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Invasion of mouse fibroblasts and macrophages by human isolates of *Agrobacterium*

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Introduction

In the past few decades there has been growing evidence that species of *Agrobacterium* can be opportunistic animal pathogens. One of these species is *Agrobacterium tumefaciens*, which for years has been studied as a plant pathogen. The results of one published experiment suggested that an *Agrobacterium* toxin could kill mice (12). Other strong evidence for *Agrobacterium* as an opportunistic human pathogen is the over fifty published clinical cases in which humans were infected by *Agrobacterium* species (3, 13-25). In almost all of these cases the patient was immunocompromised in some way and they often had an invasive procedure such as a catheter (3).

Agrobacterium is a genus of gram-negative aerobic bacteria, within the α-Proteobacteria, that is typically found in the soil. There are four species of *Agrobacterium*: *tumefaciens, radiobacter, rubi,* and *rhizogenes.* All except *A. radiobacter* have previously been viewed as only plant pathogens (10, 11). Most *Agrobacterium* strains that have been isolated from humans are non-tumorigenic on plants and thus have been referred to as *A. radiobacter;* however, this does not reflect the true biological and taxonomic subdivisions within the genus and as a result new classification schemes have been developed (4, 10 & references therein). The nomenclature of *Agrobacterium* is controversial with some arguing that that A. *tumefaciens* and *A. radiobacter* are one in the same (4).

Most research has focused on the infection of plants by A. *tumefaciens,* which causes crown gall disease. This disease results when the bacterium

transfers a piece of DNA containing "oncogenes" into plant cells. The plant cells then express these genes which results in the formation of a tumor. The infection site on the plant is usually a wound, which is similar to the infection of *Agrobacterium* in immunocompromised individuals. In plants A. *tumefaciens* injects DNA and associated proteins into plant cells. The bacterial DNA incorporated into plant chromosomes comes from a large plasmid called the Tumor inducing (Ti) plasmid. Thus, genes on the Ti plasmid are eventually expressed in the plant cell. These transformed plant cells divide and enlarge rapidly (outside of their normal growth pattern) which leads to a tumor (gall). (1, 2, 5)

While much is known about plant infection little is known about how *Agrobacterium* infects humans or any animal. The first case of *Agrobacterium* infection was reported in 1980 in a patient who had prosthetic aortic valve endocarditis (3). Since this first case *Agrobacterium* has been recognized as an opportunistic human pathogen. In a later medical case *Agrobacterium radiobacter* was reported as the first case in which *Agrobacterium* had caused bacterimic pneumonia. This patient was a 33 year-old HIV-positive woman (3). These are just two of the more than fifty cases of infection by *Agrobacterium* (3, 13-25). The majority of these patients had underlying hematological malignancies (i.e. leukemia), solid tumors (ovarian or breast cancer), or end-stage renal disease that required continuous ambulatory peritoneal dialysis (3). Other patients had aplastic anemia, HIV infection, leukopenia, or sub-acute combined

immunodeficiency (3, 4). Most of these patients presented with a single site of infection, but there were exceptions. For instance, one patient presented with both cellulitis and bacteremia. Also few of these patients lacked an invasive device, with most people having catheters (3).

A review of *Agrobacterium* isolates from January 1984 to July 1990 at the University of Minnesota Hospital and Clinics showed that there were 47 *Agrobacterium* isolates from 15 patients. All of these patients had significant underlying diseases including malignancies and severe blood dycrasias (8 of the cases) (10). In Denmark from 1986 to 1988 six strains of *Agrobacterium* were isolated from patients(9).

In all patients infected with *Agrobacterium* the outcome has been favorable. No deaths have been directly attributed to *Agrobacterium* infection. This is in part because most patients respond well to antibiotic therapy, especially after removal of the intravenous or intraperitonear device (3). The *Agrobacterium* that infected the woman with HIV was only resistant to amikacin, but was susceptible to aztreonam, cefazolin, ceftriaxone, ceftazidime, ciprofloxacin, gentamicin, imipenem, mezlocillin, piperacillin, ticarcillan plus clavulanic acid, trimethoprim/sulfamethoxazole, and tobramycin (3, 4). Gentamicin can kill *Agrobacterium,* yet it cannot cross the cell membrane. Thus, is a useful antibiotic for invasion assays with *Agrobacterium.*

To begin understanding the pathogenicity of A. *tumefaciens* on animals we wanted to know if the bacterium could invade animal culture tissue cells. Dr.

Brad Goodner obtained three strains of *Agrobacterium* that were isolated from human patients. These three clinical isolates were a gift from Dr. D. Bruckner at the UCLA Medical School Hospital (1). First, it was necessary to confirm that the "*Agrobacterium*" strains isolated from humans were really species of *Agrobacterium*. This was necessary because the clinical identification of the human isolates of *Agrobacterium* focused mainly on classic microbiological tests and on arrays of catabolic substrates. There is only one clinical report in which the Ti plasmid was looked for. In that human isolate there was no Ti plasmid. The Ti plasmid is believed to be necessary for the infection of plants by *Agrobacterium*. Thus, Dr. Goodner and his lab used pulse field gel electrophoresis on analysed uncut genomic DNA. This showed that the human isolates do have two chromosomes: one linear and one circular. The "circular chromosome" molecule was about the same size as C58, which was the plant tumor isolate sequenced by Dr. Goodner's lab and their collaborators at Cereon Genomics. The "linear chromosome" molecule was sometimes larger and sometimes smaller than C58. It is important to note that this is also true when C58 is compared to other plant strains. Another pulse field gel of PacI-digested genomic DNA showed that the overall size is extremely similar between the human isolates and C58. Interestingly one human isolate had a PacI digestion pattern almost identical to the digestion pattern of C58 (1).

Further work was done by Dr. Goodner's lab to determine the identity of the plasmids that were present in addition to the chromosomes. They wanted to

know if the plasmids were Ti or Ri plasmids. It was found that C58 induced tumor formation on all three plant hosts, while all three human isolates could only induce large tumors on carrots and were not virulent on potato or tomato.

The main question is "Can *Agrobacterium* invade animal cells?" We asked this question because all previous clinical reports on the infection of humans by *Agrobacterium* species do not determine the mechanism of invasion for this pathogen. The invasion of host cells is not *a priori* required to be an animal pathogen. However, species of *Brucella* and *Bartonella*, which are close relatives of *Agrobacterium*, do invade host cells. Thus, we predicted that *Agrobacterium* also invades host cells.

Materials & Methods

A classic experimental strategy for detecting invasion of tissue culture cells was used. It was fist developped in the study of *Yersinia pseudotuberculosis* (6).

Bacterial strains, media, and growth conditions.

A *tumefaciens* A348 is a commonly used tumorigenic strain with pTiA6 in the C58 chromosomal background (26). A *tumefaciens* strains UCLA654, UCLA779, and UCLA802, were each isolated from a human patient. These strains were obtained from David A. Bruckner, Chief of the UCLA Division of Laboratory Medicine (4). *E.coli* RR1 is a commonly used lab strain . All strains were grown in a modified Luria-Bertani (LB) medium (only 5 g NaCl/liter) at 30°C for A *tumefaciens* and 37°C for *E.coli*.

Murine tissue culture cell lines, media, and growth conditions.

The RA264.7 murine macrophage cell line (TIB 71, American Type Culture Collection, Rockville, MD) and theL929 murine fibroblast cell line (CCL-1, ATCC) were cultured in RPMI-1640- medium without antibiotics and supplemented with 10% heat-inactivated fetal calf serum, 1% L-glutamine, 1% minimal essential medium vitamins, and 1% nonessential amino acids. These cells were maintained at 37°C in 5% CO_2 in 75-cm2 tissue culture flasks and were subcultured twice weekly.

Invasion Assay

RAW or L929 cells were seeded in microliter dishes at 10^5-10^6 cells/ml and allowed to adhere for 2 hours. Overnight cultures of bacteria were diluted to 10^6-10^7 cells/ml in RPMI-1640 medium and a small portion retained to determine the titer of the diluted culture. Equal volumes of diluted bacteria were added to each microliter dish well and the dish was incubated at 37°C. At different times after addition of the bacteria, the supernatant was gently removed and the tissue culture cells washed twice with fresh RPMI-1640 medium. Fresh medium containing gentamicin sulfate at 200ug/ml was added to each well and the dish incubated at 37°C for 3 hours. The tissue culture cells were washed twice with fresh medium minus gentamicin and then lysed in 150ul of fresh medium containing 1% Triton X-100. For testing survival after invasion, cells were washed after gentamicin treatment and maintained in fresh medium plus gentamicin at 10ug/ml for 24 hours before washing and lysis. 100ul of each

plated directly on a LB agar plate and the remainder diluted before plating onto a LB agar plate. (1,6)

Electron microscopy

Standard procedures were used for electron microscopy. RAW or L929 cells were fixed. First they were in a 2-6% buffered solution for 1-2 hours. Next cells were washed in buffer three times at ten minutes each wash. In the final fixation step cells were incubated in a 1-2% buffered solution of OsO_4. Cell dehydration began with two changes of 25% ethanol at ten minutes each. This was repeated with 50%, 70%, and 90% ethanol. Then three changes of 100% ethanol were done.

As preparation for embedding the cells were placed in a solution of 50% ethanol: 50% Propylene oxide (PO) for ten minutes. Then 2 changes of 100% PO were done at 15 minutes each. Next 50% PO : 50% EPON was done for 10-20minutes. Two changes of 100% EPON occurred prior to imbedding the cells in 100% EPON in a BEEM capsule overnight. The capsules were embedded overnight in a 60°C oven. Using an Ultramicrotome sectioner the capsules were sectioned into 100 ul thick sections. Sections were placed on grids and stained. First the grids were stained using a 7% Uranyl Acetate solution and then they were stained with Lead Citrate Stain. Grids were rinsed between and after stainings with de-ionized water. Observations were made using a JEOL JEM 1010 TEM.

Results

Multiple invasion assays have been done using *Agrobacterium* and either macrophage or fibroblast cells. Data shown here is a sampling of the data collected from the invasion assays. These results show that human isolates of *Agrobacterium* can infect animal cells, both fibroblasts and macrophages.

RR1, an *E.coli* strain, was not able to invade mouse fibroblast cells as predicted. Thus, RR1 served as a control. Also A348, a derivative of the plant isolate C58, was not able to invade animal cells. These cultures had 0% bacteria remaining after both the 0 and 2 hour time points and 24 hour survival. In contrast all three human strains were able to invade, which can be seen in Tables 1 and 2. After two hours of invasion the three human strains had between 2.6 and 6.7 % of the bacteria inside them. The 24-hour survival of bacteria after a 2-hour invasion greatly varied. In UCLA654 the amount of bacteria present was 864.9%, which is almost nine times greater than the amount of bacteria added to the cell culture at the beginning of the invasion assay. The other human strains UCLA779 and UCLA802 had 0.2% and 9.8% respectively, of the original *Agrobacterium* remaining after the 2-hour invasion and 24-hour survival.

Invasion assays done with RAW macrophages confirm the results of fibroblast experiments as shown in Tables 3 and 4. A348 was not able to invade macrophages, which is shown by nearly 0% bacterial invasion for each time point and survival. Data obtained also shows that all three human isolates can invade macrophage cells, but they do so at varying degrees. UCLA654 in one trial had

4.7% bacterial invasion after 2 hours. In this same trial UCLA654 had 1811% survival after 24 hours (Table 4). Both of the other human isolates, UCLA779 and UCLA802 were able to invade, but did so to a lesser extent. These strains had between 0.3% and 2.9% invasion after two hours. While their 24-hour survival ranged from 0% to 0.3 for UCLA779 and 0.6% to 1.5% for UCLA802.

Confirmation of invasion in fibroblasts by *Agrobacterium* is shown by TEM pictures. Figure 1 shows the *Agrobacerium* already present in large vesicles of a fibroblast. While Figure 2 shows the bacteria entering a host cell.

Table 1. Invasion assay using mouse L929 fibroblasts as the host cells (100ul of 10^5 cells/ml in each assay well.) Numbers shown are the average of two replicates.

Bacterial Strain	# Cells added to each well	% of original cell number remaining in lysate after: 0 hr invasion	2hr invasion	2 hr invasion & 24 hr survival
RR1	8.4×10^4	0%	0%	0%
A348	6.3×10^4	0%	0.1%	0%
UCLA654	3.7×10^4	0.8%	3.4%	864.9%
UCLA779	3.7×10^4	0%	3.4%	0.2%
UCLA802	4.9×10^4	0%	2.6%	9.8%

Table 2. Invasion assay using mouse L929 fibroblasts as the host cells (100ul of 10^5 cells/ml in each assay well.) Numbers shown are the average of two replicates

Bacterial Strain	# Cells added to each well	% of original cell number remaining in lysate after: 0 hr invasion	2hr invasion	2 hr invasion & 24 hr survival
RR1	8.0×10^5	-	0%	-
UCLA654	4.0×10^6	-	6.7%	-

Table 3. Invasion assay of RAW macrophages as the host cells (1 ml of 10^5 cells/ml in each assay well.)

Bacterial Strain	# Cells added to each well	% of original cell number remaining in lysate after: 0 hr invasion	2hr invasion	2 hr invasion & 24 hr survival
RR1	1.9×10^6	0%	0.1%	0%
A348	2.9×10^7	0%	0.1%	0%
UCLA654	8.0×10^6	0.1%	2.1	0.3%
UCLA779	1.6×10^7	0%	0.3%	0%
UCLA802	1.5×10^7	0%	1.8%	0.6%

Table 4. Invasion assay of RAW macrophages as the host cells (1 ml of 10^5 cells/ml in each assay well.)

Bacterial Strain	# Cells added to each well	% of original cell number remaining in lysate after: 0 hr invasion	2hr invasion	2 hr invasion & 24 hr survival
RR1	8.4×10^4	0%	6.0%	1.2%
A348	6.3×10^4	0.1%	0.1%	0%
UCLA654	3.7×10^4	1.5%	4.7%	1811%
UCLA779	3.7×10^4	0.1 %	2.9%	0.3%
UCLA802	4.9×10^4	0.1%	1.8%	1.5%

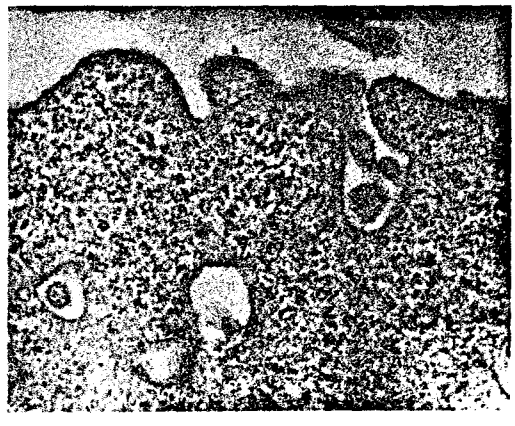

Figure1: *Agrobacterium* is present in the large vesicles of a fibroblast.

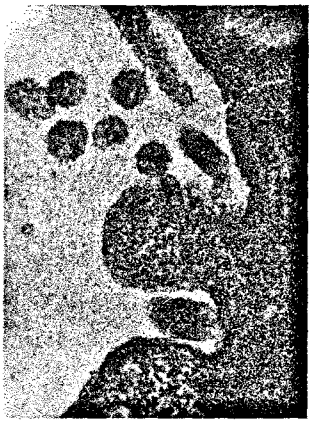

Figure 2: *Agrobacterium* entering a fibroblast (host cell).

Discussion

Agrobacterium strains that were isolated from humans invaded both macrophages and fibroblasts. However, an *Agrobacterium* strain (A348) isolated from a plant tumor was not able to invade macrophages or fibroblasts. Invasion assay data in Tables 1 through 4 demonstrate this. Transmission Electron Microscopy (TEM) pictures of *Agrobacterium* inside a fibroblast provide additional confirmation of bacterial entry into these animal cells. Due to problems with TEM preparation methods TEM pictures for *Agrobacterium* inside macrophages are not currently available.

The percentage of bacteria that invade is very low; however, the bacteria only had two hours to invade the cells. Given more time it is probable that additional bacteria would enter the cells and the invasion percentages would be higher. Human isolates of *Agrobacterium* showed invasion percentages similar to those seen for *Y. pseudotuberculosis* and other known invasive pathogens in

similar experiments (1, 6). Thus, even low amounts of bacterial invasion in this assay are biologically significant.

Macrophage cells are professional bacterial killers. Thus, some bacterial cells may get in, but soon be killed. While inside the macrophage the bacteria is exposed to both oxidative and non-oxidative defense mechanisms. UCLA654 was not only able to survive after entry into macrophages, but it was also able to replicate. This is shown by the more than 18 times bacteria present after a 2-hour invasion and 24 -hour survival in one trial. Thus, this human strain may have some ability to avoid the macrophage's defense measures.

Now that it is known that *Agrobacterium* invade human cells the next logical step is to determine the mechanism they use for invasion. Currently nothing is known about the mechanism or the genes required for infections by *A. tumefaciens*. However, there is a great deal known about the invasion of *Bartonella* into animal cells. Recently new phylogenetic realignments have shown that a close genetic relationship exists between *Agrobacterium* and *Bartonella*. In the pre-antibiotic era *Bartonella bacilliformis* was an extremely lethal pathogen. These infections were limited to a specific geographical area, mainly Peru. *B. bacilliformis* targets both red cells (it is the only bacterium known to do this) and endothelial cells. Some evidence suggests that common factors exist between *A. tumefaciens* plant virulence and *Bartonella* virulence on animals. *Bartonella* invades the animal host and then survives intracellularly within the animal host (7, 1). A two-gene operon in *Bartonella, inv*AB is required for invasion (8,1). This

two-gene locus was shown to confer the ability to invade human erythrocytes upon non-invasive *Escherichia coli* (8 & refs therein, authors in 1995).

Dr. Goodner's lab found a strong *inv*A homolog during the genomic sequencing project for the plant tumor isolate C58; however, no *inv*B homolog was found. It is postulated that this is why the plant strain, C58, cannot invade animal cells. It is possible that the human strains do have an *inv*AB operon, which allows them to enter animal cells. Determining if the *inv*AB genes are important for *Agrobacterium* invasion of cells is an important first step in finding the mechanism of the opportunistic human pathogen.

Dr. Goodner's lab found an *inv*A gene in human strain UCLA802 in addition to the plant strain C58. In mutant strains of these bacteria the *inv*A gene was knocked out. The invasion assays are being repeated to determine if *Agrobacterium* lacking the *invA* gene can infect human cells. It is predicted that C58 strains both with and without the *inv*A gene will not infect animal cells because in all previous trials it has not. The wildtype UCLA802 strains are predicted to be able to invade animal cells, while it is thought that the mutant UCLA 802 strains will not be able to invade animal cells because they lack the *inv*A gene. After completion of these invasion assays an *inv*B homolog will be searched for in the three human strains.

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