Title: Spontaneous Integration of Human DNA Fragments into Host Genome

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Introduction

A trio of recent publications in the journal NEURON reports the presence of hundreds of diverse de novo gene mutations indicating that autism spectrum disorder (ASD) may be a disease of genomic instability, with a significant environmental component. Altered double strand break formation and repair pathways (DSB) may be a commonality among the diverse genetic mutations that have been documented in ASD. US birth year change points in AD are apparent in 1980, 1988 and 1996, coinciding with the switch to or introduction of childhood vaccines contaminated with human endogenous retrovirus K (HERVK) and human fetal DNA fragments (6). We hypothesize that the HERVK and human fetal DNA contaminants could contribute to the genomic instability of ASD as demonstrated by de novo mutations.

Cell free DNA can be taken up by healthy cells via receptor mediated uptake or may spontaneously penetrate cell membranes that have altered permeability, for instance, during inflammatory reactions. Nuclear uptake of cell free DNA fragments is thought to provide a source for maintenance of DNA integrity during rescue of collapsed replication forks or base lesion repair. Spontaneous extracellular DNA uptake has also been exploited for gene therapy as well as for cellular gene correction (2,4,5,7,8, and 9). While free DNA uptake has been used advantageously, the process has also been associated with generation of mutations and chromosomal aberrations (3).

Vaccines manufactured using human fetal cells contain residual DNA fragments (50-500 bp) (Table I). It is possible that these contaminating fragments could be incorporated into a child's genome and disrupt normal gene function, leading to autistic phenotypes. In this study we demonstrate foreign DNA uptake in human cells and genomic integration by incubating the cells with Cy3-labeled human Cot1 (placental) DNA fragments which represents contaminating residual human fetal DNA in vaccines.

Table 1. Levels of residual human double stranded DNA (Picogreen assay) and human single stranded DNA (Oligreen assay) in Rubella vaccine (MeruvaxII) and Hepatitis A vaccine (HAVRIX).

Vaccine name	Double Stranded DNA (ng/vial)	Single Stranded DNA (ng/vial)	Length (bps)
Meruvax II (Rubella)	142.05	35.00	240
HAVRIX (Hepatitis A)	276.00	35.74	Not measurable

Materials and Methods: Human Cot1 DNA (Invitrogen) was labeled with Mirus *Label* IT Cy^{TM}3 *Labeling Kit* (Mirus).

U937 cells (monocytes) were grown in Dublecco's Mofication of Eagle's Medium (DMEM) supplemented with 15% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution at 37°C under a humidified atmosphere containing 5% CO^2/95% air. HL-60 cells (myeloblast) were grown in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% FBS and 1% antibiotic-antimycotic solution at 37°C under the same condition. 750ng of Cy3 labeled Human Cot1 DNA was incubated per1.0×10^7 cells for 24 hours and 48 hours.

Cellular and nuclear DNA uptake was analyzed under fluorescent microscope. Genomic DNA of U937 cells was purified by ethanol precipitation removing short fragment of nucleic acids including unincorporated Cy3 labeled Human Cot1 DNA. The amount of Cy3 labeled human Cot1 DNA incorporated into U937 chromosomes was calculated with relative fluorescent unit (RFU) measured by a fluorimeter.

Loosely adherent NCCIT (teratocarcinoma) cells were grown with a cell density 3×10^4 per well of a 24-well plate which a German glass cover slips was placed in each well at 37°C under a humidified atmosphere containing 5% CO^2/95% air. HFF1 (Human Foreskin Fibroblast 1) cells were grown with the same condition except DMEM supplemented with 15% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution was used as a medium.

Methods and Results

BE (2)-C (neuroblastoma) cells were grown in the same condition except medium used was a 1:1mixture of Eagle's Minimum Essential Medium (EMEM) and F12 Medium supplemented with 10%FBS and 1% antibiotic-antimycotic solution. M059K (Glioblastoma-Double Stranded Break repair proficient) and M059J (Glioblastoma-Double Stranded Break repair deficient) were also grown with the same condition except the medium used was a 1:1 mixture of DMEM and Ham's F12 Medium supplemented with 10% FBS, 0.05mM non-essential amino acids, and 1% antibiotic-antimycotic solution. After cells were cultured in each condition for 2 to 3 days 500ng Cy3 labeled Human Cot1 DNA was added and incubated at 37°C under a humidified atmosphere containing 5% CO^2/95% air by gently shaking for 24 hours and 48 hours. After incubation nucleus was stained with Hoechst, German glass cover slips were placed on glass slides, and cellular and nuclear DNA uptake was analyzed under fluorescent microscope.

To model inflammation, all adherent cell lines were activated with lipopolysaccharide (LPS). And, saponin permeabilization was also tested for HFF1 cells . Three concentrations of LPS, $1ng/10^4$cells, $10ng/10^4$cells, and $100ng/10^4$cells were tested in the wells of each cell line previously mentioned. Cells were incubated with Cy3 labeled Human Cot1 DNA and LPS at 37°C under a humidified atmosphere containing 5% CO^2/95% air by gently shaking for 24 hours and 48 hours. As well as cells incubated without LPS, these cells were also stained with Hoechst before cellular and nuclear DNA uptake was analyzed under fluorescent microscope.

HFF1 cells were incubated with 0.02% saponin , 300ng DAPI, and 500ng Cy3 labeled human Cot 1 DNA for 24 hours, 48 hours, and 72 hours. Cells were viewed under fluorescent microscope as well.

Results (Table 2):

Spontaneous cellular and nuclear DNA uptake was evident in HFF1, NCCIT and U937 (Fig1, 3, 7 and 8). DNA uptake in BE (2)-C and M059K was not measurable because of high auto fluorescence of the cells. No Cy3 signal was observed in HL-60. With inflammation caused by LPS cellular DNA uptake was observed in HFF1, NCCIT, M059J, and U937 (Fig 2, 4, 5 and 6).

The amount of labeled Cy3 human Cot1 DNA incorporation in U937 genomic DNA was 0.0111 +/- 0.0034pg (n=12) per cell in 24 hours, which was approximately 0.167% of total U937 genomic DNA. DNA incorporation in NCCIT cells was 0.0026pg/cell in 24 hours and 0.04pg/cell in 48 hours which is 0.6% of total NCCIT genomic DNA.

Table 2: DNA uptake in Various Cell lines

	Spontaneous Cellular uptake	Spontaneous Nuclear uptake	Incorporation in Genomic DNA	Cellular /Nuclear Uptake with LPS or saponin
HFF1	Yes	Yes	Not Done	Increase/Increase
NCCIT	Yes	Yes (variable)	0.0026pg per cell 24 hrs 0.04pg per cell 48 hrs	Same/Same
BE(2)-C	No	No	Not Done	No/No
M059K	No	No	No	No/No
M059J	No	No	Not Done	Yes/No
U937	Yes	Yes	0.011 +/- 0.003pg per cell 24 hrs	Same/Same
HL60	No	No	No	No

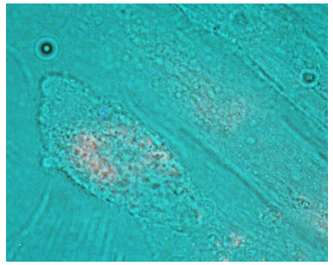

Fig 1. HFF1 spontaneous cellular and nuclear DNA uptake (bright field & Cy3 red combined).

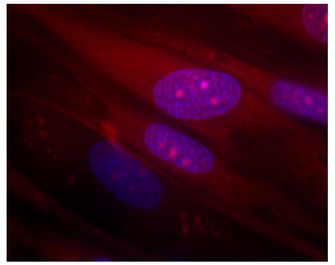

Fig 2. HFF1 cellular and nuclear DNA uptake after permeabilization with saponin. (Cy3 red & nucleus blue combined)

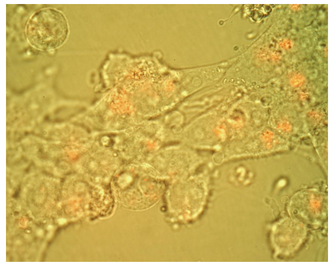

Fig 3. NCCIT spontaneous cellular DNA uptake (bright field & Cy3 red combined)

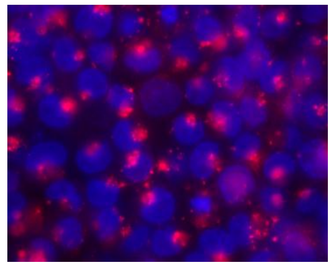

Fig 4. NCCIT cellular DNA uptake after lipopolysaccharide activation (Cy3 red & nucleus blue combined)

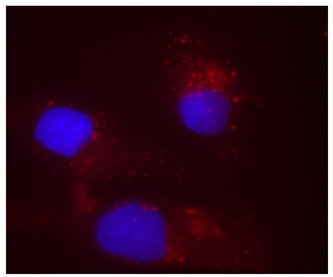

Fig 5. M059J cellular DNA uptake after lipopolysaccharide activation ($10ng/10^4$ cells).
(Cy3 red & nucleus blue combined).

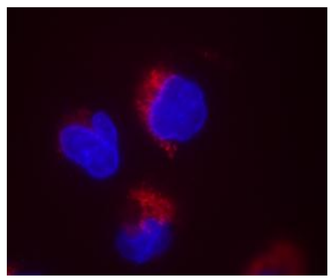

Fig 6. M059J cellular DNA uptake after lipopolysaccharide activation ($100ng/10^4$ cells).
(Cy3 red & nucleus blue combined).

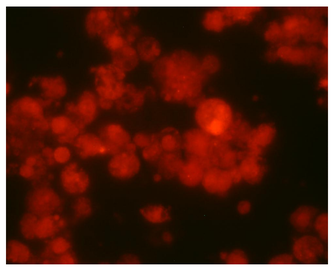

Fig 7. U937 spontaneous cellular/nuclear DNA uptake (Cy3 red)

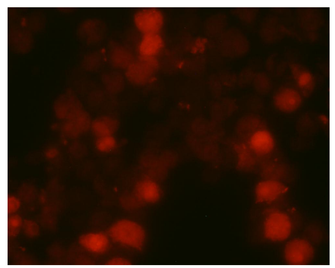

Fig 8. Purified U937 **nuclei** containing Cy3 labeled DNA before DNA purification (Cy3 Red)

Discussion

Our measured genomic incorporation (0.003 to 0.04 pgs) of 0.2% - 0.6% of the whole genome in 24 to 48 hours seems high at first glance. However, our numbers are consistent with previous reports showing that exogenous DNA replaced up to 1% of the whole genome within 30 minutes (6). Although HL-60 cells did not spontaneously take up exogenous DNA in our experiments, the cell line has been used in the past as a model for spontaneous DNA uptake (8).

Cellular and nuclear DNA uptake in human foreskin fibroblast (HFF1) cells and in NCCIT cells suggests that embryonic and neonatal cell are more susceptible to DNA uptake than cells from a more mature source. These results indicate the need for further study of DNA incorporation from exogenous sources to compare the susceptibility of infants and toddlers versus teens and adults.

Increased DNA uptake after LPS activation suggests that systemic inflammation or immune responses could increase susceptibility for exogenous DNA uptake. Human diploid cell produced vaccines are contaminated by exogenous DNA fragments and a retrovirus, and vaccines elicit systemic inflammation and immune activation. Our future research goals are to localize the sites of DNA integration, to demonstrate phenotype changes caused by foreign DNA integration in factor dependent cell lines, and to determine the biological and/or pathological activities of Human Endogenous Retrovirus K (HERVK) fragments in vaccines.

Table3: Cell Description

Cells	Source	Morphology	Transfection host
U937	Histocytic Lymphoma	Monocyte	Yes
HL60	Leukemia	Myeloblast	Yes
BE(2)C	Neuroblastoma	Neuroblast	No
M059K	Glioblastoma	Fibroblast	No
M059J	Glioblastoma	Fibroblast	No
HFF1	Foreskin	Fibroblast	No

Conclusion

Not only damaged human cells, but also healthy human cells can take up foreign DNA spontaneously. Foreign human DNA taken up by human cells will be transported into nuclei and be integrated into host genome, which will cause phenotype change. Hence, residual human fetal DNA fragments in vaccine can be one of causes of autism spectrum disorder in children through vaccination. Vaccine must be safe without any human DNA contaminations or reactivated viruses, and must be produced in ethically approved manufacturing processes.

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