RELEVANCE OF STX2-PHAGE IN THE DEVELOPMENT OF HEMOLYTIC UREMIC SYNDROME (HUS)

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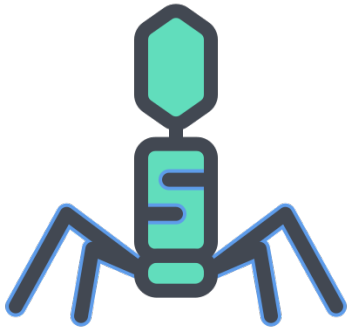
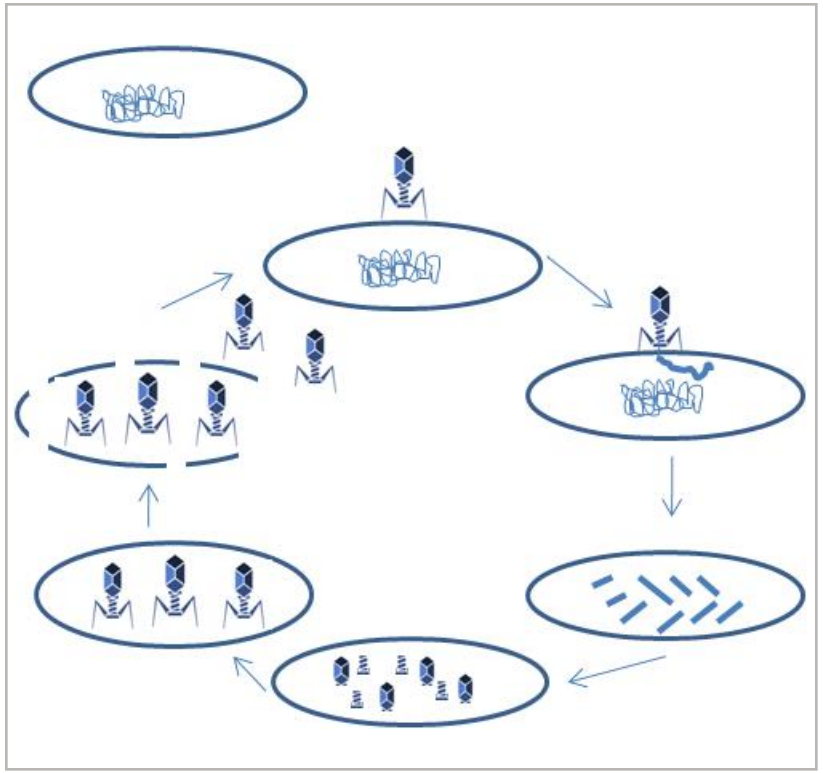
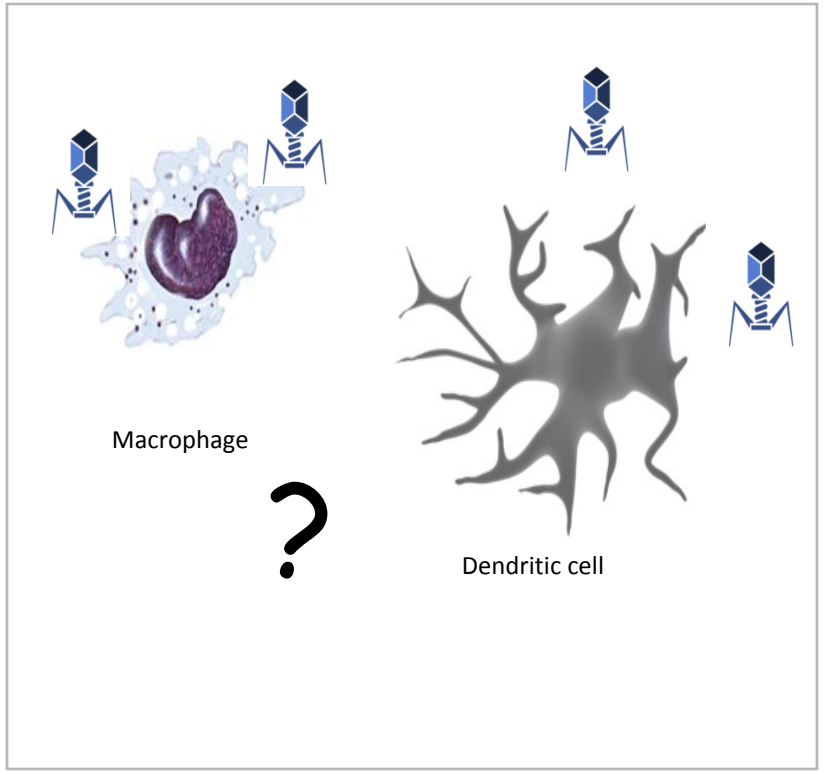


BACTERIOPHAGES AND EUKARYOTIC CELLS

Bacteriophages: bacterial virus



What is happening in eukaryotic environment?



BACTERIOPHAGES AND EUKARYOTIC CELLS

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Bacterial Virus Gene Expression in Human Cells

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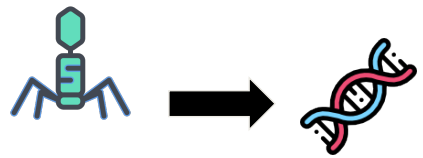
Human fibroblasts, from a patient with congenital lack of α-D-galactose-1-phosphate uridyl transferase activity, have been infected with transducing bacteriophage that harbours either wild type or defective transferase gene. Infection only by the former phage initiates transferase synthesis.

Gal-1-P it is possible to follow this reaction (Fig. 2). This activity was linear with time in the conditions used and proportional to the amount of cell lysate used (Fig. 3). All activity was lost by boiling the cell lysate. Uninfected galactosemic cells had an activity ≤0.2 nmol of UDP-Gal/60 min/mg of protein (Table 1). No difference in enzyme activity could be detected between uninfected galactosemic fibroblasts and fibroblasts infected with λ virus or λpgal T(⁻), with a mutation in transferase structural gene. The λpgal T⁺ DNA-infected cells had enzyme levels which were generally greater than the λpgal T⁺ whole virus-infected cells. Infected cell cultures have

VIROLOGY 47, 638–643 (1972)



Lambda Phage Transcription In Human Fibroblasts

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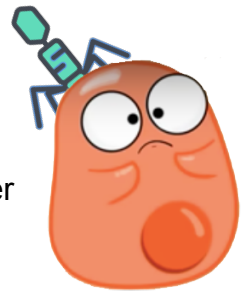
Accepted October 27, 1971

Lambda bacteriophage was used to infect human fibroblasts and viral specific RNA was detected by hybridization with lambda DNA. A correlation was found between the amount of virus used in each infection and the percentage of lambda-specific RNA produced. The percentage of lambda-specific RNA (0.2%) reached a maximum after 4 days. Cells monitored for 41 days following infection continue to produce lambda-specific RNA.

Is able to express genes under eukaryotic promoter



So, once inside the cell, host cell is able to express genes under eukaryotic promotors

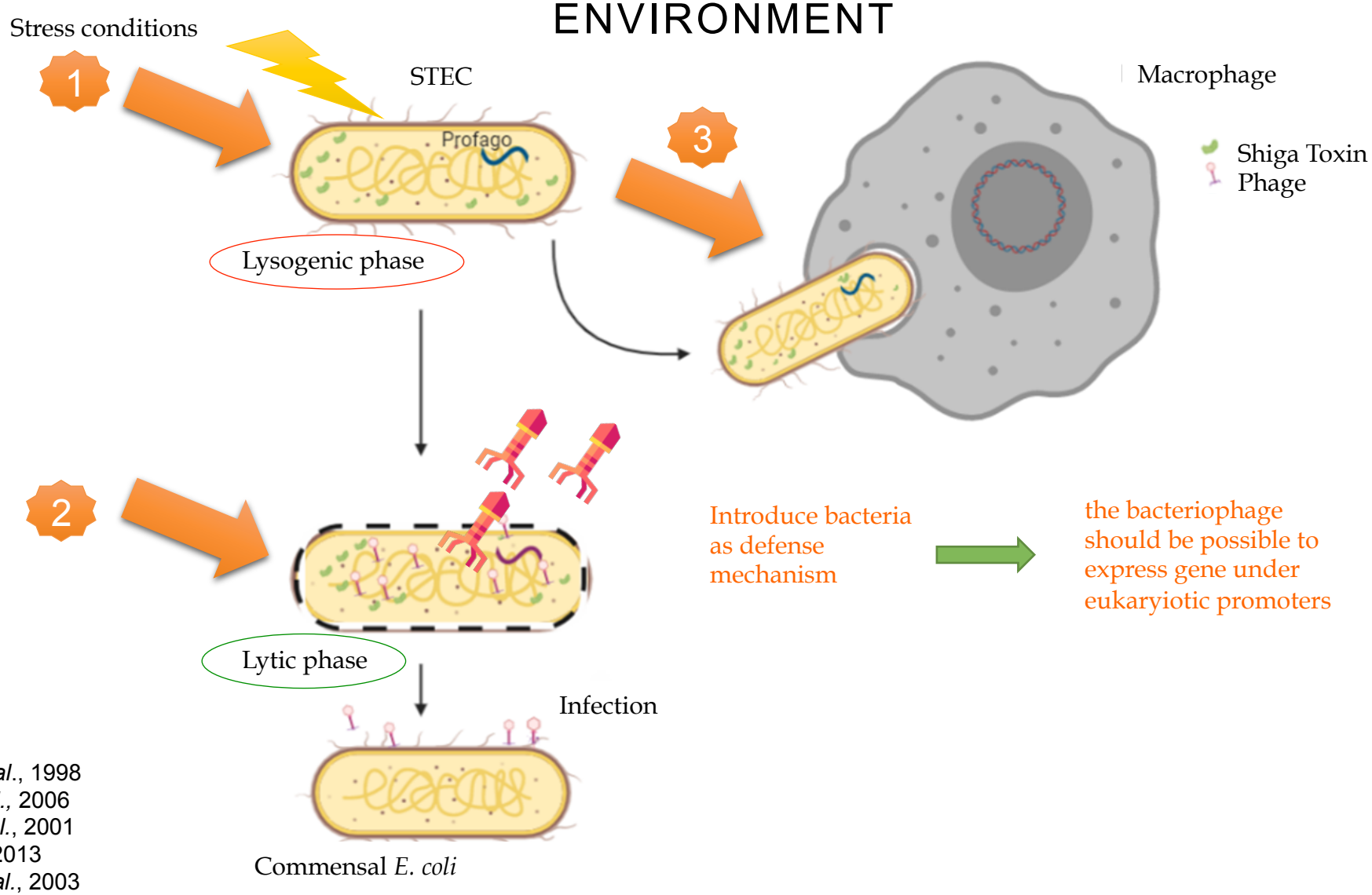
PREVIOUS IDEAS

THAT WE MAKE US ARRIVE OUR OBJETIVES

- Shiga toxins (Stx) are the main virulence factors in enterohemorrhagic Escherichia coli (EHEC) infections causing diarrhea, hemorrhagic colitis, and Hemolytic Uremic Syndrome (HUS)
- Stx genes are located in the genomes of prophages that resemble the coliphage lambda
- Under stress condition, the bacteriophage release and Stx is express
- Bacteriophage could infect others bacterias present in the gut (Gamage *et al.*, 2003)
- Bacteriophage induction is required for HUS development (Tyler *et al*., 2013).

HYPOTHESIS

THE MECHANISM OF THE BACTERIOPHAGE INDUCTION IN A EUKARYOTIC ENVIRONMENT



Introduce bacteria as defense mechanism → the bacteriophage should be possible to express gene under eukaryiotic promoters

Acheson *et al*., 1998
Cornick *et al.,* 2006
Schmidt *et al.*, 2001
Tyler *et al.*, 2013
Gamage *et al.*, 2003

OUR HYPOTHESIS + PREVIOUS IDEAS

WE DECIDED OUR OBJETIVES

1. Studies about internalization into mammalian cells

2. Studies focus in the expression of Stx in eukaryotic cells

3. Studies focused on the bacteriophage as therapeutic target

OPEN ACCESS Freely available online

PLOS ONE

Functional Capacity of Shiga-Toxin Promoter Sequences in Eukaryotic Cells

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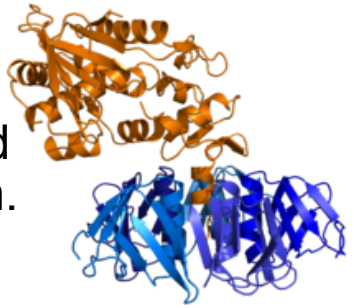
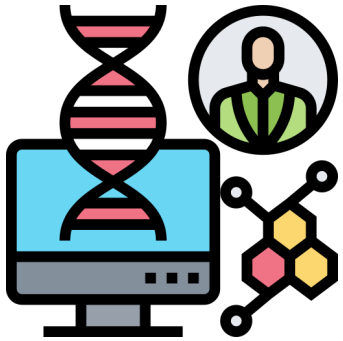
Evaluate the Ability of the eukaryotic machinery to recognize genetic sequences as Stx2 promoters

To transcribe a Stx2-like protein and produce the functionally active toxin.



Tested by **GFP** expression

PLASMID CONSTRUCTIONS



In Silico analysis of promoters

7 regions (pr1-pr7) with high score for putative eukaryotic promoter sequence

Plasmid pStx2

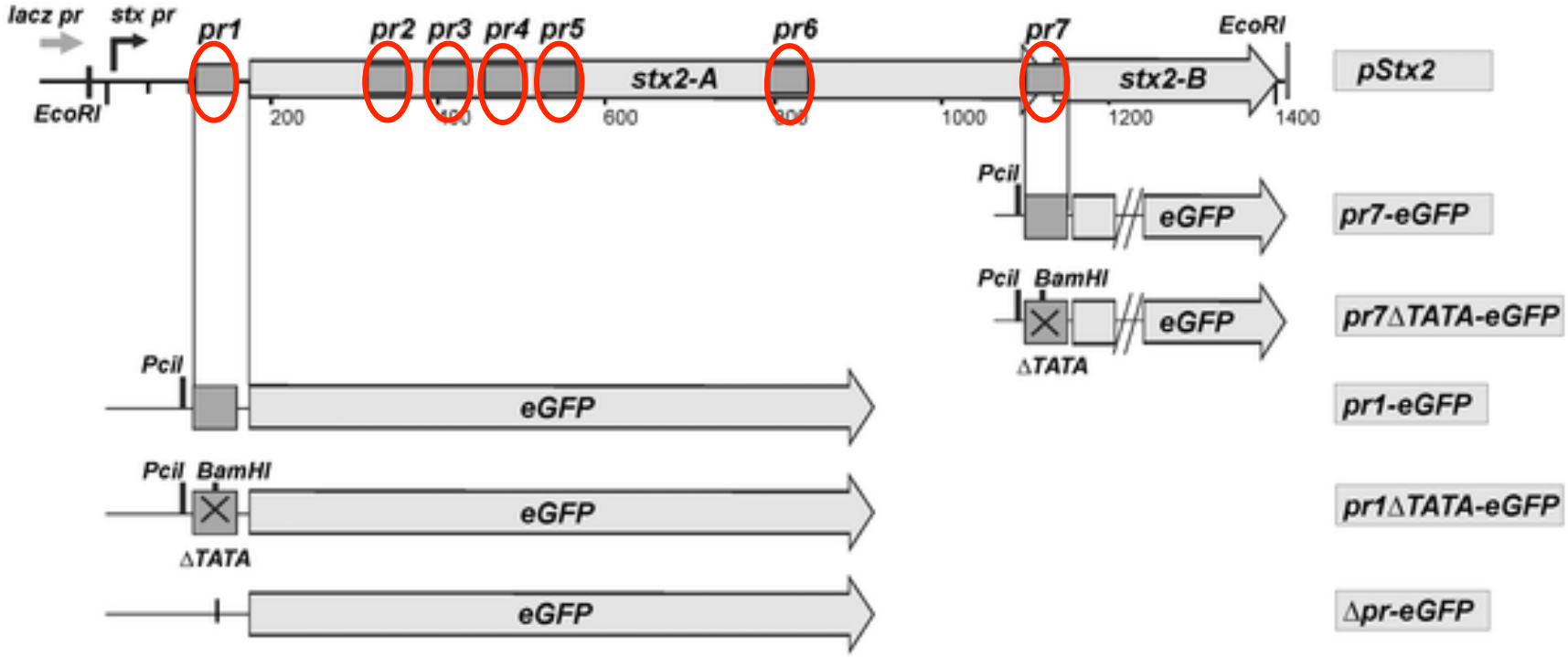
1 - Cloned the gen of Stx2 in pGEM-T

2 - Change

pr1 → pr1-eGFP

pr7 → pr7-eGFP

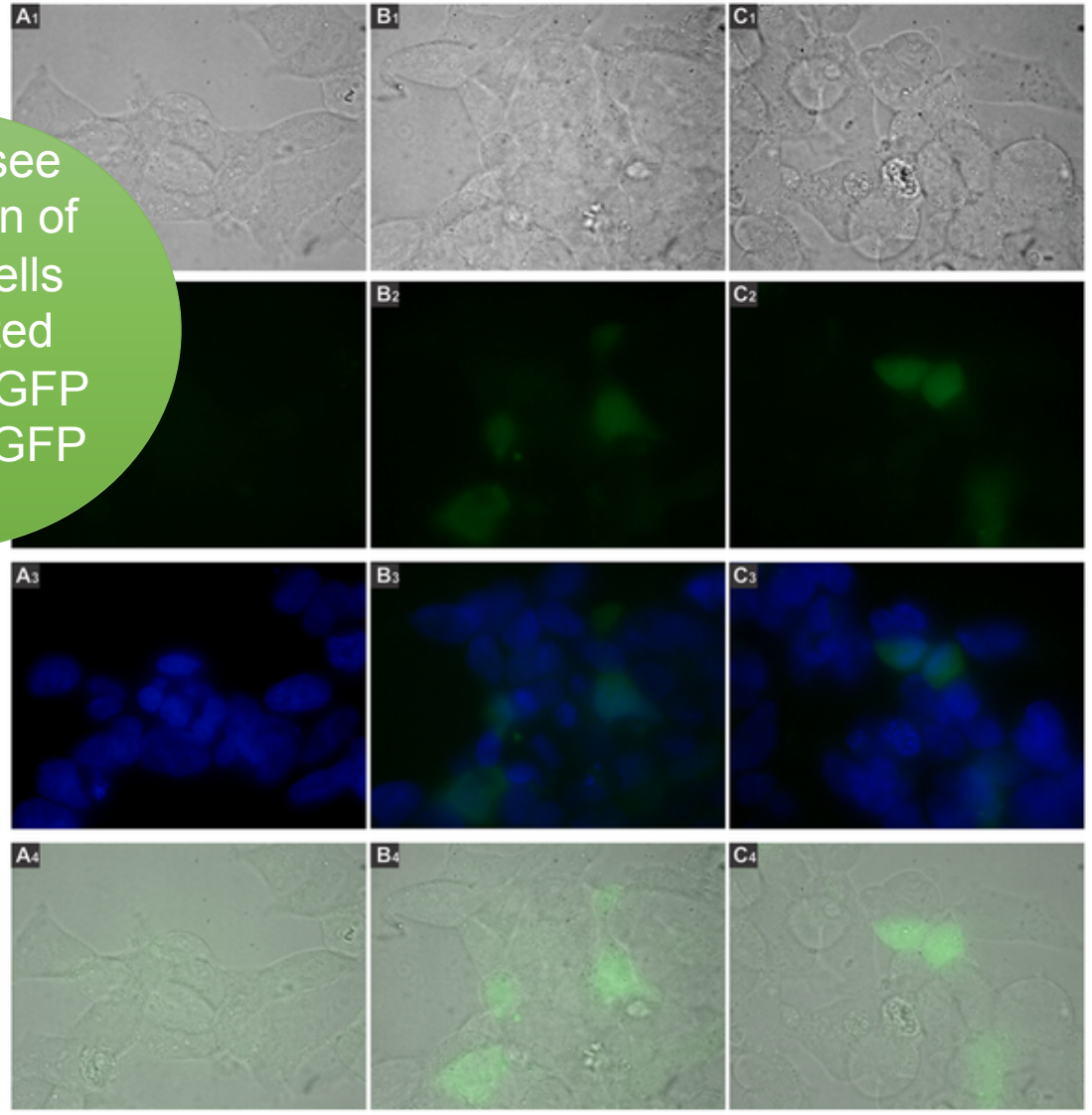
To see **GFP** expression



PLASMID CONSTRUCTIONS

GFP activity driven by the pr1 or pr7 regions

We can see expression of GFP in cells transfected with pr1-eGFP and pr7-eGFP



Analyzed by fluorescence microscopy using the Nikon Eclipse TE2000 microscope equipped with a CCD camera, with 1000X magnification.

293T cells were transfected with the next plasmids - (Incubated for 48h)

pr1-eGFP
pr7-eGFP } Treatment groups

Δpr-eGFP → As negative control

Numbers

- 1 correspond to images visualized with white light
- 2 correspond to images visualized with green filter
- 3 correspond to merge between DAPI and green filter
- 4 correspond to merge between white light and green filter

A. Cells transfected with the Δpr-eGFP plasmid.

B. Cells transfected with pr1-eGFP.

C. Cells transfected with pr7-eGFP.

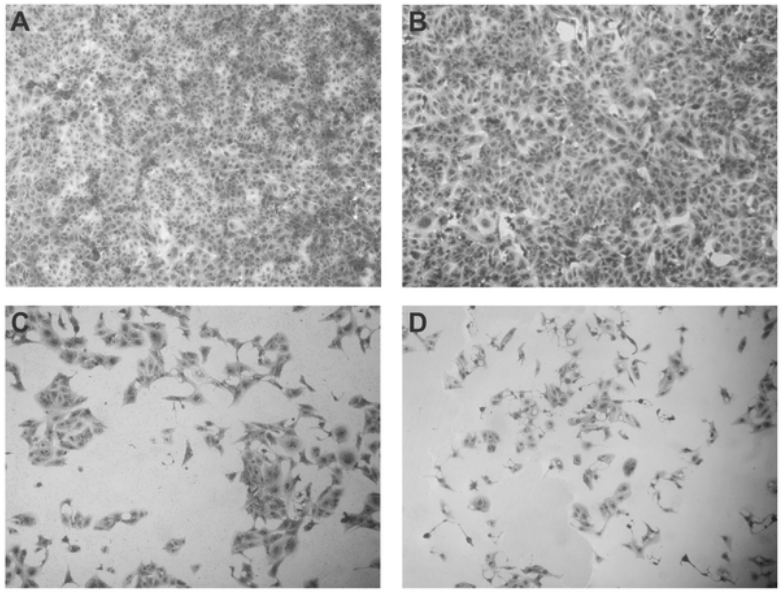
pStx2: CYTOTOXICITY ON SUSCEPTIBLE EUKARYOTIC CELLS

Cytotoxicity on Vero Cells

Vero cells were incubated with purified Stx2 or supernantants of cells transfected with pStx2 plasmid.

48 h

Cells were stained with Crystal Violet



Analyzed by optical microscopy. Representative pictures using 200X original magnification are shown.

A. Non-treated Vero cells.

B. Cells transfected with the pGEM-T plasmid.

C. Cells transfected with the pStx2 plasmid.

D. Cells incubated with purified Stx2.

Evaluate the specificity of citotoxicity

We evaluated of the neutralization of Stx2 citotoxic activity

How we did that?

Vero cells were incubated with a dilution of culture supernatants derived from vero cells transfected with pGEMT or pStx2

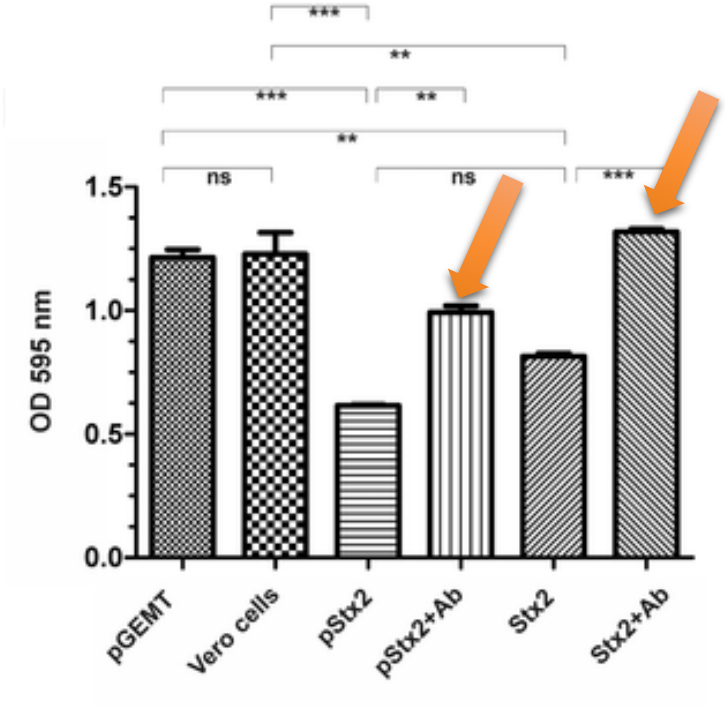
The samples were pre incubated with anti Stx2-antibodies

48 h

Cells were stained with Crystal Violet

pStx2: Cytotoxicity on susceptible eukaryotic cells

OD of the samples with antibodies is more higher than the citotoxic samples.



This results demonstrated that Stx is expressed in eukaryiotic keeping this structure, function and the capacity to bind the receptor.

CONCLUSIONS - IN VITRO ASSAYS

- pr1 and pr7 sequences are recognized by eukaryotic cells
- Stx2 expressed by eukaryotic cells is biologically active
- Significant neutralization of the cytotoxic activity was observed using mouse polyclonal anti-Stx2 antibodies, confirming that the cytotoxicity was specifically induced by Stx2.
- TATA box depletion did not show GFP expression

What would happen *In vivo*?

So, for this reason we did the same but *In vivo* in a Murine model.

RESEARCH ARTICLE

Promoter Sequence of Shiga Toxin 2 (Stx2) Is Recognized *In Vivo*, Leading to Production of Biologically Active Stx2

Leticia V. Bentancor,[a,b] Maria P. Mejías,[a] Alípio Pinto,[c] Marcos F. Bilen,[b] Roberto Meiss,[a] Maria C. Rodriguez-Galán,[d] Natalia Baez,[d] Luciano P. Pedrotti,[d] Jorge Goldstein,[c] Pablo D. Ghiringhelli,[b] Marina S. Palermo[a]

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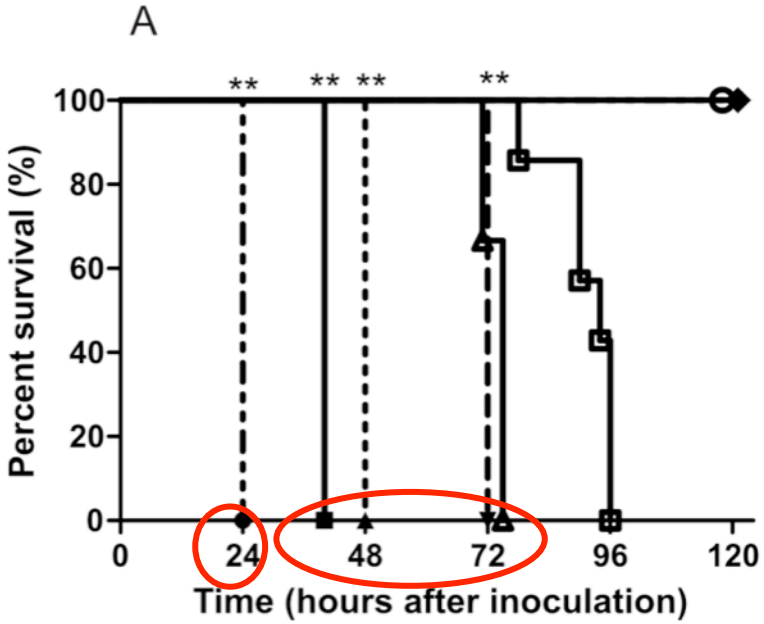
pStx2: HYDRODYNAMIC INOCULATION IN MOUSE



- pStx2 3.5ug
- pStx2 1ug
- pStx2 0.5ug
- pStx2 0.25ug
- pGEMT control
- pStx2ΔAB
- 1LD100
- 3LD100

The survival rate of mice infected with dosses of pStx2 plasmid

Plasmid construction in which the active site of Stx2 has been deleted

The dosis of pStx2 is correlative with quickly death of mice infected.

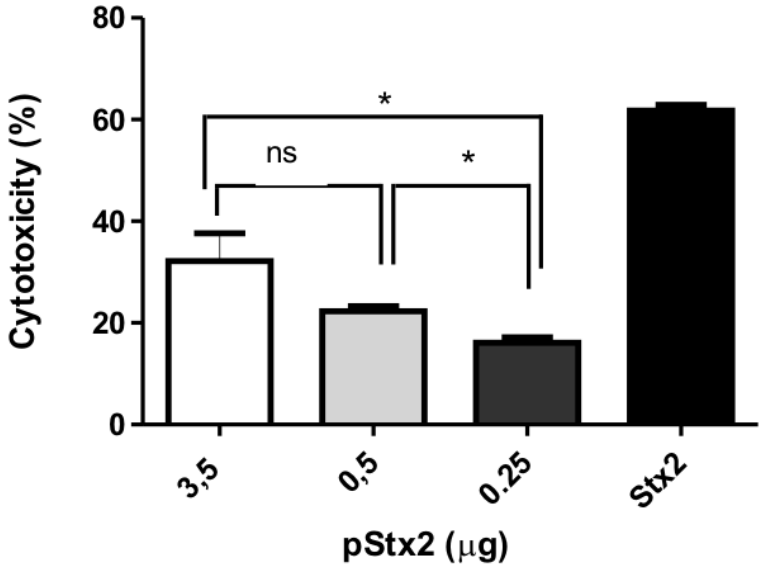
With 3.5 ug the mice were dead after 24 hours.

Letal doses depended of concentration

Positive control (Letal dosis of Stx2)

- 1ld100 the mice were dead after 72 hours
- 3ld100 the mice were dead after 96 hours

IN VITRO EVALUATION OF STX2 ACTIVITY IN PLASMA



This result shown the functionally active toxin in mice serum

In vitro evaluation of Stx2 activity in plasma.

(A) Vero cells were incubated with plasma from mice inoculated with different doses of pStx2 obtained at 24 h post-inoculation. Each bar represents the mean ± SEM for 4 mice. *, $P < 0.03$, t test.

CONCLUSIONS - *IN VIVO* ASSAYS

- Stx2 is expressed in vivo after pStx2 HBT, driven by its own wild stx2 promoter.

- Active Stx2 thus produced is able to target the kidney and the brain and reproduces the lethal damage induced by purified Stx2 or secondary to EHEC infection.

WHICH IS THE ROLE OF BACTERIOPHAGE 933W IN HUS DEVELOPMENT?

IS BACTERIOPHAGE 933W ENOUGH TO DEVELOP THE SYNDROME?

frontiers in Microbiology

ORIGINAL RESEARCH
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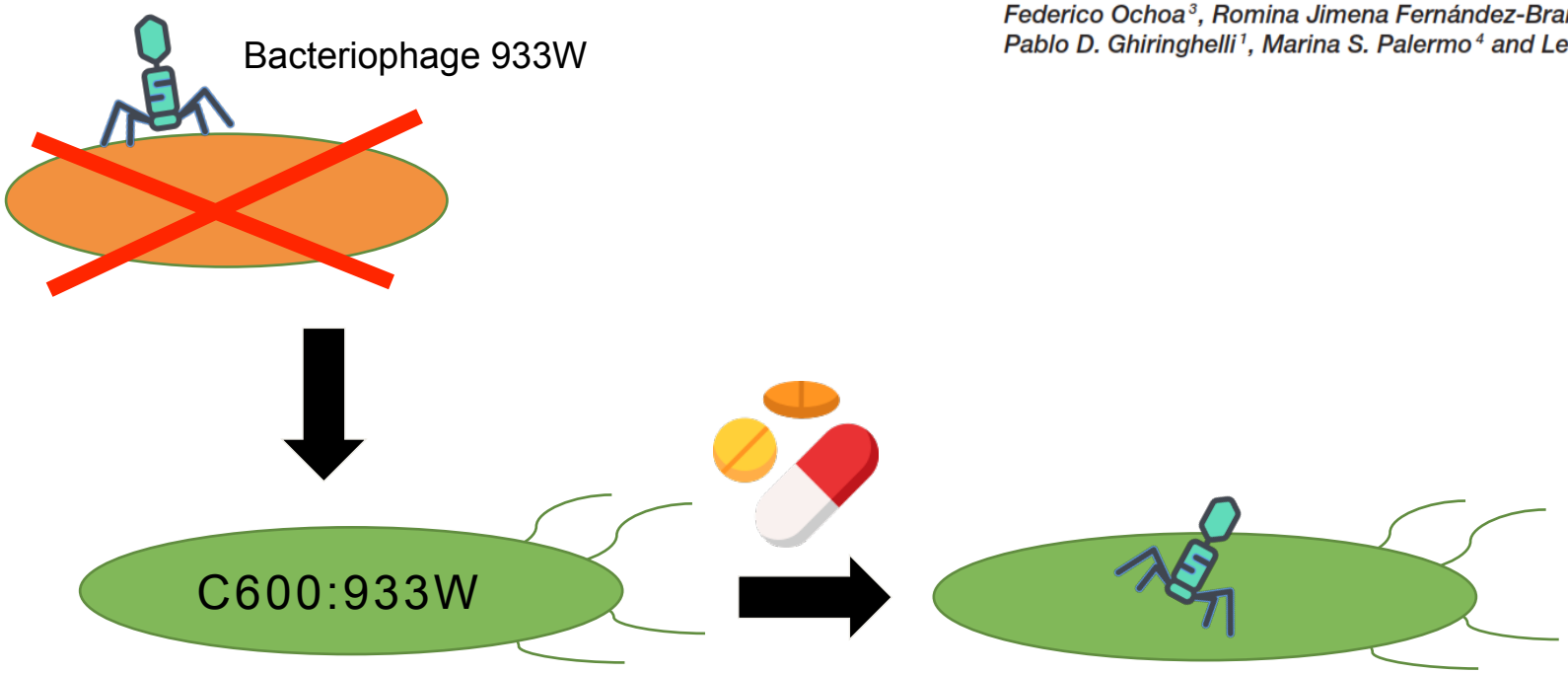
Relevance of Bacteriophage 933W in the Development of Hemolytic Uremic Syndrome (HUS)

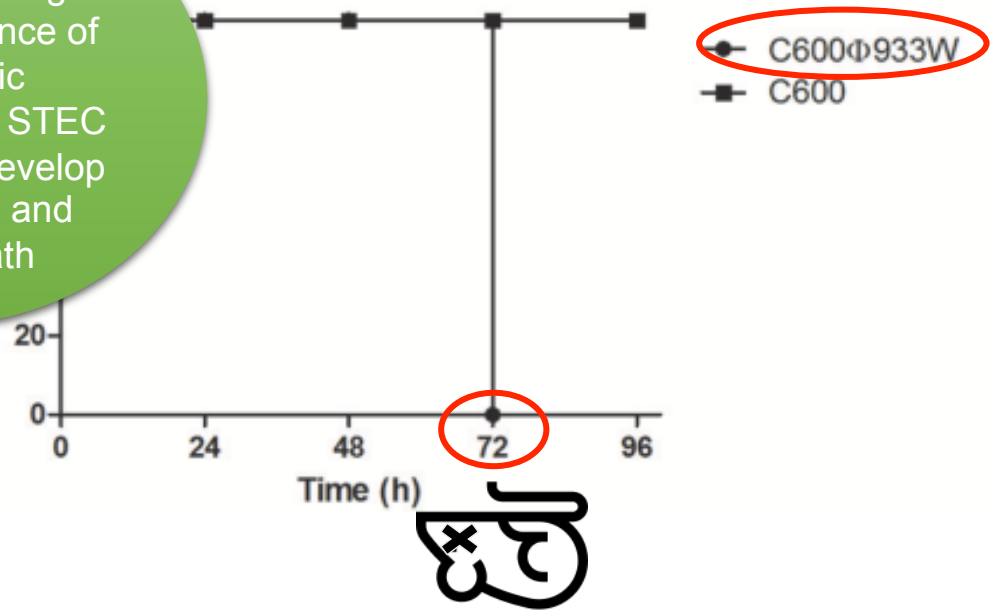
Manuel E. Del Cogliano[1†‡], Alipio Pinto[2†‡], Jorge Goldstein[2], Elsa Zotta[3], Federico Ochoa[3], Romina Jimena Fernández-Brando[4], Maite Muniesa[5], Pablo D. Ghiringhelli[1], Marina S. Palermo[4] and Leticia V. Bentancor[1]*



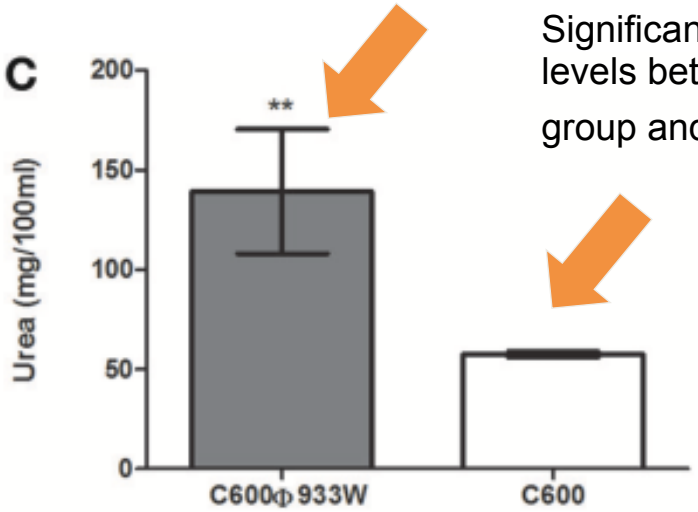
We worked with a non pathogenic *Eschericia coli* strain carrying the lysogenic bacteriophage 933W (C600:933W) and we induced the release of the bacteriophage using antibiotics.

SURVIVAL CURVE AND PHYSIOLOGICAL PARAMETERS OF HUS

This result shown that bacteriophage 933W in absence of pathogenic background of STEC is enough to develop HUS in mice and cause death



Survival rates of mice infected with E. coli C600Φ933W are shown. Mice infected with E. coli C600 were used as controls.

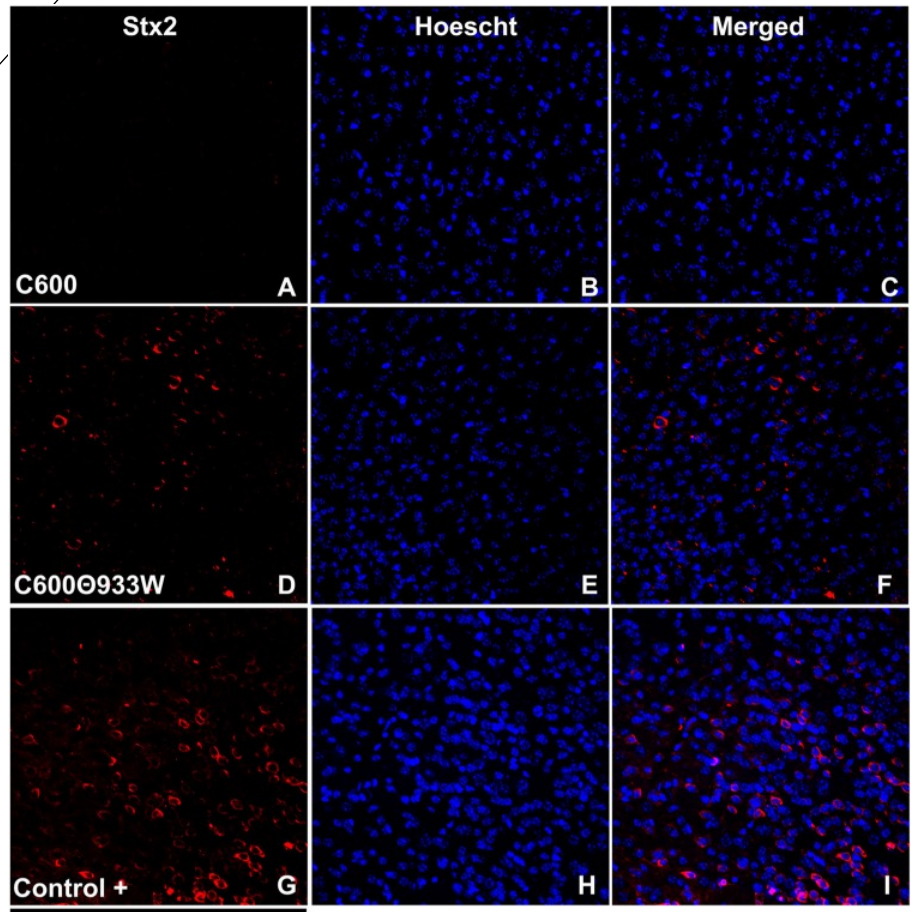


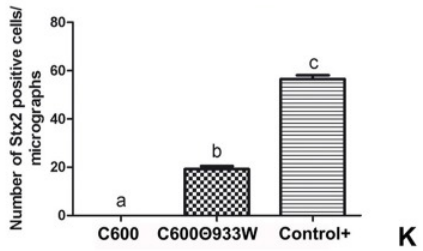
Significant difference of urea levels between the treated group and the control

(C) Stx-induced renal damage. Plasmatic urea levels at 72 h post-infection were measured as a parameter of renal damage. Each bar represents the mean ± SEM of 4–6 mice/group. **P = 0.0095

Parameter of kidney damage we analyze urea levels

DETECTION OF STX2 IN BRAINS





We performed an immunofluorescence assay using a polyclonal anti-Stx2 antibody.

Were observed inmunopositive cells in mice infected with C600:933W
→ Like in + Control

Immunopositive cells for Stx2 were not found in control mice infected with C600

Detection of Stx2 in brains of mice infected with E. coli C600Φ933W.

(A–C) A representative brain section from control mouse infected with *E. coli* C600.

(D–F) Brain section from mouse infected with *E. coli* C600Φ933W.

(G–I) Brain section from a positive control mouse after 4 days of treatment with 1 µg of Stx2.

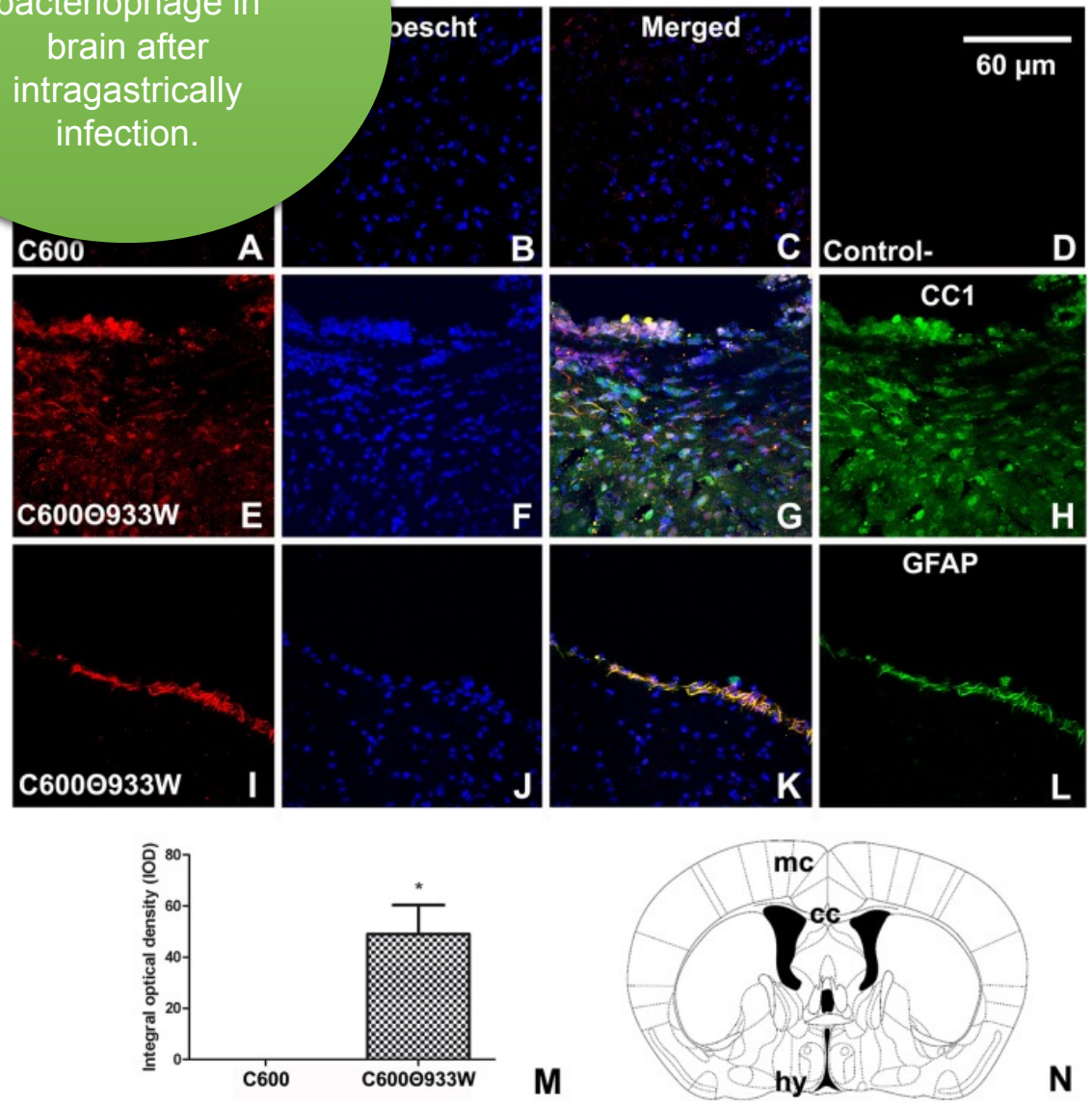
(J) Negative control.

(K) Number of Stx2-immunopositive cells in the mouse motor cortex (mc) per micrograph. Stx2 immunopositive cells are indicated by arrows in (F,I). (L)

The area observed is located in the mouse motor cortex. The scale bar in “J” applies to all micrographs.

BACTERIOPHAGE DETECTION IN BRAINS

This is the first report showing bacteriophage in brain after intragastrically infection.



We used antibodies for phage detection

Bacteriophage 933W was observed in mice infected with C600:933W

The controls were negatives

Bacteriophage detection in brains of mice infected with E. coli C600Φ933W.

(A,C,D) Brain from control mouse infected with E. coli C600.

(E,L) Brain from mouse infected with E. coli C600Φ933W 72 h after inoculation.

(E–H) External capsule;

(I–L) hypothalamus.

(B) Negative control omitting the secondary antibody.

(N) Bacteriophage were detected in the mouse corpus callosum (cc) and hypothalamus (hy) (the red square shows where the micrographs were taken).

(M) Quantification of bacteriophages in the cerebral parenchyma by integral optical density (IOD). lv, lateral ventricle; gl, glia limitans. The scale bar in “B” applies to all micrographs.

CONCLUSIONS BACTERIOPHAGE *IN VIVO*

- In absence of other pathogenic bacterial factors, bacteriophage 933W provoked intestinal, renal and brain damage associated with HUS development.
- It is the first detection of bacteriophage in brain.
- The new animal model described here is proposed as a useful tool to study bacteriophages as a therapeutic target, independently of the bacterial background, and opens an innovative approach to treating infections caused by toxin-encoding bacteriophages.

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