as the host should conform to the definition of Class 1 etiologic agents given in ref. 5 (i.e., "Agents of no or minimal hazard...."), and the plasmid or phage vector should not make the host more hazardous. In addition, it is recommended that the newly developed host-vector systems offer some distinct advantage over the *E. coli* K-12 host-vectors--for instance, thermophilic organisms or other host-vectors whose major habitats do not include humans and/or economically important animals and plants. Appendix A gives a detailed discussion of the *B. subtilis* system, the most promising alternative to date.

At the initial stage, the host-vector should exhibit at least a moderate level of biological containment comparable to EK1 systems, and be capable of modification to obtain high levels of containment comparable to EK2 and EK3. The type of confirmation test(s) required to move a host-vector from an EK2-type classification to an EK3-type will clearly depend upon the preponderant habitat of the host-vector. For example, if the unmodified host-vector propagates mostly in, on, or around higher plants, but not appreciably in warm-blooded animals, modification should be designed to reduce the probability that the host-vector can escape to and propagate in, on, or around such plants, or transmit recombinant DNA to other bacterial hosts that are able to occupy these ecological niches, and it is these lower probabilities which should be confirmed. The following principles should be followed in using the containment criteria given for experiments with E. coli K-12 host-vectors as a guide for other prokaryotic systems. Experiments with DNA from prokaryotes (and their plasmids or viruses) should be classified according to whether the prokaryote in question exchanges genetic information with the host-vector or not, and the containment conditions given for these two classes with E. coli K-12 host-vectors applied. Transfer of recombinant DNA to plant pathogens can be made safer by using nonreverting, doubly auxotrophic, non-pathogenic variants. Experiments using a plant pathogen that affects elements of a local flora will require more stringent containment than if carried out in areas where they are not common.

Experiments with DNAs from eukaryotes (and their plasmids or viruses) can also follow the criteria for the corresponding experiments with E. coli K-12 vectors if the major habitats of the given

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host-vector overlap those of E. coli. If the host-vector has a major habitat that does not overlap those of E. coli (e.g., root nodules in plants), then the containment conditions for some eukaryotic recombinant DNAs should be increased (for instance, higher plants and their viruses in the preceding example), while others may be reduced.

4. Experiments with eukaryotic "host-vector" systems

1. Maxine Singer: The use of SV40 virus is troublesome. If it is not to be banned altogether, it should probably be used only at P4 levels.

2. Peter Barton Hutt: In the case of SV40, if it is to be used at all, it should be done only at the P4 level. Because this virus is known to cause cancer in animals, the burden is on the scientist to show there is no possibility whatever of harm to man when it is used in these experiments. If it is to be used, its use should be fully defended in the preamble in the guidelines.

PP 34-39 <a> Animal host-vector systems - Because host cell lines generally have little if any capacity for propagation outside the laboratory, the primary focus for containment is the vector, although cells should also be derived from cultures expected to be of minimal hazard. Given good microbiological practices, the most likely mode of escape of recombinant DNAs from a physically contained laboratory is carriage by humans; thus vectors should be chosen that have little or no ability to replicate in human cells. To be used as a vector in a eukaryotic host, a DNA molecule should display all of the following properties:

(1) It should not consist of the whole genome of any agent that is infectious for humans or that replicates to a significant extent in human cells in tissue culture.

(2) Its functional anatomy should be known--that is,
there should be a clear idea of the location within the
molecule of:

- a) the sites at which DNA synthesis originates
and terminates,
- b) the sites that are cleaved by restriction
endonucleases,
- c) the template regions for the major gene products.

(3) It should be well studied genetically. It is desirable
that mutants be available in adequate number and variety, and
that quantitative studies of recombination have been performed.

(4) The recombinant should be defective, that is, its pro-
pagation as a virus is dependent upon the presence of a comple-
menting helper genome. This helper should either (a) be integrated
into the genome of a stable line of host cells (a situation that
would effectively limit the growth of the vector to that particular
cell line) or (b) consist of a defective genome or an appropriate
conditional lethal mutant virus (in which case the experiments
would be done under non-permissive conditions), making vector and
helper dependent upon each other for propagation. However, if
none of these is available, the use of a non-defective genome
as helper would be acceptable.

Currently only two viral DNAs can be considered as meeting
these requirements: these are the genomes of polyoma virus and
SV40.

Of these, polyoma virus is highly to be preferred. SV40 is known to propagate in human cells, both in vivo and in vitro, and to infect laboratory personnel, as evidenced by the frequency of their conversion to producing SV40 antibodies. Also, SV40 and related viruses have been found in association with certain human neurological and malignant diseases. SV40 shares many properties, and gives complementation, with the common human papova viruses. By contrast, there is no evidence that polyoma infects humans, nor does it replicate to any significant extent in human cells in vitro. However, this system still needs to be studied more extensively. Appendix B gives further details and documentation.

Taking account of all these factors, it is proposed that:

1. Polyoma Virus

- a Recombinant DNA molecules consisting of defective polyoma virus genomes plus DNA sequences of any non-pathogenic organism, including Class 1 viruses (5), can be propagated in or used to transform cultured cells in P3 conditions; appropriate helper virus can be used if needed. Whenever there is a choice, it is urged that mouse cells, derived preferably from embryos, be used as the source of eukaryotic DNA. Polyoma virus is a mouse virus and recombinant DNA molecules containing both viral and cellular sequences are already known to be present in virus stocks grown at a high multiplicity. Thus, recombinants formed in vitro between polyoma virus DNA and mouse DNA are presumably not novel from an evolutionary point of view.

b. Such experiments can be done, under P4 conditions, if the recombinant DNA contains segments of the genomes of Class 2 animal viruses (5). Once it has been shown by suitable biochemical and biological tests that the cloned recombinant contains only harmless regions of the viral genome (see Section IIIB-2-c-i) and that the host range of the polyoma virus vector has not been altered, experiments can be continued under P3 conditions.

How will this be done? even tests in vitro.

2. SV40 Virus

- a. Defective SV40 genomes, with appropriate helper, can be used in P4 conditions as a vector for recombinant DNA molecules containing sequences of any non-pathogenic organism or Class I virus (5), (i.e., a shotgun type experiment); established lines of cultured cells should be used.
- b. Such experiments can be carried out in P3 conditions if the non-SV40 DNA segment is (a) a purified⁹ segment of prokaryotic DNA lacking toxigenic genes, or (b) a segment

⁹The DNA preparation is defined as purified if the desired DNA represents at least 99% (w/w) of the total DNA in the preparation, provided that it was achieved or verified by more than one procedure.

of eukaryotic DNA whose function has been established, which does not code for a toxic product, and which has been previously cloned in a prokaryotic host-vector system.

c. A recombinant DNA molecule consisting of defective SV40 DNA lacking substantial segments of the late region, plus DNA from non-pathogenic organisms or Class I viruses (5), can be propagated as an autonomous cellular element in established lines of cells under P3 conditions provided that there is no exogenous or endogenous helper, and that it is demonstrated that no infectious virus particles are being produced. Until this has been demonstrated, the appropriate containment conditions specified in 2. a. and 2. b. shall be used. / how?

d. Recombinant DNA molecules consisting of defective SV40 DNA and sequences from non-pathogenic prokaryotic or eukaryotic organisms or Class I viruses (5) can be used to transform established lines of non-permissive cells under P3 conditions. It must be demonstrated that no infectious virus particles are being produced; rescue of SV40 from such transformed cells by co-cultivation or transfection techniques must be carried out in P4 conditions.

3. Efforts should be made to ensure that all cell lines are free of virus particles and mycoplasma.

Since SV40 and polyoma are limited in their scope to act as vectors, chiefly because the amount of foreign DNA

that the normal virions can carry probably cannot exceed 2×10^6 daltons, we urge that consideration be given to the development of systems in which recombinants can be cloned and propagated purely in the form of DNA, rather than in the coats of infectious agents. Plasmid forms of viral genomes or organelle DNA should be explored as possible cloning vehicles in eukaryotic cells.

PPP39-41 Plant host-vector systems

1. Milton Zaitlin: In the guidelines plants have only a peripheral role; however, they could become extraordinarily important in recombinant DNA studies. Cloning of higher plant DNA and incorporating this DNA into bacteria is well covered by the guidelines. However, the guidelines are silent on the incorporation of foreign DNA into the genome of higher plants. There are five DNA plant viruses that could play a significant role here. These viruses are transmitted by aphids and are a problem with regard to physical containment. Physical containment in the guidelines is designed to prevent bacteria from getting out of the facility and to this, negative air pressures are specified. But positive air pressures are required to avoid drawing insects into the facility. Guidelines might be modified to specify positive air pressures under these circumstances. The guidelines also are silent on how to deal with a new plant once it is produced. Procedures need to be outlined for testing new plants for any undesirable characteristics they might have. The guidelines, therefore, should include restrictions on the release of plants from containment facilities until they can be tested through several generations. Plants should be tested to insure they are not toxic to animals which might be likely to eat them. In plants where the capacity for nitrogen fixation is introduced, testing must be done to insure that these plants don't have a competitive advantage over other plants and become pests in themselves.

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2 Cells in tissue cultures, seedlings, or plant parts (e.g., tubers, stems, fruits, and detached leaves) or whole mature plants of small species (e.g., Arabidopsis) can be handled under the P1-P4 containment conditions that we have specified previously. However, work in most plants poses additional problems. P2 physical containment conditions can be provided by: (i) the best insect-proof greenhouses, (ii) appropriate disinfection of contaminated plants, pots, soil, and runoff water, and (iii) adoption of the other standard practices for microbiological work. P3 physical containment can be sufficiently approximated by confining the operations with whole plants to growth chambers like those used for work with radioactive isotopes, provided that (i) such chambers are modified to produce a negative pressure environment with the exhaust air appropriately filtered, and (ii) that other operations with infectious materials are carried out under the specified P3 conditions. The P2 and P3 conditions specified earlier are therefore extended to include these cases for work on higher plants.

The host cells for experiments on recombinant DNAs may be cells in culture, in seedling or plant parts, or in whole plants. Cells in whole plants that cannot be adequately contained should not be used as hosts for shotgun experiments at this time, and attempts to infect whole plants with DNA recombinants cloned elsewhere should not be initiated until their effects on host cells in culture, seedlings, or plant parts have been studied.

Organelle or plasmid DNAs or DNAs of viruses of low pathogenicity to plants may be used as vectors. In general, the same preference criteria for selecting host-vectors given in the preceding section on animal systems apply to plant systems, where organelle and plasmid DNAs can be grouped together as offering the potential of highly contained vectors that should be investigated.

Experiments on recombinant DNAs formed between the initial moderately contained vectors and DNA from cells of species in which the vector DNA can replicate, either autonomously or as an integrated segment of the cell's genome, should use P2 physical containment--provided that the source of the DNA is itself not pathogenic or known to carry pathogenic agents, or to produce products dangerous to plants. In the latter cases, of if the vector is an unmodified virus of low pathogenicity, the experiments should be carried out under P3 conditions.

Experiments on recombinant DNAs formed between the above vectors and DNAs from other species can also be carried out under P2 if that DNA has been purified¹⁰ and determined not to contain harmful genes. Other-

¹⁰The DNA preparation is defined as purified if the desired DNA represents at least 99% (w/w) of the total DNA in the preparation, provided that it was achieved or verified by more than one procedure.

wise, the experiments should be carried out under P3 conditions if the source of the inserted DNA is not itself a pathogen, or known to carry such pathogenic agents, or to produce harmful products--and under P4 conditions if these conditions are not met.

The development and use of host-vector systems that exhibit a high level of biological containment permit a decrease of one step in the physical containment specified above (P4 → P3 → P2 → P1).

<c> Fungal or similar lower eukaryotic host-vector systems

The containment criteria for experiments on recombinant DNAs using these host-vectors most closely resemble those for prokaryotes, rather than those for the preceding eukaryotes, in that the host cells usually exhibit a capacity for dissemination outside the laboratory that is similar to that for bacteria. We therefore consider that the containment guidelines given for experiments with E. coli K-12 and other prokaryotic host-vectors (Sections IIIB-1 and -2, respectively) provide adequate direction for experiments with these lower eukaryotic host-vectors. This is particularly true at this time since the development of these host-vectors is presently in the speculative stage.

IV. IMPLEMENTATION

Being Revised