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Unregulated Hazards - 'Naked' and 'Free' Nucleic Acids

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Executive Summary

A huge variety of naked/free nucleic acids are being produced in the laboratory and released unregulated into the environment. They are used as research tools, in industrial productions and in medical applications such as gene therapy and vaccines. These nucleic acids range from oligonucleotides consisting of less than 20 nucleotides to artificial constructs thousands or millions of basepairs in length, typically containing a heterogeneous collection of genes from pathogenic bacteria, viruses and other genetic parasites belonging to practically every kingdom of living organisms. Most of the nucleic acids and constructs have either never existed in nature, or if they have, not in such large amounts. They are, by definition, xenobiotics -- substances foreign to nature -- with the potential to cause harm. Some, such as gene therapy vectors and vaccines, have already been shown to elicit toxic and other harmful reactions in preclinical trials.

Nucleic acids are now known to persist in all environments, including the digestive system of animals. Transformation by the uptake of DNA is recognized to be a significant route of horizontal gene transfer among bacteria, and there is overwhelming evidence that horizontal gene transfer and recombination have been responsible for the recent resurgence of drug and antibiotic resistant infectious diseases.

Recent investigations associated with gene therapy and vaccines leave little doubt that naked and free nucleic acids are readily taken up by the cells of all species including human beings, and may become integrated into the cell's genetic material. There is also abundant evidence that the extraneous nucleic acids taken up can have significant and harmful biological effects including cancers in mammals.

The need to establish regulatory oversight of naked/free nucleic acids at both national and international levels is long overdue. It is irresponsible to continue to exclude them from the scope of the International Biosafety Protocol.

'Naked' and 'free' nucleic acids

'Naked' nucleic acids are DNA/RNA produced in the laboratory and intended for use in, or as the result of genetic engineering [1]. 'Free' nucleic acids refer to the laboratory-produced nucleic acids transfected into cells or organisms, whether incorporated as transgenic DNA or not, and subsequently released into the environment by secretion, excretion, waste disposal, death, industrial processing, or carried by liquid streams, or in airborne dust and pollen.

A huge variety of naked nucleic acids are being produced in the laboratory (see Box 1), which are used as research tools, in industrial productions, and in medical applications such as gene therapy and vaccines. They range from oligonucleotides consisting of less than 20 nucleotides to artificial constructs thousands of basepairs in length, and artificial chromosomes millions of basepairs long. The constructs typically contain antibiotic resistance marker genes plus a heterogeneous array of genes from pathogenic bacteria, viruses and other genetic parasites belonging to practically every kingdom of living organisms on earth [2]. Most of the naked nucleic acids and constructs have either never

existed in nature, or if they have, not in such large amounts. They are, by definition, xenobiotics -- substances foreign to nature [3] -- with the potential to cause harm.

Box 1

Naked nucleic acids in genetic engineering biotechnology

DNA - BASED

Viral genomes, eg, cauliflower mosaic virus, cytomegalovirus, vaccinia, baculovirus, adenovirus, SV40, many bacteriophages

cDNA of RNA viral genomes, eg, retroviruses SIV, HIV, Rous Sarcoma virus, mouse Moloney virus

Plasmids, eg, Ti of *Agrobacterium*, many plasmids from *E. coli* and yeast, often carrying antibiotic resistance genes

Transposons, eg, many broad host range transposons from *E. coli* with antibiotic resistance genes, some from *Drosophila*, such as mariner are found in all kingdoms

Artificial vectors made by recombining viral genomes, plasmids and transposons, carrying one or more antibiotic resistance genes; used for gene amplification, DNA sequencing, transfection, gene therapy, etc. [5], many are shuttle-vectors designed for replication in more than one species, 'pantropic' vectors cross many species barriers

Naked DNA vaccines, plasmid-based, viral vector based [6]

Artificial chromosomes: yeast (YAC) plasmid (PAC) and mammalian (MAC) made from telomeric and centromeric repeat sequences [7]

Artificial constructs: transgene cassettes, often include antibiotic resistance gene cassettes

PCR amplified sequences

Oligodeoxynucleotides (antisense), hairpin-forming oligodeoxynucleotides used in gene therapy [8]

RNA - BASED

Antisence RNA used in gene therapy

Ribozymes used in gene therapy [9]

Self-replicating RNA (linked to RNA-dependent RNA polymerase) used in gene therapy [10]

RNA vaccines

RNA-DNA HYBRID

Chimeroplasty hairpins used in targeted gene mutation [11]

There is no regulation governing the release of naked nucleic acids into the environment. Many novel constructs are incorporated into transgenic micro-organisms and animal cell cultures for commercial drug production, and into crops, livestock, fish and other aquatic

organisms for food, animal feed, and other purposes. These constructs are therefore greatly amplified, and at the same time introduced into foreign genomes where recombination with host genes and the genes of the host's viral pathogens may readily occur. Transgenic wastes containing large amounts of free or potentially free transgenic DNA are being released unregulated into the environment, including those from microorganisms and cell cultures supposed to be in 'contained use' (see Box 2) [4]. Under the current EU Directive for Contained Use, contained users are allowed to release certain live transgenic microorganisms in liquid waste, and all killed microorganisms and cells containing transgenic DNA as solid waste.

Box 2

Free nucleic acids resulting from genetic engineering biotechnology

Transfected, unincorporated nucleic acids/constructs due to gene-therapy, vaccination, transgenesis, which are released into the environment by secretion, excretion, waste carcass disposal, cell death, etc.

Transgenic DNA released from live or dead cells contained in:

- Transgenic wastes from genetically engineered microorganisms in contained use
- Transgenic wastes from cell cultures in contained use
- Transgenic wastes from genetically engineered crops
- Transgenic wastes from genetically engineered fish and other aquatic organisms
- Transgenic wastes from genetically engineered farm animals
- Unprocessed transgenic food and animal feed
- Processed transgenic food for human use and animal feed
- Processed transgenic textiles such as cotton
- Transgenic dust from food processing
- Transgenic pollen
- Transgenic honey

The lack of regulation of naked/free nucleic acids is based largely on the assumption, *now proven to be erroneous*, that naked/free nucleic acids would be rapidly broken down in the environment and in the digestive system of animals [12]. Another assumption is that as DNA is present in all organisms, it is not a hazardous chemical, and hence there is no need to regulate it as such [13].

DNA persists in all environments and is readily taken up by cells of all organisms

Naked or free DNA are now known to persist in all natural environments, and high concentrations are found in the soil, in marine and fresh water sediments as well as in the air-water interface, where it retains the ability to transform microorganisms [14]. DNA also persists in the mouth [15] and the digestive tract of mammals [16], where it may be taken up and incorporated by the resident microbes, and by the cells of the mammalian host.

A genetically engineered plasmid was found to have a 6 to 25% survival after 60 min. of exposure to human saliva. The partially degraded plasmid DNA was capable of transforming *Streptococcus gordonii* , one of the bacteria that normally live in the human mouth and pharynx. Human saliva contains factors that promote competence of resident bacteria to become transformed by DNA [17].

It has long been assumed that DNA cannot be taken up through intact skin, surface wounds, or the intestinal tract, or that it would be rapidly destroyed if taken up. Those assumptions have been overtaken by empirical findings. The ability of naked DNA to penetrate intact skin has been known at least since 1990. Cancer researchers found that within weeks of applying the cloned DNA of a human oncogene to the skin on the back of mice, tumours developed in endothelial cells lining the blood vessel and lymph nodes [18].

Viral DNA fed to mice is found to reach white blood cells, spleen and liver cells via the intestinal wall, to become incorporated into the mouse cell genome [19]. When fed to pregnant mice, the viral DNA ends up in cells of the fetuses and the new born animals, suggesting that it has gone through the placenta as well [20]. The authors remark that "The consequences of foreign DNA uptake for mutagenesis and oncogenesis have not yet been investigated." [21]

Recent developments in gene therapy demonstrate how readily naked nucleic acids (see Table 2) can gain access to practically every type of human cells and cells of model mammals. Naked nucleic acids can be successfully delivered, either alone or in complex with liposomes and other carriers, in aerosols via the respiratory tract [22], by topical application to the eye [23], to the inner ear [24], via hair follicles [25], direct injection into muscle [26], through the skin [27], as well as by mouth, where the nucleic acid is taken up by cells lining the gut [28]. Naked DNA can even be taken up by sperm cells of marine organisms and mammals, and transgenic animals created [29]. Geneticists are contemplating using sperms as vectors in gene therapy.

High levels of foreign gene expression was observed in the liver cells of rats, mice and dogs when naked DNA was injected into blood vessels supplying the liver [30]. Gene expression is observed in skin cells injected with naked DNA [31], and naked DNA was integrated into chromosomes of cells and expressed in human and pig skin [32]. Researchers have found integration of a plasmid-based naked DNA malarial vaccine injected into mouse muscle in a preclinical trial, but dismissed it as "3000 times less than the spontaneous mutation rate for mammalian genome" and hence "not considered to pose a significant safety concern" [33].

Hazards of naked nucleic acids

One of the key findings is that naked viral DNA is more infectious and have a wider host range than the intact virus. Human T-cell leukaemia viral DNA formed complete viruses when injected into the bloodstream of rabbits [34]. Similarly, naked DNA from the human polyomavirus BK (BKV) gave a full-blown infection when injected into rabbits, despite the fact that the intact BKV virus is not infectious [35]. This hazard is particularly relevant to the entire range of virus-based gene therapy vectors and naked DNA vaccines under development [36]. Modifications to viral genomes can have unexpected effects on virulence and the host range [37].

The safety of gene therapy vectors is unproven [38]. The hazards include both direct toxicities and indirect effects (see Box 3) and there is a growing debate over the potential for generating infectious viruses, and harmful effects due to random insertion into the cellular genome [39], both of which are shared by naked DNA vaccines. Recombinant DNA vaccines, in both the naked and intact viral form, also tend to be more unstable and prone to recombination, increasing the likelihood of generating new viruses [40]. A viral vaccine made by deleting genes from the simian immuno-deficiency virus (SIV) was found to cause AIDS in infant and adult macaques [41], raising serious safety concerns over similar AIDS viral vaccines for humans.

Box 3

Hazards from naked nucleic acids

- Acute toxic shock from viral vectors
- Immunological reaction from viral vectors
- Autoimmune reactions from double-stranded DNA and RNA
- Non-target interference with gene function from anti-sense DNA, RNA and ribozymes
- Generation of virulent recombinant viruses
- Insertion mutagenesis
- Insertion oncogenesis
- Genetic contamination of germ cells

Gene therapy vectors and naked DNA vaccines can cause acute toxic shock reactions [42] and severe delayed immunological reactions [43]. Between 1998 and 1999, scientists from US drug companies failed to notify the FDA about six deaths that had occurred during clinical trials of gene therapy, the causes of which are yet to be determined [44]. Naked DNA can also trigger autoimmune reactions, in which the body's immune system attack and kill its own tissues and cells. New research shows that any fragment of double-stranded DNA or RNA introduced into cells can induce these reactions which are responsible for many diseases [45]. Examples of autoimmune diseases are rheumatoid arthritis, insulin-dependent diabetes and Graves disease of the thyroid. Many 'spontaneous mutations' are due to insertions of transposable elements and other invasive DNA. Insertion mutagenesis is now found to be associated with a range of cancers, including lung [46], breast [47], colon [48] and liver [49] cancers. Finally, unintended modification of germ-cells may result from gene therapy and vaccinations [50].

Not as much is known concerning naked RNA. It is to be expected that antisense RNA, like antisense DNA, will have biological effects either in blocking the function of homologous genes or genes with homologous domains. RNA may also be reverse transcribed into complementary DNA (cDNA) by reverse transcriptase, which is present in all higher organisms as well as some bacteria [51], to become incorporated into the cell's genome.

The direct uptake and incorporation of genetic material from unrelated species is referred to as *horizontal gene transfer*, or gene transfer by infection, to distinguish it from the usual *vertical* gene transfer from parent to offspring in reproduction.

The horizontal transfer of transgenic DNA

Many geneticists may accept that naked nucleic acids are transferred horizontally, especially to microorganisms, but dispute the transfer of transgenic DNA, which they regard to be no different from the host cell DNA.

There is evidence of secondary horizontal transfer of transgenic DNA to soil bacteria and fungi in the laboratory. In the case of fungi, the transfer was obtained simply by co-cultivation [52]. Successful transfers of a kanamycin resistance marker gene to the soil bacterium *Acinetobacter* were obtained using DNA extracted from homogenized plant leaf from a range of transgenic plants: *Solanum tuberosum* (potato), *Nicotiana tabacum* (tobacco), *Beta vulgaris* (sugar beet), *Brassica napus* (oil-seed rape) and *Lycopersicon esculentum* (tomato) [53]. It is estimated that about 2500 copies of the kanamycin resistance genes (from the same number of plant cells) is sufficient to successfully transform one bacterium, despite the fact that there is six million-fold excess of plant DNA present. A single plant with say, 2.5 trillion cells, would be sufficient to transform one billion bacteria.

Schluter *et al* [54] investigated horizontal gene transfer under a variety of conditions, some of which gave positive results. For example, a high gene transfer frequency of 5.8×10^{-2} per recipent bacterium was demonstrated for ampicillin resistance transgene -- re-isolated from the DNA of transgenic potato -- to *Erwinia chrysanthem*, a bacterial pathogen. This was achieved by 10^5 copies of the ampicillin resistance gene per potato genome, introduced into 6.4×10^8 bacteria by electroporation. When reduced to one copy of ampicillin resistance gene per potato genome, the gene transfer frequency was still significant at 4×10^{-6}. The total genomic DNA from the transgenic potato, estimated to carry two copies of ampicillin resistance gene per potato genome, likewise gave a transfer frequency of 9×10^{-6}. With only transgenic potato tissue, it was less than 8.7×10^{-9}, effectively nil, according to the limit of sensitivity of the protocol. The same result was obtained by co-cultivation of the transgenic tuber with bacteria for 6 weeks. The negative results were not surprising, given the limited access of the bacteria to plant DNA under those conditions. The authors then 'calculated' an extremely low frequency of gene transfer at 2.0×10^{-17} under extrapolated "natural conditions*", assuming the different factors acted independently*. The natural conditions are unknown and by the authors' own admission, synergistic effects cannot be ruled out.

Free transgenic DNA will be readily available in the rhizosphere around the plant roots, which is an 'environmental hotspot' for gene transfer [55]. Gebbard and Smalla [56] have also found evidence of horizontal transfer of kanamycin resistance from transgenic DNA to *Acinetobactor* , and positive results were obtained using just 100 m l of plant-leaf homogenate. Many other factors, such as the density of bacteria, temperature, availability of nutrients, heavy metals and pH, can greatly influence the frequency of horizontal gene transfer in nature [57]. Moreover, less than one percent of all bacteria in the environment can be isolated [58] and monitored for horizontal gene transfer, so negative results in the field must be interpreted with due caution. There is no ground to assume that horizontal transfer of transgenic DNA will not take place under natural conditions.

There are also reasons to suspect that transgenic DNA may be more likely to take part in

horizontal gene transfer than the organism's own genes (see Box 4) [59].

Box 4

Reasons to expect that transgenic DNA may be more likely to spread horizontally than non-transgenic DNA

1. The mechanisms enabling foreign genes to insert into the genome also enable them to jump out again, to re-insert at another site, or to another genome.

2. The integration sites of most commonly used artificial vectors for transferring genes are 'recombination hotspots', prone to break and join up with other DNA, and so have an increased propensity to transfer horizontally.

3. Viral promoters, such as that from the cauliflower mosaic virus, widely used to boost the expression of transgenes, also contain a recombination hotspots [60], and will therefore further enhance horizontal gene transfer.

4. The unnatural gene combinations in transgenic DNA tend to be unstable, and hence prone to recombine and transfer horizontally.

5. The metabolic stress on the host organism due to the continuous over-expression of transgenes may contribute to the instability of the insert, as it is well-known that mobile genetic elements in all genomes are mobilized to jump out of genomes during conditions of stress, to multiply and/or reinsert randomly at other sites resulting in many insertion-mutations. The foreign gene-constructs and the vectors into which they are spliced, are typically mosaics of DNA sequences from numerous species and their genetic parasites; that means they will be more prone to recombine with, and successfully transfer to, the genomes of many species and their genetic parasites [61].

The hazards of horizontal gene transfer

Horizontal gene transfer is uncontrollable. Unlike chemical pollutants which break down and become diluted out, nucleic acids are infectious, they can invade cells and genomes, to multiply, mutate and recombine indefinitely.

Horizontal gene transfer is by no means unknown to our Governments. Among the scientific advice given by the UK Ministry of Agriculture, Fisheries and Food (MAFF) to the US Food and Drug Administration (FDA) at the end of 1998 [62] are the following warnings:

- Transgenic DNA can spread to farm workers and food processors via dust and pollen.

- Antibiotic resistance marker genes may spread to bacteria in the mouth, as the mouth contains bacteria that readily take up and incorporate foreign DNA (see above). Similar transformable bacteria are present in the respiratory tracts.

- Antibiotic resistance marker genes may spread to bacteria in the environment, which then serves as a reservoir for antibiotic resistance genes.

- DNA is not readily degraded during food processing nor in the silage, hence transgenic DNA can spread to animals in animal feed.

- Foreign DNA can be delivered into mammalian cells by bacteria that can enter into the cells.

- The ampicillin resistance gene in the transgenic maize undergoing 'farm-scale' field-trials in the UK and elsewhere is very mutable, and may compromise treatment for meningitis and other bacterial infections, should the gene be transferred horizontally to the bacteria. The potential hazards of horizontal gene transfer are unlike those we have ever experienced (see Box 5).

Box 5

Potential hazards from horizontal gene transfer of naked/free nucleic acids

- Generation of new viruses that cause disease
- Generation of new bacteria that cause diseases
- Spreading drug and antibiotic resistance genes among the viral and bacterial pathogens, making infections untreatable
- Random insertion into genomes of cells resulting in harmful effects including cancer
- Reactivation of dormant viruses, present in all cells and genomes, which may cause diseases
- Multiplication of ecological impacts due to all the above

The dangers of generating new viruses and bacteria that cause diseases, and spreading drug and antibiotic resistance among the pathogens, were both foreseen by the pioneers of genetic engineering. That was why they called for a moratorium in the Asilomar Declaration of 1975. But commercial pressures cut the moratorium short, and guidelines were set up based on assumptions, every one of which has been invalidated by scientific findings since [63]. Within the past 20 years, drug and antibiotic resistant infectious diseases have come back with a vengeance. *Geneticists have confirmed that the diseases are due to new viral and bacterial strains that have been created by horizontal gene transfer and recombination*. Horizontal gene transfer is now recognized to be widespread, involving the entire biosphere, with bacteria and viruses in all environments serving as reservoir and highway for gene multiplication, gene swapping and trafficking. Has genetic engineering contributed to creating the new pathogens, and will it continue to do so through the unregulated release of naked and free nucleic acids? [64] The possible links between genetic engineering biotechnology and the recent resurgence of infectious diseases are summarized in Box 6.

Box 6

Possible links between genetic engineering biotechnology and the recent resurgence of infectious diseases

Inductive

1. Horizontal gene transfer is responsible for creating new viral and bacterial pathogens and for spreading drug and antibiotic resistance

2. Experimental evidence of horizontal gene transfers, some between very distant species, has been obtained in all natural environments and in the gut. Thesewere all accomplished with artificially constructed vectors used in genetic engineering.

3. Genetic engineering makes extensive use of antibiotic resistance genes as selectable markers, thereby increasing the spread of antibiotic resistance genes.

4. Antibiotics increases the frequency of horizontal gene transfer 10 to 10000 fold, thereby enhancing the spread of disease-causing genes as well as antibiotic resistance genes.

5. Genetically 'crippled' strains of bacteria, supposed to be 'biological contained', are nevertheless found to survive in the environment, and to swap genes with other bacteria.

6. DNA released from dead as well as live cells are not entirely broken down in the environment, nor in the gut, where it may be taken up and incorporated into the genomes of bacteria.

7. DNA from viruses is more infectious than the intact virus itself.

8. Routine chemical inactivation of genetically engineered microorganisms prior to disposal in the general environment may be ineffective, leaving a substantial proportion of viruses and bacteria in an infective state.

9. Current legal limits of 'tolerated releases' of genetically engineered microorganisms from contained use vastly exceed the minimal infective dose of pathogens and potential pathogens.

10. Non-pathogens are transformed into pathogens by horizontal gene transfer of unit blocks of virulence genes.

11. Horizontal gene transfers are bi-directional. Released non-pathogens can be readily converted into pathogens in one step, by acquiring unit-blocks of virulence genes.

Deductive

12. Genetic engineering is based on facilitating horizontal gene transfer between distant species by constructing artificial vectors that break down species barriers.

13. The artificial vectors constructed for genetic engineering are combinations of viral pathogens and other invasive genetic elements that can generate new cross-species viral pathogens.

14. The artificial vectors and other constructs for genetic engineering are inherently unstable and prone to recombination, thereby enhancing horizontal gene transfer and recombination.

15. Special 'shuttle vectors' made by genetic engineering are essentially unstoppable, as they contain signals for transfer and replication in different species; and helper functions for mobilization and transfer can be supplied by viruses and other genetic parasites which occur naturally in bacteria in all environments.

Circumstantial

16. The accelerated emergence of infectious diseases and of drug and antibiotic resistance coincide with the development of commercial genetic engineering biotechnology.

17. Many horizontal gene transfer events responsible for the spread of virulenc and antibiotic resistance are recent, as inferred from the high degree (>99%) of similarity in sequences of genes found in unrelated species.

Dormant and relict viral sequences have been discovered in the human and other animal genomes at least 20 years ago [65]. Viral sequences have also been discovered recently in plant genomes [66]. Viral transgenes are found to recombine with defective viruses to generate infectious recombinants [67]. Recombination between exogenous and endogenous viral sequences are associated with animal cancers [68]. It is not inconceivable that the cauliflower mosaic viral promoter, which is in practically all first generation of transgenic plants, may recombine with dormant/relict viral sequences in the genome to regenerate infectious viruses [69], in view of the fact that viral promoters have modules in common. Recombination hotspots may be associated with all transcriptional promoters [70], including those of animal viruses, such as the SV40 and cytomegalovirus, used in animal and human genetic engineering [71]. This possibility should be addressed by empirical investigations, particularly in view of the recent claim that a significant part of the toxicity of certain transgenic potatoes fed to young rats may be due to the transgenic construct or the transformation process, or both [72].

In the light of the existing evidence, the most dangerous naked/free DNA may be coming from the wastes of contained users of GMOs which are discharged into our environment. These include constructs containing cancer genes from laboratories in research and development of cancer and cancer drugs, virulence genes from bacteria and viruses in pathology labs and *all kinds of other novel constructs and gene combinations that did not previously exist in nature, and may never have come into being but for genetic engineering*.

Despite the growing body of evidence of hazards from the innumerable exotic naked nucleic acids that are created and released in increasing amounts into the environment from the burgeoning biotech industry, there is no effective regulatory oversight, nor is there any indication that our Government is prepared to establish effective regulatory oversight (see Box 7).

Box 7

Current regulatory oversight is seriously out of date and does not address the dangers of naked or free nucleic acids

- there is no monitoring for horizontal gene transfer in current field trials, including the farm-scale field trials supported by the Government
- there is no requirement for industry to monitor for horizontal gene transfer in seeking approval for field trials or commercial release
- there is no requirement for industry to monitor for horizontal gene transfer prior or subsequent to discharge of transgenic wastes from contained use into the environment
- current regulation allows certain live genetically engineered bacteria to be discharged into the environment without any pretreatment
- undegraded transgenic DNA from killed bacteria and viruses are routinely discharged into the environment,
- there is no requirement for industry to report on health impacts of transgenic DNA, which may spread drug and antibiotic resistance among pathogens, create new viral and bacterial pathogens as well as cause cancer; nor is there any monitoring of such health impacts being conducted by our Government
- Nucleic acid sequences and constructs, including artificial viral vectors, and other genetic parasites are not subject to regulation, and may be freely discharged into the environment
- current regulation of contained use still regard DNA as an ordinary, non-hazardous chemical which can be released unchecked into the environment

Conclusion

The naked/free nucleic acids created by genetic engineering biotechnology are potentially the most dangerous xenobiotics to pollute our environment. Unlike chemical pollutants which dilute out and degrade over time, nucleic acids can be taken up by all cell to multiply, mutate and recombine indefinitely. The need for regulatory oversight at both national and international levels is long overdue. It is irresponsible to continue to exclude naked/free nucleic acids from the scope of the Biosafety Protocol.

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