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Acceptability of cell substrates for production of biologicals

Report of a
WHO Study Group

World Health Organization
Technical Report Series
747

World Health Organization, Geneva 1987

ISBN 92 4 120747 7

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ISSN 0512-3054

PRINTED IN SWITZERLAND

7122 – Schüler S.A. – 7000

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Geneva, 18–19 November 1986

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ACCEPTABILITY OF CELL SUBSTRATES FOR PRODUCTION OF BIOLOGICALS

Report of a WHO Study Group on Biologicals

A WHO Study Group on Biologicals met in Geneva on 18 and 19 November 1986. The meeting was opened on behalf of the Director-General by Dr S. Litvinov, Assistant Director-General.

1. BACKGROUND AND INTRODUCTION

The acceptability of cell substrates for vaccine production has been a subject of controversy ever since the 1950s, when the decision was made that primary cell cultures of non-human primate origin were acceptable for the production of certain vaccines such as poliomyelitis vaccine. This decision set an important precedent, which has had an impact on the consideration of alternative cell systems. For example, it took a decade or more for human diploid cell substrates to be accepted by certain national control authorities, even though all of the available scientific evidence indicated that products derived from them were safe to use.

The first reconsideration of the acceptability of substrates other than primary and diploid cells occurred in 1978 at Lake Placid, USA, when human lymphoid (lymphoma-derived) cells were proposed as a source of α-interferon. The major reason for considering a human malignant cell as a substrate for the production of interferon was that the product could be generated in very large quantities and in such a way that the manufacturing process could ensure the elimination of cellular contaminants that might be of concern. This research and development effort laid the groundwork for methods that might be used to ensure the safety of products derived from a variety of other continuous cell lines.

As advances were made during the past 10 years in basic biological research, and as it became clear that recombinant DNA techniques would provide an opportunity to develop biological products that it had not previously been possible to manufacture, the usefulness of continuous cell lines as substrates became apparent. The development of hybridoma technology also heightened interest

in the use of such cell lines. As a result, a series of meetings was held in Europe and North America to discuss the subject further. Products for clinical trials were then developed in cell lines by various groups, and certain national control authorities began to approve the experimental use of these products. More recently, national control authorities have also licensed certain products (e.g., lymphoid interferon, monoclonal antibodies, and inactivated rabies and poliomyelitis vaccines). In addition, the WHO Expert Committee on Biological Standardization approved the use of non-tumorigenic and virus-free continuous cell lines for the production of inactivated poliomyelitis vaccine in 1981. In fact, the general trend since 1978 has been towards the acceptance of continuous cell lines for the production of various biologicals. However, when a group of consultants met to consider the development of WHO Requirements for hepatitis B vaccine produced by recombinant DNA techniques in continuous cell lines, they suggested that it would be useful for WHO to convene a group of experts to consider issues associated with the use of eukaryotic and prokaryotic cells in the production of biologicals, with particular emphasis on the potential risks from contaminating DNA, in order to provide international guidance on the acceptability of various cell substrates and production strategies. The Director-General therefore convened the Study Group on Biologicals to provide advice to WHO on these issues.

Background documents providing both experimental data and theoretical information were discussed and led to the formulation of the conclusions provided later in this report (section 3). A major concern was the long-term risk of malignancy represented by heterogeneous contaminating DNA, especially if it were to contain potentially oncogenic coding or regulatory sequences. This concern was especially real because many healthy people, including infants, might be vaccinated with, or otherwise receive, products derived from continuous cell lines.

There is some evidence for the safety of certain continuous cell lines as substrates for the production of biologicals. For example, an inactivated virus vaccine for foot and mouth disease has been prepared in the BHK-21 cell line, and it is estimated that more than 100 million doses have been administered to cattle over a 20-year period. Carcass inspection has failed to reveal any ill-effects attributable to the vaccine, suggesting that in the short term (2–4 years) this vaccine has been safe. Although clinical experience with

biologicals produced for human use in continuous cell lines has been more recent, and therefore more limited, than experience with veterinary products, it was noted that about 19 million doses of inactivated poliomyelitis vaccine produced in Vero cells had already been administered to children in the three years since 1983.

2. ISSUES CONSIDERED BY THE STUDY GROUP

The issues were of two main types: firstly, the acceptability of developing a biological product in a new cell system when the same generic product is already being manufactured by an approved method (see *a* below); and secondly, the degree of risk associated with certain classes of possible contaminants in the product, including heterogeneous contaminating DNA (see *b–f* below); viruses (see *g* below); and transforming proteins (see *h* below).

The specific questions discussed by the Study Group were as follows:

(*a*) What position should be taken on the acceptability of a product generated in a continuous cell line when an alternative production method exists in another cell system? For example, α-interferon can be produced in *Escherichia coli* as well as in human lymphoid cells; and hepatitis B vaccine can be produced from human plasma and in yeast, as well as in continuous cell lines.

(*b*) Is there an absolute amount of DNA per dose of product (or accumulated dose for repetitively administered biologicals) below which the probability of a biological effect is so small as to be effectively zero? Would nuclease treatment enhance confidence in the safety of the product, or simply add another concern, perhaps even more significant than that related to the DNA?

(*c*) What type of DNA should be the focus of attention in tests to quantify the amount of DNA in a product or in a given step in the manufacturing process? If regulatory sequences are used in constructing the recombinant cell, should specific efforts be made to look for such sequences? Are highly repetitive and widely dispersed sequences, such as *Alu* in human DNA, adequate as a generic probe?

(*d*) Are viral sequences, if they exist in any given cell line, of special concern, or can they be dealt with in a generic sense with the rest of the cellular DNA?

(*e*) What are the experimental models of choice for assessing the transforming potential of DNA from cells being considered for use, and how long should the studies last?

(*f*) How valuable are validation studies of the ability of a manufacturing process to eliminate or inactivate unwanted viruses and DNA as compared with studies on the final product?

(*g*) Are the screening procedures now contained in WHO Requirements for the characterization of continuous cell lines adequate to detect viruses of concern?

(*h*) Should continuous cell lines be characterized regarding their ability to produce transforming proteins? What are the assay systems of choice? If transforming proteins are found, how significant is the risk to the recipient of the product and what levels would be acceptable?

(*i*) What criteria of purity should be applied to the final product to provide a reasonable assurance of acceptability?

3. DISCUSSION AND CONCLUSIONS

Several features make continuous cell lines particularly suitable as substrates for the production of a wide range of biologicals for human use. These include:

—low cost (relative to other mammalian substrates such as primary cells, which must be intensively tested at each harvest);
—avoidance of the use of primates as the source of primary cells for production;
—ability to prepare, standardize, and store cell seeds;
—ease of preparing suspension cultures on a very large scale;
—susceptibility to infection by a variety of viruses of human importance;
—relative ease of transfection with recombinant DNA plasmids and subsequent cloning of high-yield transformants;
—high likelihood of correct post-translation processing of mammalian proteins encoded by transfected DNA, which enhances the probability of correct conformational structure of the product;
—secretion (with or without genetic manipulation) of products into the medium.

These properties are important not only for the production of biologicals in developed countries, but could greatly facilitate the

transfer of vaccine-producing capability to developing countries, which is an important goal of WHO.

There are, however, potential risks associated with the human use of biologicals produced in continuous cell lines. Before describing and analysing these risks, the Study Group reviewed general issues associated with the acceptance of new biologicals.

3.1 Risk assessment during research and development

There are several important steps in the progression of a novel approach from concept to practice. The first step is an evaluation of the risks that the new drug, vaccine, or cell substrate does or might pose to human beings. In most countries, if not all, preclinical safety data are required before the administration of a new drug or biological product to human subjects. Indeed, international as well as national ethical norms have been established to protect human subjects from unreasonable risks during the experimental phases of the development of new therapeutic and prophylactic products.

Before products derived from continuous cell lines were first approved for human use, different committees assessed the associated benefits and risks, taking into consideration not only the data in support of product safety but also the relative benefits of the new products in comparison to existing products. Before proceeding with human clinical studies, the committees evaluated the safety of each product on the basis of all the data available, taking into account the capacity of various steps in the manufacturing process to remove and inactivate potentially harmful contaminants. Some national control authorities also made a decision on the acceptability of proceeding with experimental studies in human subjects.

Those clinical study committees, and in some cases national control authorities as well, concluded that it was reasonable to proceed with human studies with a variety of products derived from continuous cell lines. They did not expect that the use of a continuous cell line would result in any real risk for the human subjects. It would be inimical to the basic concepts of medicine knowingly to impose the risk of cancer on patients with serious diseases or on healthy people, particularly since alternatives exist to many of the products that are now available from continuous cell lines or that are expected to be approved soon. The general view of the groups that have considered these issues so far is that, whatever the potential risks might have been, the data in support of the safety

of the products justified their acceptance. Nevertheless, it is prudent to be alert to unexpected risks, and appropriate long-term follow-up studies of groups of recipients of these new products should be considered as they are introduced into general use. Taking this into consideration, the Study Group concluded that the basic decision on the safety of a product derived from a continuous cell line must be made at the point of approving the first clinical trial. That initial judgement needs to be modified only if new and relevant data subsequently come to light.

3.2 Products manufactured by alternative methods

The Study Group also considered the acceptability of using continuous cell lines for products such as α-interferon, poliomyelitis vaccine, and hepatitis B vaccine, which can be produced by alternative methods. Both the individual and society are continually faced with choices involving relative safety and benefits. The selection of acceptably safe options from among various alternatives has played a key role in the history of biological product development, and has included a consideration of issues such as which virus strains and which cell substrates to use for vaccine production.

The Study Group concluded that, when reliable data are provided in support of the safety of a product derived from a continuous cell line, that product should be considered acceptable. The existence of an approved manufacturing process for the same product in another cell system is not considered to be relevant to the acceptance or rejection of a product derived from a continuous cell line. Every product should stand on its own merits regarding safety and efficacy.

3.3 Potential risks associated with biologicals produced in continuous cell lines

The main potential risks associated with the use of biologicals produced in continuous cell lines fall into three categories: heterogeneous contaminating DNA, viruses, and transforming proteins. A summary of the risk assessment for each follows. More comprehensive statements on heterogeneous contaminating DNA and transforming proteins are provided as Annexes 1 and 2, respectively.

3.3.1 *Heterogeneous contaminating DNA*

The Study Group concluded, on the basis of the experimental data available, that the risk associated with heterogeneous contaminating DNA in a product derived from a continuous cell line is negligible when the amount of such DNA is 100 pg or less in a single dose given parenterally. The assessment of the safety of any product with respect to DNA should take into consideration: (*a*) the elimination of the biological activity of DNA by various steps in the manufacturing process; and (*b*) the reduction in the amount of DNA during the purification of the product in the manufacturing process. A given product may be considered safe on the basis of reliable data on either or both of these operations. Because of the extremely low probability of a biological effect of 100 pg of heterogeneous contaminating DNA per dose of product given parenterally, nuclease treatment of products during manufacture would probably add more concerns that it would remove.

The use of special DNA sequences, such as viral regulatory sequences, in the construction of recombinant cells is considered acceptable because there is no evidence that such sequences would impose any additional risk beyond that of heterogeneous contaminating cellular DNA in general. Nevertheless, the manufacturing process should be validated to demonstrate that such sequences are not concentrated in the crude harvest or at other steps. It is expected, however, that recombinant DNA products will be highly purified and the amount of DNA per dose will be less than 100 pg; in some cases it may even be undetectable.

When a product is likely to be contaminated by DNA with important biological activity, detection of that DNA should be attempted by appropriate techniques, some of which are referred to in Annex 1. Newer techniques for detecting DNA are under development.

Viral inactivating agents have been used for many years in the preparation of safe and effective vaccines for poliomyelitis, rabies, and hepatitis B. Some data suggest that these viral inactivating agents may also destroy the biological activity of DNA, thus providing an additional level of confidence in the safety of these products even when the amount of DNA in a parenteral dose of the product is above 100 pg. However, the Study Group concluded that more specific data were needed on the effects of these inactivating agents under manufacturing conditions in order to draw firm conclusions on their DNA-inactivating potential.

3.3.2 *Viruses*

The Study Group reviewed the potential risk to human recipients of products manufactured in cells containing viral agents. These agents may include complete viruses with known patterns of replication such as simian virus 40, virus particles such as the type A retroviruses that can be visualized by electron microscopy, and persisting viral genomes or parts of genomes, for example those of the hepatitis B and Epstein-Barr viruses. Cells may be divided into three risk categories with respect to their potential for carrying viral agents pathogenic for human beings:

High risk:	Blood and bone marrow cells derived from human or non-human primates; caprine and ovine cells; hybridomas when at least one fusion partner is of human or non-human primate origin.
Medium risk:	Mammalian non-haematogenous cells such as fibroblasts and epithelial cells.
Low risk:	Human diploid cell lines and cells derived from avian tissues.

In making these risk distinctions, the following points were noted:

(*a*) Human lymphocytes and macrophages may carry latent viruses, such as human retroviruses, which may become activated when the cells are exposed to growth-stimulating factors during *in vitro* cultivation. The existence of simian retroviruses infectious for human cells places haematogenous cells from non-human primates in the high risk group. If primary human leukocytes are used for the production of interferon or other biologicals, the manufacturing process should be demonstrated to inactivate the known human retroviruses. In addition, it will be important that products manufactured in primate non-haematogenous cells be tested for viruses that are pathogenic in human subjects and are known to be harboured by the species from which the cells were originally derived. Caprine and ovine cells have been shown frequently to be contaminated with lentiviruses and "slow viruses" associated with subacute spongiform encephalopathies.

(*b*) Primary monkey kidney cells have been used to produce millions of doses of poliomyelitis vaccines over the past 20 years, and although latent viruses such as simian virus 40 were discovered in such cells, control measures were introduced to eliminate any risk

associated with the manufacture of vaccines in cells containing those endogenous viruses. Additional controls may be needed as new viral agents are identified.

(*c*) Continuous lines of non-haematogenous cells from human and non-human primates may contain viruses or have viral genes integrated into their DNA. In either case, virus expression may occur under *in vitro* culture conditions.

(*d*) Rodent and avian tissues and cells are well known to harbour viruses, but there is no evidence for transmission of disease to human beings by products from these sources. For example, large quantities of yellow fever, measles, and live influenza vaccines have been produced for many years in eggs that contain avian leukosis viruses, but there is no evidence that these products have had any harmful effects in their long history of use for human immunization. On the other hand, lymphocytic choriomeningitis virus and haemorrhagic fever viruses harboured by rodents have caused disease in humans by direct infection.

(*e*) Human diploid fibroblasts have been used for vaccine production for over 10 years, and although initial concern was expressed about the possibility of the cells containing a latent human virus, no evidence for such an agent has been found, and vaccines produced from this class of cell have proved to be safe.

Taking into account this classification of cells according to their potential for transmitting viruses pathogenic for human beings, the Study Group agreed that different degrees of concern, and therefore testing, were appropriate for products manufactured from the various types of cell mentioned.

Nevertheless, it was emphasized that when either diploid cell lines or continuous cell lines are used for production, a cell seed lot system must be used and the cell seed must be characterized as specified in the appropriate WHO Requirements. Efforts to identify viruses, viroids, and similar structures should constitute an important part of the characterization of such cell banks. For example, the possible presence of contaminating viroid-like RNA, such as the Delta agent, should receive attention and appropriate methods should be used for its detection.

When cell lines of rodent or avian origin are examined for the presence of viruses, the major emphasis in risk assessment should be placed on the results of studies in which transmission to target cells or animals is attempted. Risk to human recipients should not be

assessed *solely* on ultrastructural evidence for the presence of viral agents in the cells.

There may be as yet undiscovered microbial agents for which there is no current evidence or means of detection. However, during the long history of use of animals, tissues, and cells to manufacture products for human administration, the rare cases of viral contamination detected have all been due to activation of known latent viruses, either in the starting material or in the product, rather than to "new" agents that transmitted disease to human beings.

The Study Group stressed the importance of validating the ability of a manufacturing process to eliminate and to inactivate those viruses that may pose a risk to human beings when cells or cell lines carrying such viruses are proposed for use in the manufacture of biologicals for human administration. As with heterogeneous contaminating DNA, a wide margin of safety should be established in any inactivation or purification process. In addition, cells from human beings or animals with diseases of unknown origin and animal cells that may contain "slow viruses" should not be used to produce biologicals for human administration.

3.3.3 *Transforming proteins*

The apparent risk from oncogene-encoded proteins is limited to growth factors, since they are the only such proteins that can exert their biological effects via the cell surface. Growth factors may be secreted by cells used to produce biologicals, but the risks from growth factors are limited, since their growth-promoting effects are usually transient and reversible, they do not replicate, and many of them are rapidly inactivated *in vivo.*

Growth factors do not ordinarily appear to be oncogenic. Given the levels at which known growth factors are secreted by cultured cells, it would be necessary for them to be concentrated from the culture medium before they would be expected to be biologically active *in vivo*. In exceptional circumstances, however, growth factors can contribute to oncogenesis, but even in these cases, the tumours apparently remain dependent upon continued administration of the growth factor.

In summary, the Study Group did not consider that the presence of contaminating known growth factors in the concentrations at which they are ordinarily to be found constitutes a serious risk in the preparation of biological products from continuous cell lines.

3.4 General conclusions

Although it is possible to estimate an upper limit of contamination of a final product with heterogeneous DNA, and although all reported experiments have indicated that picogram amounts of such DNA are biologically inactive in a variety of tests, the *complete* absence of DNA or its associated risk cannot be claimed for products derived from continuous cell lines any more than for products derived from primary and diploid cell cultures. However, the probability of DNA from continuous cell lines or other cell systems inducing malignancy and other diseases is considered to be extremely low. In addition, there are well-documented and effective methods available for the preparation of safe products. For example, using appropriate purification and inactivation procedures, it has been possible to prepare a safe hepatitis B vaccine from the blood of infected patients, although this source of material carries very high risks. Many national control authorities have approved such hepatitis B vaccines because reliable data show that the manufacturing process has resulted in safe and effective products which meet WHO Requirements.

The importance of validating the efficiency with which various steps in a manufacturing process inactivate and/or eliminate unwanted material such as cellular DNA and viruses was emphasized. Validating the ability of a process to yield a product with certain specifications and establishing the consistency of that process are essential in providing the basis for an acceptable biological derived from continuous cell lines. Once a process has been validated and consistency of production has been established, limited tests appropriate for each product should suffice, as has been the usual practice with biologicals in the past. It was noted that this is the approach proposed in the WHO Requirements for hepatitis B vaccine produced by recombinant DNA techniques.

The Study Group concluded that, in general, continuous cell lines are acceptable as substrates for the production of biologicals, but that differences in the nature of the products and in the characteristics of the manufacturing processes must be taken into account in making a decision on the acceptability of a given product. There is, therefore, no reason to exclude continuous cell lines from consideration as substrates for biological products. In this regard, there was agreement with the actions taken to date by committees in approving the use of various products derived from continuous

cell lines only when they were satisfied that the manufacturing process in question yielded a product with no detectable risk attributable to the cell substrate.

In addition to those already available, a variety of other biologicals will eventually be produced in continuous cell lines. These can currently be grouped broadly into three classes: vaccines, biologically active proteins, and monoclonal antibodies. The dose and frequency of administration of these products may vary widely among the three major groups as well as within a group. This emphasizes the importance of taking into consideration the specific details of a product such as the dose, route of administration, and frequency of administration when assessing the ability of a manufacturing process to provide a safe product.

The risks from heterogeneous contaminating DNA were considered negligible for preparations given orally. For such products the principal requirement is the elimination of contaminating viruses and toxic proteins. If the manufacturing principles advocated by the Study Group for parenteral products are followed, the risk from oral administration of these products is greatly reduced.

4. RECOMMENDATIONS

1. WHO should promote establishment of several banks of cell seeds for continuous cell lines to assist Member States and manufacturers, when requested, in developing working cell banks that conform to WHO Requirements for the characterization of continuous cell lines for the production of biologicals.
2. WHO should take all possible steps to encourage the replacement of biologicals derived from neural tissues with biologicals derived directly from cell culture, including continuous cell lines, or made by recombinant DNA techniques.
3. WHO should encourage and coordinate studies to determine the effect of various inactivating agents, such as propiolactone and formalin, on the biological activity of DNA.
4. National control authorities should consider establishing multidisciplinary groups to assist them in assessing the safety and acceptability of substances produced by modern biological techniques, since the issues are often new and complex, and require the collective wisdom of experts from several different disciplines.

5. The Study Group recognizes that the matters considered in this report, especially the malignant transformation of human cells, are fast-moving fields of research. The Study Group therefore recommends that the WHO Secretariat should keep a close watch on further developments, and requests the Director-General to convene groups of scientists as appropriate.

5. ACKNOWLEDGEMENTS

The Study Group acknowledges the valuable contribution to its work made by the following staff members of the World Health Organization and the International Agency for Research on Cancer: Dr F. Assaad, Director, Division of Communicable Diseases, WHO, Geneva, Switzerland; Dr J. Dunne, Chief, Pharmaceuticals, WHO, Geneva, Switzerland; Dr J. Esparza, Microbiology and Immunology Support Services, WHO, Geneva, Switzerland; Dr Y. Ghendon, Microbiology and Immunology Support Services, WHO, Geneva, Switzerland; Dr V. Grachev, Biologicals, WHO, Geneva, Switzerland; Dr R. Montesano, International Agency for Research on Cancer, Lyon, France; Dr Y. Pervikov, Microbiology and Immunology Support Services, WHO, Geneva, Switzerland; and Dr P. Sizaret, Biologicals, WHO, Geneva, Switzerland.

Annex 1

HETEROGENEOUS CONTAMINATING DNA

The major risk associated with heterogeneous contaminating DNA in biological preparations intended for human use is connected with its potential pathogenic activity. After consideration of the data available and of calculations of the tumour-inducing potential of residual DNA concentrations, the Study Group concluded that there is negligible risk from heterogeneous contaminating DNA at a concentration of 100 pg or less per parenteral dose. This value is based on experimental results obtained in tests in which the pathogenic activity of DNA sequences was measured in susceptible animals. The following DNA sequences were examined:

—DNA of oncogenic viruses, including polyoma virus, simian virus 40, adenovirus, and Rous sarcoma virus;
—cloned genomic DNA from hepatitis B virus;
—chromosomal DNA from tumour cells and cloned mutant c-*ras* genes.

DNA of oncogenic viruses, including polyoma virus, simian virus 40, adenovirus, and Rous sarcoma virus

Viral DNA injected into experimental animals is weakly oncogenic (Table 1). The results can be summarized as follows:

Polyoma virus

Injection of 0.5–2 μg of polyoma virus DNA into newborn hamsters or rats resulted in the induction of tumours in 10–80% of the animals (*1–3*).

Simian virus 40

Subcutaneous injection of 1 μg or 2 μg of simian virus 40 DNA into newborn hamsters gave rise to sarcomas in 33–55% of the animals (*2, 4*).

Adenoviruses

Doses of 3–5 μg of simian adenovirus 7 DNA were reported to induce tumours in newborn and 21-day-old hamsters (*5, 6*). Injection

Table 1. Tumorigenicity of tumour virus DNA in animals

DNA		Animals tested	Route of injection	Tumour induction[a]	Reference
Source	Amount (μg)				
Polyoma virus	0.5	Newborn hamster	i.p.	10% (5/52)	*1*
	1	Newborn hamster	s.c.	11% (2/22)	*3*
	2	Newborn rat	s.c.	60% (33/55)	*2*
	0.2	Newborn rat	s.c.	22% (2/9)	*2*
	2[b]	Newborn hamster	s.c.	80% (4/5)	*2*
Simian virus 40	1–2	Newborn hamster	s.c.	33% (11/33)	*4*
	2	Newborn hamster	s.c.	55% (4/7)	*2*
	2[c]	Newborn hamster	s.c.	53% (9/17)	*2*
	1–10 (sub-genomic DNA)	Newborn hamster	s.c.	0% (0/131)	*11*
Adenovirus					
Simian adenovirus 7	3	Newborn hamster	s.c.	28% (7/25)	*5*
	5	Newborn hamster	s.c.	30% (24/82)	*6*
	2.5	Newborn hamster	s.c.	7% (4/59)	*6*
Human adenovirus 12	4	Newborn hamster	s.c.	4% (2/50)	*7*
Rous sarcoma virus (v-*src*)	2	Chicken	Wing web	65% (11/17)	*8*

[a] Percentage of animals in which tumours developed; actual numbers are given in parentheses.
[b] This plasmid expressed the polyoma middle T protein only. The latency periods for tumour induction were 5–10 times longer than with wild-type polyoma DNA. The middle T gene did not induce tumours in newborn rats.
[c] A combination of the genes for simian virus 40 large T antigen and for the polyoma small T antigen.

into newborn hamsters of 4 μg of human adenovirus 12 DNA or an equivalent amount of the cloned transforming DNA fragment resulted in tumour formation in 2 out of 50 animals (*7*).

Rous sarcoma virus

Injection of 2 μg of a subgenomic proviral DNA fragment of Rous sarcoma virus containing the viral *src* gene into the wing web of chickens caused tumours in 65% of the animals (*8*). These tumours regressed after several weeks.

These and other results indicate that DNA from oncogenic viruses is able to induce tumours in animals, but usually only when injected in doses between 1 and 10 μg.

Cloned genomic DNA from hepatitis B virus

Intrahepatic injection into chimpanzees of 5 μg of cloned DNA from hepatitis B virus caused hepatitis B, but intravenous injection of this DNA did not cause disease (*9*).

Chromosomal DNA from tumour cells and cloned mutant c-*ras* genes

Experiments with chromosomal DNA from tumour cells and with cloned activated *ras* genes have recently been conducted in several laboratories or are in progress (Table 2). So far, all tests have been negative, both with chromosomal DNA isolated from T24 bladder carcinoma cells and with the cloned activated H-*ras* or K-*ras* genes, at least during the observation periods used. The amounts of DNA injected varied from 10 to 500 μg for chromosomal DNA and from 2 to 50 μg for cloned activated *ras* genes (*3, 10*).

Calculations concerning DNA concentration and tumour-inducing potential

Experiments have shown that 2 μg of DNA from polyoma virus or simian virus 40 can induce tumours in about 50% of animals tested. If 2 μg of DNA is defined as the "tumour-inducing dose", then a residual amount of 100 pg of tumour virus DNA in a biological preparation would correspond to

$$\frac{100}{2 \times 10^6} = 0.5 \times 10^{-4} \text{ tumour-inducing dose.}$$

The contaminating DNA, however, normally consists of chromosomal DNA and not of pure viral DNA. If the chromosomal

Table 2. Tests for oncogenicity of cellular DNA containing an activated *ras* gene and of cloned activated *ras* genes[a]

DNA		Animals tested	Route of injection	Tumour induction[b]	Reference
Type	Amount (μg)				
T24 genomic DNA	10 (H-*ras*)	Newborn rat	s.c.	0% (0/20)	*10*
Rat EJ-6 genomic DNA[c]	500 (H-*ras*)	Newborn hamster	s.c.	0% (0/8)	*3*
Chromatin from T24 cells	~1000	Rhesus monkey	Intramuscular, i.v., intracerebral (single or multiple doses)	In progress	(Food and Drug Administration, USA)
Cloned activated H- or K-*ras* genes	2–10 (K-*ras*)	Newborn rat	s.c.	0% (0/10)	*10*
	2–10 (H-*ras*)	Newborn rat	s.c.	0% (0/14)	*10*
	50 (H-*ras*)	Newborn hamster	s.c.	0% (0/15)	*3*

[a] The observation periods in these tests did not exceed two years.
[b] Percentage of animals in which tumours developed; actual numbers are given in parentheses.
[c] Rat EJ-6 cells are a line of rat embryo cells transformed by the H-*ras* oncogene from EJ bladder carcinoma.

DNA contains an activated oncogene and there is only one copy per genome, the oncogene will represent only $1/10^6$ of the total DNA.[1] Then 100 pg of heterogeneous contaminating chromosomal DNA would contain $100 \times 10^{-6} = 10^{-4}$ pg of activated oncogene, which corresponds to

$$\frac{10^{-4}}{2 \times 10^6} = 0.5 \times 10^{-10} \text{ tumour-inducing dose.}$$

The risk associated with this amount of DNA is so small that it can be safely regarded as being negligible.

Several points should be considered in these risk estimations. Firstly, all calculations are based on the assumption that the risk factor for tumour induction decreases linearly with decreasing DNA concentration. This may not necessarily be correct, since an amount of DNA that has no measurable biological effect in a standard assay because it is present at too low a concentration may still have an effect under certain conditions or in certain organs or tissues. Secondly, it is not clear whether the risk associated with consecutive exposures to DNA will act in a cumulative way or not. Thirdly, the possibility should be considered that preparations of DNA that do not produce tumours in experimental systems may in human beings induce changes that could increase the incidence of tumours developing after long latent periods. Fourthly, experiments with short-lived animals do not permit assessment of the long-term effects of acquired DNA sequences.

Quantification of heterogeneous contaminating DNA or oncogenic sequences

Residual cellular DNA in biological products is generally quantified by nucleic acid hybridization techniques. For these tests the following probes have been used:

(*a*) Highly repetitive species-specific sequences that occur in high copy numbers in the cellular genome, such as the *Alu* sequences in human DNA.

(*b*) The specific gene sequences of interest and known to be present in the cell, such as those of a virus or of an oncogene.

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[1] If the amount of genomic DNA per cell has a mass of 10 pg and an oncogene measures 10 kilobases ($= 10^{-5}$ pg), then the oncogene will represent $1/10^6$ of the genome.

In addition it may be useful to have available a technique that can detect DNA in general, irrespective of its sequence.

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Annex 2

TRANSFORMING PROTEINS

Proteins encoded by cellular transforming genes (c-*onc*) or by their mutated versions can induce the proliferation of many different cell types. Some onc proteins exert their biological effects from outside the cell, but most are active only intracellularly. *Onc* genes were first detected as the transforming genes of acute transforming retroviruses (*1, 2*). The sequences of the cellular homologues (proto-*onc*) of retroviral oncogenes have been highly conserved during evolution, and are similar in invertebrates and human beings. Many proto-*onc* appear to possess little or no oncogenic potential. However, when inserted into a retrovirus vector, at least some inappropriately regulated proto-*onc* or genes carrying structural mutations have an oncogenic potential similar to that of the viral oncogenes.

The profound alterations in cell growth that these genes can induce have prompted the question of what might be the tumorigenic risk of contamination by oncogenes or their protein products when cell-derived materials are administered to human beings. What, then, are the theoretical and actual hazards that might arise from oncogene-encoded proteins? The potential problems raised by contamination with normal or mutated proto-*onc* themselves have been discussed previously (*3*). In this discussion, the term oncogenes will include any gene whose encoded product can directly stimulate cell proliferation. Under this broad definition it will be possible to discuss growth factors that could not be included if a more stringent definition were used (*4, 5*).

Theoretical risks from DNA versus protein

In addition to obvious chemical differences between nucleic acids and proteins, at least two important qualitative distinctions between genes and their products are directly relevant to their oncogenic potential. Malignant transformation is believed to represent a more or less irreversible change in the proliferative state of the cell. Inadvertent administration of oncogene DNA could in theory cause changes that mimic this process, if the gene were taken up by the cell, since stable acquisition of the DNA could lead to the constitutive expression of its encoded onc protein. The introduction of the

transforming gene may therefore cause an irreversible genotypic change in the cell, which results in the continuous exposure of the cell to the onc protein in a biologically relevant site, whether it is extracellular or intracellular. By contrast, the inadvertent administration of protein is theoretically less hazardous because, unlike DNA, it does not have the capacity for self-renewal. Its effects are therefore likely to be limited strictly by the amount that is administered, and will last only as long as the protein remains biologically active.

A second significant difference is that administered onc proteins can be active only if they exert their biological effects via the surface of the cell, since the uptake by the cell of onc proteins administered by standard techniques is inherently so inefficient that they could not, even theoretically, achieve high enough intracellular levels to be biologically active (*6*). This means that most onc proteins would not be potentially oncogenic as contaminants, since most of them are active as intracellular (as opposed to extracellular) proteins. Therefore only those onc proteins that are active extracellularly will be considered further. However, the general principles that follow should still be relevant to considering the potential hazards of onc proteins that would act intracellularly if administered under conditions favouring their cellular uptake in a biologically active form, for example within lipid-bound vesicles.

Growth factors

The onc proteins that can induce proliferation from outside the cell are usually referred to generically as peptide growth factors (*7*). The platelet-derived growth factor is the prototype for this group of molecules that can deliver an extracellular, growth-stimulatory signal (*8, 9*). This group also includes epidermal growth factor, the closely related transforming growth factor alpha, transforming growth factor beta, fibroblast growth factor, insulin, somatomedins (insulin-like growth factors), transferrin, and gastrin-releasing peptide (bombesin-like). Growth factors may also include molecules that initially were not believed to possess growth-promoting properties, such as tumour necrosis factor (*10*). Growth factors can be assayed quantitatively either biologically using susceptible cells or by specific radioimmunoassays.

The simian sarcoma virus is the only transforming retrovirus whose oncogene, v-*sis*, is known to encode a growth factor; v-*sis* is derived from one of the two genes for platelet-derived growth factor.

In contrast to the intracellularly active protein products of several cellular transforming genes, no activated versions of platelet-derived growth factor or other growth factors with increased growth-promoting properties (compared with the normal versions) have been described. In particular, v-*sis* does not have a higher transforming activity than its cellular proto-*onc*. Growth factors such as platelet-derived growth factor may, under physiological circumstances (as in wound healing or clot formation following wound healing), be present in sufficiently high concentrations locally to induce cell growth in response to injury. It is important to note that the effects of growth factors are reversible and that these factors are not known to be specifically mutagenic. Inadvertent administration of growth factors as contaminants would represent exposure to products that are expressed by normal cells under certain physiological conditions. Tumours might arise only if "pharmacological" (i.e., non-physiological) doses were given repeatedly, and such tumours would presumably stop growing when the growth factor was no longer administered.

Each growth factor transmits its growth signal via specific receptors located on the cell surface. Therefore, a growth factor can act only on those cells that display the appropriate receptor. Receptors for many growth factors are found in only a subset of cells, although receptors for transforming growth factor beta are virtually ubiquitous in their distribution; this wide distribution among many different cell types may be related to the many apparent functions of transforming growth factor beta, which apparently stimulates cell growth under some circumstances and inhibits it under others.

As noted above, growth factors are synthesized physiologically by normal cells. Their synthesis may be elevated in various pathological states, and many tumour cells constitutively synthesize large amounts of various growth factors (*11*). The expression of certain oncogenes whose proteins act intracellularly has been shown to stimulate the production of secreted growth factors. The inappropriately elevated secretion of growth factors by tumour cells that display cell-surface receptors for those factors forms the basis for the hypothesis that some tumours persist via an autocrine mechanism. Increased levels of growth factor receptors, as reported for some tumours, may render the tumour cells more sensitive to growth induction by growth factors via paracrine or autocrine mechanisms (*12*).

Oncogenic potential of growth factors

It does not appear that growth factors, either singly or in combination, can by themselves induce the fully tumorigenic phenotype in primary cells. However, a combination of growth factors can induce established non-neoplastic rodent cells to become fully anchorage independent for growth, a phenotype that is often highly correlated in fibroblasts with tumorigenicity (*13*). Single growth factors can induce anchorage independence and tumorigenic growth of some established cells (*14*).

Oncogenic effects of growth factors have been demonstrated *in vivo,* but only under exceptional circumstances. In a strain of mice with a high incidence of mammary tumours whose continued growth depends in part on epidermal growth factor, surgical removal of the salivary glands, which are the major source of endogenous epidermal growth factor, led to a significant reduction in the incidence of mammary tumours (from 62% to 12%; *15*). Administration of "pharmacological" amounts of epidermal growth factor (5 μg per animal) every other day partially restored (to 33%) the normal tumour incidence. The results confirm that in this strain of mice tumour formation depends in part on epidermal growth factor. In another experiment, co-inoculation into a nude mouse of estrogen-independent and estrogen-dependent cells from mouse mammary tumours rendered the latter cells hormone independent, probably because of the production of autostimulatory growth factors (*16*).

In the above examples, growth factors were tumorigenic under circumstances in which the animals presumably contained markedly abnormal cells; the addition of growth factors probably represented only one of several important changes that resulted in tumorigenesis. The oncogenic potential of the simian sarcoma virus appears to be extremely limited, despite the constitutive expression of v-*sis* in infected cells; when the virus does induce tumours, they are slowly growing fibroblastic masses that do not appear to be locally invasive, nor do they metastasize (*17*).

Quantitative considerations

Although some growth factors may be bound specifically to serum proteins, most have an extremely short half-life in the blood (2 min for platelet-derived growth factor). A short half-life markedly limits potential *in vivo* effects. *In vivo* administration of transforming growth factor alpha to newborn mice (which are highly susceptible to it) at daily subcutaneous doses of greater than 0.3 μg/g of body

weight accelerated incisor eruption and eyelid opening (known biological effects of epidermal growth factor), but doses greater than 0.3 μg/g of body weight did not have any growth-promoting effects (*18*). Usually 0.1–20 ng/ml of a growth factor are required for significant growth-promoting effects on responsive cells *in vitro,* although some factors, such as fibroblast growth factor, may be active at concentrations of several picograms per millilitre.

Even those cells that secrete large quantities of growth factors produce concentrations of no more than 50–75 ng/ml of a given growth factor in the culture medium. Because of dilution in body fluids, it would be almost impossible for administration of unconcentrated supernatant material to have any detectable oncogenic activity *in vivo*. Potential problems might arise, however, if growth factors were concentrated along with the product of interest. It seems reasonable to conclude provisionally that doses of growth factor at the level of 10 μg/kg of body weight daily would be without oncogenic effect, even in susceptible individuals. The murine mammary tumour system (in conjunction with salivary gland removal) might be a fruitful one to study further with different doses of epidermal growth factor or transforming growth factor alpha to obtain more definitive information on the risks of *in vivo* administration to a highly susceptible animal.

Summary and conclusions

The theoretical risk from oncogene-encoded proteins is limited to growth factors, which may be secreted by cells in which biological products might be manufactured. Because these peptides do not replicate, their effect is finite. In addition, their effects are reversible. Growth factors do not ordinarily appear to be oncogenic. Even under circumstances in which they contribute to oncogenesis, repeated administration of high concentrations of growth factors (several micrograms per kilogram) appears to be required for them to serve as cofactors in the carcinogenic process, and the resulting tumours appear to remain dependent upon the continued presence of the growth factor for their growth.

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