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Research Letter

RESEARCH LETTER – Pathogens & Pathogenicity

Rescue of fish exposed to a lethal dose of pathogen, by signals from sublethally exposed survivors

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One Sentence Summary: Fish were challenged with a low dose of a bacterial disease, cohabited with bystander fish, resulting in cellular communication leading to protection against subsequent infection by the same disease.

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ABSTRACT

Rainbow trout (*Oncorhynchus mykiss*, Walbaum) were challenged intraperitoneally with a sublethal dose of *Vibrio anguillarum* VIB1 and allowed to recover. Then, after 7 days, naïve fish, (designated as 'bystander' fish) which had never been exposed to the pathogen, were introduced to the same tank. These swam with the adapted (recovered) fish for 7 days before both groups and a control (never exposed directly to the pathogen or to recovered fish) group were exposed to a lethal dose of VIB1. Mortality records were 100% in the control group within 3 days, 47% in the adapted group and 60% in the unchallenged bystander group, which swam with the adapted group. In both the latter groups, the time to death of the non-surviving fish was attenuated. This inter-animal communication of signals has previously been documented for animals exposed to ionizing radiation. Assays of tissues from control, challenged and 'bystander fish exposed to the pathogen showed that a signal as yet unidentified but similar to that seen in bystanders to irradiated fish was being produced. This signal caused a sharp and transient increase in intracellular calcium and a decrease in clonogenicity in a well-characterized reporter assay.

Key words: *Vibrio anguillarum*; rainbow trout; bystander effect; sublethal exposure; disease

INTRODUCTION

Adaptive responses to environmental and pathogenic stressors leading to the development of protective responses are well accepted as an evolutionary mechanism facilitating change in biology (Sonneborn 2005; Hastings 2007; McBryan *et al.*, 2013). Generally, direct exposure to the stressor is required, and the mechanism is considered to involve mutational or epigenetic change tailored to ensure survival of the population in the face of changed environmental conditions (Mothersill and Seymour 2012a; Rosenberg *et al.*, 2012). Recent evidence using physical stressors, i.e. ionizing or ultraviolet radiation, has suggested that signals can be transferred from exposed organisms, i.e. fish, to buddies swimming in the same area. Similar effects have been observed for rodents (Surinov *et al.*, 1998; Mothersill *et al.*, 2006; Mothersill and Seymour 2012b). To date, this phenomenon has only been confirmed after exposure to radiation; although there is one unconfirmed report in the literature (Sin 1992) that goldfish which recovered from a protozoan parasitic infection had cross-resistance to a different protozoan parasite causing velvet disease and could 'protect' other goldfish from this. No

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mechanism was suggested, but surface antibody sharing was suspected.

Vibrio diseases are an important cause of mortality in aquaculture, with traditional efforts to control outbreaks including immunoprophylaxis and chemotherapy (Austin and Austin 2012). However, few vaccines are available, and the use of chemotherapeutants engenders the development and spread of resistance, which poses unacceptable risks to users and inhabitants of aquatic ecosystems (Austin and Austin 2012). In this report, a new approach is presented which could avoid these issues. The approach relies on a mechanism well established in plants where inter-plant communication of information vital to survival is well known in situations of herbivore stress or insect attack. In some cases, allelopathic processes have been identified (Singh *et al.*, 2011; Soares *et al.*, 2014). Recently, our group showed that similar communication could occur between fish resulting in both adaptive and adverse molecular and cellular effects in individuals swimming with irradiated fish (Mothersill *et al.*, 2006; Smith *et al.*, 2007; Mothersill and Seymour 2012. This effect is known as an inter-animal bystander effect in the radiation field. The mechanisms remain controversial and the nature of the signal is unknown, but calcium signaling and downstream activation of common stress pathways such as MAPK are involved (Lyng, Seymour and Mothersill 2000). In the absence of clear impacts on survival or reproduction, the relevance of the radiation effect to radiation risk is unclear. In the experiments reported here, we can for the first time clearly link a survival endpoint *in vivo* to mechanistic pathways which result in communication of information between fish. The result is reduced mortality following a lethal challenge to *Vibrio anguillarum* VIB1.

MATERIALS AND METHODS

Bacterial culture

Vibrio anguillarum VIB1 was selected because of its taxonomic status and pathogenicity to rainbow trout (*Oncorhynchus mykiss*, Walbaum) and also because of its lack of ability to survive in fresh water (Austin *et al.*, 1995). The culture was maintained in 15% (v/v) glycerol as cryopreservant at −70°C. Working cultures were prepared in tryptone soya broth (Oxoid, Basingstoke, UK) supplemented with 1% (w/v) NaCl with incubation at 25°C for up to 48 h. Cultures were centrifuged, washed and resuspended in 0.9% (w/v) saline, and cell numbers determined by use of a haemocytometer slide and microscopic observation at a magnification of x400 on a Euromex BioBlue light microscope (Arnhem, The Netherlands). Cell numbers were adjusted to 10^5 and 10^7 ml^{-1} with saline. These concentrations had been established as causing sublethal and lethal effects respectively in earlier experiments using rainbow trout.

Fish experiments

Rainbow trout of 25 g average weight were obtained from a commercial fish farm in Scotland, and held under quarantine conditions for 10 days and verified to be free of pathogens, notably *V. anguillarum*, while monitoring the health of the animals using standard procedures (Austin and Austin 1989). The animals were randomly placed in groups of 15 in polypropylene tanks containing aerated free-flowing (flow rate = ∼0.6 l min^{-1}) freshwater at 13°C. One group was exposed to a sublethal challenge of *V. anguillarum* [0.1 ml containing 10^5 cells ml^{-1} by intraperitoneal (i.p.) injection] and a second group was an unexposed control group. Seven days later, 15 naive fish were marked with small nicks on the dorsal fin and added to each tank. Then after a further 7 days, all fish were challenged with a lethal dose of *V. anguillarum* (0.1 ml containing 10^7 cells ml^{-1} by i.p. injection). The tank water was routinely examined throughout the experiment for the presence of culturable cells of *V. anguillarum* by spreading 0.5 ml volumes of water and sedimentary material (including faeces) from the bottom of the tanks onto groups of five plates of *V. anguillarum* medium (Alsina *et al.*, 1994) with incubation at 25°C for up to 7 days when the presence of bright yellow colonies were distinctive for the pathogen. Identification followed the approach of Austin *et al.* (1995). The numbers of dead fish per day and time to mortality were recorded. Throughout the experiment the fish were fed to appetite twice daily using a commercial salmonid diet (Skretting, Northwich, UK) appropriate to the size of the fish. Dead fish were examined microbiologically to confirm the presence of *V. anguillarum* (after Austin *et al.*, 1995).

Demonstration of bystander signal

Fish were sampled after recovering from the sublethal challenge. Control fish, recovered fish and fish which swam with the recovered fish were sacrificed by overdose of anaesthetic (MS-222; Sigma-Aldrich, Basingstoke, UK) and exsanguination, and the caudal fin was removed. The tissue samples were placed in 5 ml volumes of RPMI-1640 tissue culture growth medium, containing 10% (v/v) foetal calf serum (Life Technologies, Burlington, ON, Canada) and stored for 24 h at 15°C to allow signal generation into the medium following the method established for tissue explants (Mothersill *et al.*, 2001). The medium was then harvested and filtered using Acrodisc (Pall, Port Washington, NY, USA) 0.22 μm filters.

The resulting conditioned medium was tested to determine the presence and strength of the bystander signal using a well-established clonogenic reporter assay using living human epithelial cells (HaCats) seeded in clonogenic densities 6 h prior to medium transfer. Flasks were then left undisturbed for 10–12 days in a 37°C, 5% CO_2, 95% humidity incubator to allow for colony formation (Liu *et al.*, 2006). A rapid transient calcium flux from outside to inside the reporter cell was also measured in response to conditioned medium exposure. This assay is also well established for detecting and quantifying the existence and strength of bystander signals (Lyng, Seymour and Mothersill 2000; Liu *et al.*, 2006). The calcium flux assay detects changes in calcium concentration through the cell membrane using the Fura-2/AM fluorescent dye. The protocol was developed by Lyng, Seymour and Mothersill (2000), and adapted for the laboratory settings at McMaster University. Briefly, living human epithelial cells (HaCats) were seeded on glass-bottom dishes (MatTek Corporation) 24 h in advance. The cell culture medium was then discarded and cells were gently washed three times with a combination of Hank's balanced salt solution + 20 mM of HEPES. Cells were immediately loaded with 8.4 μM of Fura-2/AM for 45 min at room temperature. Dye was discarded and cells were then washed three more times, after which 300 μl of buffer was added. The dishes were then placed on an Olympus inverted fluorescent microscope (Olympus Canada, Richmond Hill, Canada) and 10 cells were selected using a 40 × oil objective. Then 100 μl of explant-conditioned medium was added to the cells 1 min after acquisition started. Images were captured with a CCD CoolSnap HQ camera (Photometrics, Tucson, Arizona) and the ratio of calcium-bound versus calcium-free Fura-2/AM was measured at 340 and 380 nm, respectively. The ratio of emission between those wavelengths is correlated with the flux of

calcium through the cellular membrane. The difference between groups for both clonogenic survival and calcium flux was identified through one-way ANOVA and significance between the mean of each treatments against the mean of the controls was confirmed using the Holm-Sidak's multiple comparison test, with a 99% level of confidence.

RESULTS

All fish survived the initial challenge with the low dose of *V. anguillarum*. During this period, there was not any evidence of clinical disease nor were culturable cells of the pathogen recovered from the tank water or the debris at the bottom of the tanks. The results for fish mortality following a lethal challenge to *V. anguillarum* VIB1 are included in Fig. 1. It is apparent that the directly exposed fish, which were not pre-exposed, all died, and the presence of the pathogen in the dead fish was confirmed by culturing and examination of key phenotypic traits. However, both the recovered fish and those swimming with recovered fish showed less than the expected 100% mortality and those which died survived longer than the lethal challenge only group.

The results for the clonogenic reporter assay are shown in Fig. 2. Clearly, bystander signals are being produced by tissues from the sublethally exposed group and the swimming partners but not by tissues from the controls. The calcium flux assay data are presented in Fig. 3a. Again, the results are similar to those seen with radiation stress—there is a very small sustained increase in intracellular calcium after application of medium from the controls to reporter cells but the area under the curve from Fig. 3a shown in Fig. 3b demonstrates that such increase is not relevant. In fact, the sublethally exposed fish and the swimming partners produced strongly significant values against the controls.

DISCUSSION

Until now, radiation-induced bystander effects have been considered an oddity of unknown relevance in the radiobiology field (Morgan and Bair 2013; Mothersill and Seymour 2013). This report means that a totally different stress caused by disease exposure induces a similar mechanism and that adaptive responses can be communicated to individuals other than those directly exposed to the pathogen. Apart from obvious practical applications in aquaculture, this finding could lead to new thinking about adaptive mechanisms. Currently most models require the interaction of the stressor with the host to achieve an adaptive response (Choi *et al.*, 2013; Matsumoto *et al.*, 2009). Even where signaling and cellular communication mechanisms are accepted, the mechanisms usually involve circulating messengers in the body such as hormones or blood-borne factors that induce epigenetic or post-translational changes in the targeted organism (Mancuso *et al.*, 2008; Ilnytskyy *et al.*, 2009). These models do not require any formal departure from Darwinian and post-Darwinian principles of evolution through a process of adaptation and natural selection. However, the idea that adaptations can be induced in organisms that have not been stressed by physical exposure to a stressor could point to a population level mechanism where the benefit of response to a stressor does not involve the often deleterious effects of direct exposure (Zhao and Robbins 2009; Adam-Guillermin *et al.*, 2012).

To summarize, we have demonstrated that mechanisms inducing protection against a lethal exposure to a fish pathogen—*V. anguillarum* VIB1—can be transmitted to naïve fish, resulting in reduced severity of disease and attenuated pathogenicity in the fish, some of which ultimately succumb to the challenge. The mechanism appears to be similar to that seen with physical stressors and involves calcium signaling. This widens the importance of this adaptive protective mechanism biology, and also suggests possible new avenues for vaccine development in aquaculture.

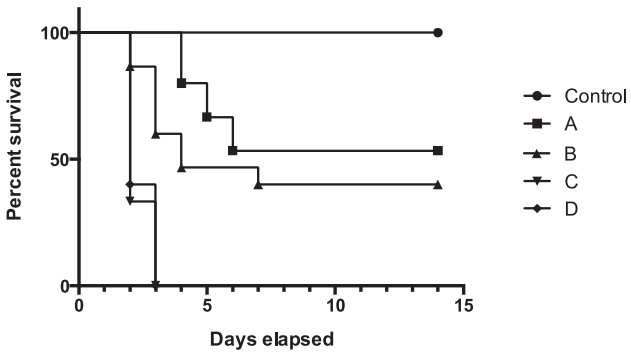

Figure 1. Mortality data for each fish group. Controls were never exposed to the pathogen. Group A were sublethally exposed, allowed to recover and then exposed to a lethal dose of *V. anguillarum*. Group B were swim buddies of group A but were not exposed to the sublethal dose; they received the lethal dose at the same time as group A. Group C received the lethal dose with not adapting sublethal exposure. Group D were added as swim buddies to group C and also received the lethal dose.

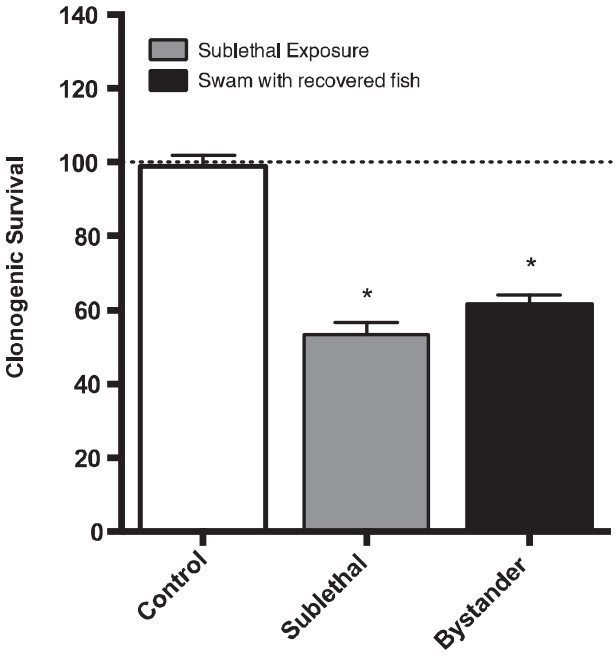

Figure 2. Clonogenic survival of HaCat cells after having been independently grown with explant-conditioned culture medium from the three fish groups. Direct and bystander groups are significantly different from the control ($P < 0.01$). Error bars indicate SEM.

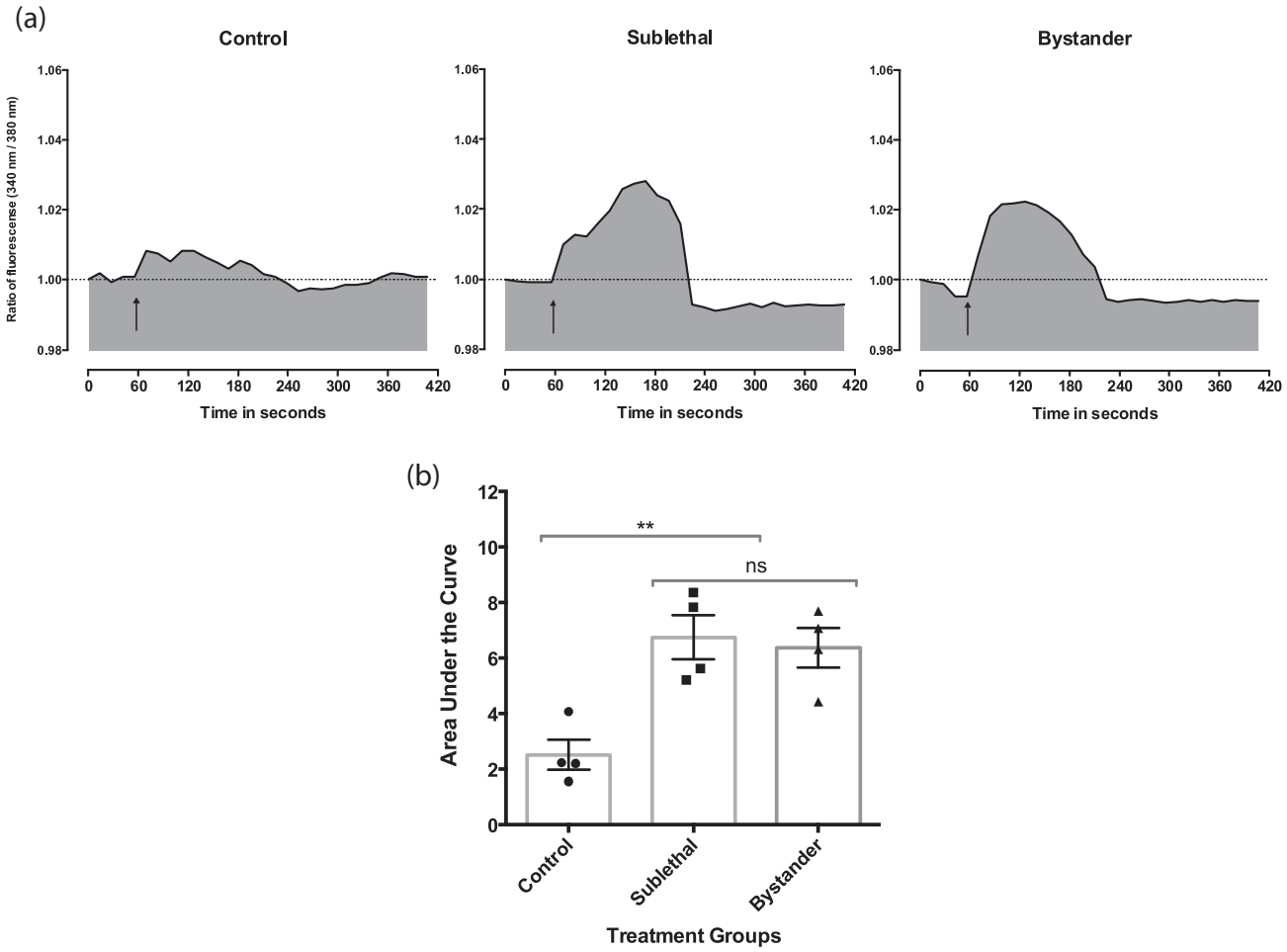

Figure 3. (a) Ratiometic analysis showing the calcium (Ca^{2+}) status of HaCat cells after being exposed to explant-conditioned culture medium from the three fish groups. Arrows indicate the inflexion point produced by the addition of the medium at 60 seconds. (b) Area under the curve (AUC) obtained from the ratiometic calcium images. AUC was calculated for each sample within the three fish groups. Significance was demonstrated using one-way ANOVA and the Holm-Sidak's multiple comparison test ($P < 0.01$). Error bars indicate SEM, 'ns' means 'not significant'.

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Conflict of interest statement. None declared.

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