Quantitative Assessment of the Risks of Residual Cell-Substrate DNA

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Types of Perceived Risks Associated with Residual Cell-Substrate DNA,

Oncogenic Risk

Potential Risk of Infectivity Following Inoculation of Cell-Substrate DNA

- Cell-substrate DNA may encode an infectious genome
  - DNA virus (e.g., polyomavirus, herpesvirus, papillomavirus)
  - Provirus of a retrovirus (e.g., HIV, HTLV)

WHO Requirements for Residual Cell-Substrate DNA Levels

- WHO Study Group, Geneva, 1986
  Residual DNA from continuous cell lines: 0.1 ng or less

- WHO Expert Committee on Biological Standardization, 1998
  Residual DNA from continuous cell lines: 10 ng or less

Proposed Mechanisms of Oncogenic Risk by Cell-Substrate DNA

- Introduction of a Dominant Activated Oncogene.
- Insertional Mutagenesis
  - Disruption of a Cellular Tumor Suppressor Gene
  - Activation of a Cellular Dominant Oncogene (Promoter/Enhancer Insertion)
- DNA Methylation Pattern Changes Following Integration

Nature of DNA Inoculum

- Linear versus Circular
- Single-Stranded versus Double-Stranded
- State of DNA - Free versus Chromatin
- Size Distribution of DNA
Routes of DNA Inoculation

- Intramuscular (i.m.)
- Intradermal (i.d.)
- Subcutaneous (s.c.)
- Intranasal (i.n.)
- Oral
- [Intraperitoneal (i.p.)]

Assumptions for DNA Activity (1)

- For a given DNA, the level of the response of a cell to that DNA is proportional to the amount of that DNA.
- The activity of a gene integrated in chromosomal DNA or as part of plasmid or phage vector is equivalent or lower.
- The amount of uptake of a given gene by a cell and the expression of this gene in the cell is related to the concentration of the gene in the DNA.

Assumptions for DNA Activity (2)

- Because a single-copy gene is represented at approximately one millionth of the haploid mammalian genome, the amount of DNA corresponding to a single-copy gene is 10^6-fold less abundant for equivalent amounts of cellular DNA compared with plasmid DNA with the same gene.
- For the uptake and expression of a gene in mammalian genomic DNA equivalent to 1 pg of plasmid DNA, 1 x 10^6 pg or 1 g of mammalian DNA is needed to elicit an equivalent biological effect.
- Conversely, if a single oncogene is present in 1 pg of mammalian DNA, the equivalent amount of the same oncogene if cloned in a plasmid vector is 1 x 10^4 pg, or 1 x 10^3 ng (1 pg).

Oncogenicity of ras DNA in Mice

Burns et al. (1991)

- Activated H-ras (T24) gene (10 pg) inoculated by scarification of mouse skin
- Lymphangiosarcomas developed in 33/34 animals within 12 months; usually within 12 weeks
- Normal-c-ras failed to induce tumors (0/10 animals)

Conclusion

10 pg (1.1 x 10^12 molecules) of activated ras is oncogenic in adult mice

Oncogenicity of src DNA in Chickens

Fung et al. (1993)

- Cloned RSV DNA (2 pg) induced tumors in 6/6 chickens inoculated s.c. in their wing-web
- Cloned c-src DNA (2 pg) induced tumors in 7/10 chickens inoculated s.c. in their wing-web

Halpem et al. (1990)

*Cloned v-src DNA (20 pg) induced tumors in chickens
52/60 (87%) inoculated s.c. in their wing-web
8/36 (22%) inoculated i.v.

Conclusion

2 pg (2.5 x 10^11 molecules) of cloned v-src oncogenic in chickens

Oncogenicity of Polyoma Virus DNA in vivo

- Polyoma virus DNA in newborn hamsters
  - I.p. 0.5 μg supercoiled 5/52 (15%)
  - s.c. 0.5 μg supercoiled 14/73 (19%)
  - s.c. 0.5 μg linear 29/164 (45%)
- Cloned polyoma virus DNA in newborn hamsters
  - s.c. 0.5 μg supercoiled 11/20 (55%)
  - s.c. 2 μg supercoiled 0/31 (0 %)
  - s.c. 2 μg linear 33/55 (60 %)
  - s.c. 0.2 μg linear 2/9 (22%)

Conclusion

0.2 pg (1.9 x 10^10 molecules) of polyoma virus DNA is oncogenic in newborn hamsters
**In vivo Infectivity of Cloned Viral Genomes - Retroviruses (1)**

<table>
<thead>
<tr>
<th>Viral DNA</th>
<th>Dose/Route</th>
<th>Infected/Total Genomes for Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIV(Has)</td>
<td>200 µg i.m.</td>
<td>3/1 6.1 x 10^11</td>
</tr>
<tr>
<td>SIV(Rup)</td>
<td>500 µg i.m.</td>
<td>3/3 3.5 x 10^10</td>
</tr>
<tr>
<td>SIV(Puma)</td>
<td>300 µg i.m.</td>
<td>2/2 2.3 x 10^11</td>
</tr>
<tr>
<td>BLV(W6/sL)</td>
<td>10 µg DOTAP i.d.</td>
<td>6/5 5.5 x 10^11</td>
</tr>
<tr>
<td>CAEV(Suza)</td>
<td>100 µg DOTAP i.d.</td>
<td>1/1 5.3 x 10^11</td>
</tr>
<tr>
<td>FcLV(Rag-Human)</td>
<td>50 µg DOTAP i.d.</td>
<td>12/13 3.6 x 10^12</td>
</tr>
<tr>
<td>FIV(Schwe)</td>
<td>30 µg i.d.</td>
<td>1/3 6.4 x 10^12</td>
</tr>
<tr>
<td>FIV(Schwe)</td>
<td>50 µg i.m.</td>
<td>2/2 3.1 x 10^12</td>
</tr>
<tr>
<td>FIV(Schwe)</td>
<td>100 µg i.m.</td>
<td>3/3 7.8 x 10^11</td>
</tr>
<tr>
<td>FIV(Schwe)</td>
<td>500 µg i.m.</td>
<td>3/3 2.1 x 10^11</td>
</tr>
</tbody>
</table>

**Summary of in vivo Infectivity with Cloned Viral Genomes**

- Retroviruses: 15-500 µg i.m., 1.1 x 10^12 - 2.3 x 10^13 genomes
- Polyoma Virus: 5 x 10^5 µg - 1 µg s.c., 1.3 x 10^7 genomes

**Conclusions**
- Infectivity of different retroviral DNAs is similar
- Depending on the route of inoculation, 15 µg can be infectious
- Infectivity of polyoma virus DNA is higher (ca. 50 pg)

**Activity of DNA Administered Intranasally**

- Influenza virus HA gene DNA vaccine inoculated intranasally
- Different amounts of DNA were administered in PBS (10 µL)
- After 4 weeks, a second intranasal inoculation of 1.5 µg was given

<table>
<thead>
<tr>
<th>H-ADN A</th>
<th>Immune Response</th>
<th>Primary Inoculation</th>
<th>Secondary Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µg</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>0.02 µg</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>0.4 µg</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>6 µg</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

**Conclusion**

Even 0.02 µg DNA (i.e., 20 ng) administered intranasally elicits an immune response, since a secondary inoculation triggers the response. Therefore, 20 ng of DNA can be biologically active when administered intranasally.

**In vivo Infectivity of Cloned Viral Genomes - Retroviruses (2)**

<table>
<thead>
<tr>
<th>DNA</th>
<th>DNA/Dose/Route</th>
<th>Infected/Total Genomes for Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murine FrCas 3</td>
<td>38 µg i.p</td>
<td>16/16 2.7 x 10^12</td>
</tr>
<tr>
<td>0 µg</td>
<td>OH 4</td>
<td>0.0005</td>
</tr>
<tr>
<td>38 µg i.p.</td>
<td>9/12</td>
<td>1.6 x 10^12</td>
</tr>
<tr>
<td>3.8 µg i.p.</td>
<td>7/10</td>
<td>3.9 x 10^7</td>
</tr>
<tr>
<td>0.38 µg i.p.</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>0.038 µg i.p.</td>
<td>0/6</td>
<td>0/6</td>
</tr>
</tbody>
</table>

**Comparison of Oncogenicity and Infectivity**

<table>
<thead>
<tr>
<th>DNA</th>
<th>Oncogenicity</th>
<th>Infectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyoma Virus</td>
<td>0.2 µg (1.3 x 10^5+µg)</td>
<td>ID, 1.3 x 10^4+µg (2.3 x 10^7 genomes)</td>
</tr>
<tr>
<td>SV40</td>
<td>1 ID (1.7 x 10^11 genomes)</td>
<td>ND</td>
</tr>
<tr>
<td>Retroviruses</td>
<td>NR</td>
<td>15 - 30 µg</td>
</tr>
<tr>
<td>v-src</td>
<td>2 µg (2.5 x 10^11 molecules)</td>
<td>NR</td>
</tr>
<tr>
<td>Activated ras</td>
<td>10 µg (9.1 x 10^11 molecules)</td>
<td>NR</td>
</tr>
</tbody>
</table>

**Activity of DNA Administered via the Oral Route (1)**

<table>
<thead>
<tr>
<th>Polyoma Virus DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice fed polyoma virus DNA (1 µg or 0.5 µg)</td>
</tr>
<tr>
<td>0/25 became infected with 1 µg</td>
</tr>
<tr>
<td>0/30 became infected with 0.5 µg</td>
</tr>
<tr>
<td>Mice administered polyoma virus DNA (1 µg or 0.5 µg) through a gastric tube</td>
</tr>
<tr>
<td>S/10 became infected with 1 µg</td>
</tr>
<tr>
<td>-11/16 became infected with 0.5 µg (ID, 0.81 µg)</td>
</tr>
</tbody>
</table>

**Conclusion**

Unlike that small amounts of DNA will survive passage through stomach intact.
Activity of DNA Administered via the Oral Route (2)

<table>
<thead>
<tr>
<th>Phage DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice fed M13 DNA (50 μg); fate of DNA followed</td>
</tr>
<tr>
<td>DNA detected in: feces (size 100 bp - 1700 bp; M13 DNA 7.25 kb)</td>
</tr>
<tr>
<td>blood (size 194 bp - 976 bp)</td>
</tr>
<tr>
<td>small intestine</td>
</tr>
<tr>
<td>large intestine</td>
</tr>
<tr>
<td>leukocytes (size &lt;712 bp); found in 0.1% cells</td>
</tr>
</tbody>
</table>

Conclusions
- Small amounts of DNA can pass through the stomach
- No full-length DNA detected
- DNA can be found integrated into the mouse chromosome

Estimates of DNA Risk: Oncogenicity

Cloned Activated Oncogene
For a single dominant activated oncogene per cell, 1 μg cell DNA has 152,000 oncogenes (DNA in 152,000 cells is 1 μg)

Number of molecules required for oncogenicity:
- polyoma virus DNA 3.6 x 10^10
- activated ras DNA 9.1 x 10^11

Therefore, the number of tumors expected from 1 μg of residual cell DNA with a single dominant oncogene per cell is between:
- 152,000/3.6 x 10^10 = 4.2 x 10^-6
- 152,000/9.1 x 10^11 = 1.7 x 10^-7

Therefore, for 10 ng DNA, the probability of an oncogenic event is between:
- 1 in 2 x 10^8 (polyoma virus) and 1 in 6 x 10^8 (act. ras)

Conclusions from Estimates of DNA Risk
- Infectious risk of DNA can be more important than oncogenic risk
- i.m. and s.c. routes
  - 10 ng DNA provides an estimated risk:
    - Polyoma virus DNA: 1 in 2 x 10^7 for oncogenic event
    - 1 in 7 x 10^7 for infectious event
    - Activated ras DNA: 1 in 6 x 10^7 for oncogenic event
    - 1 in 3 x 10^8 for infectious event
- Oral route
  - 10 ng DNA provides an estimated safety margin of:
    - 1 in 10^6 for an 'expression' event
- Oral route
  - 1 μg polyoma virus DNA administered orally is not infectious;
  - therefore, for 10 ng of mammalian DNA with 1 copy of polyoma virus DNA, the safety margin is at least 1 in 10^8

Estimates of DNA Risk: Infectivity

Polyoma Viral DNA
Viral genomes required for infection: 1 x 10^7
The probability of an infectious event for 10 ng of mammalian DNA with a single copy of polyoma virus DNA is:
- 1 in 2 x 10^8

Retrovirus Cloned Proviral DNA
Viral genomes required for infection: 4 x 10^11
The probability of an infectious event for 10 ng of mammalian DNA with a single provirus is:
- 1 in 3 x 10^9

Potential Mitigating Factors Regarding DNA Risks
- Uptake, expression, and integration are inefficient processes
  - integration requires cell division
  - integration required for maintenance of DNA
- Degradation of DNA
  - Vaccine manufacture procedure
  - in vivo
- Host immune response to transfected cells.
- Multistep nature of carcinogenesis
- Transformation of human cells is more difficult than transformation of rodent cells

CBER Activities to Address the Risks of Residual Cell-Substrate DNA

CBER/NCI/CDERDNA Oncogenesis Study
- To develop sensitive animal models to detect oncogenic activity of DNA
  - newborn NIH Swiss anh C57Bl6 mice, athymic (nude) mice, and KG/ODC mice
  - myc and activated ras genes

CBER DNA Infectivity Study
- To develop quantitative in vitro assays to assess:
  - the infectivity of proviral DNA of retroviruses
  - the integrated and extrachromosomal genomes of DNA viruses