

*Short communication*

## Antibody-dependent enhancement and persistence in macrophages of an arbovirus associated with arthritis

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Ross River virus (RRV) is the aetiological agent of epidemic polyarthritis (EPA) a predominantly rheumatic disease afflicting up to 5000 Australians annually. We show here for the first time that macrophages can be productively infected by RRV. Subneutralizing titres of anti-RRV IgG (but not IgM) also showed classical antibody-dependent enhancement (ADE) of RRV infection in macrophage and monocyte cell lines. No correlation between development of EPA and the pre-existence of ADE titres was apparent, nor could sera

raised against a related arbovirus, Barmah Forest, enhance RRV infection. Tumour necrosis factor- $\alpha$ , implicated in the immunopathogenesis of rheumatoid arthritis, was not secreted by RRV-infected monocytes or macrophages. Macrophage cell lines infected with RRV were, however, capable of producing virus for over 50 days. RRV-induced arthritis may therefore be due to the persistent productive infection of macrophages, perhaps established by a brief period of ADE early in infection.

Ross River virus (RRV) is a mosquito-borne alpha-virus endemic to Australia and New Guinea and is the aetiological agent of epidemic polyarthritis (EPA). In Australia, where EPA is a notifiable disease, up to 5000 cases are reported annually. In 1979/1980 an explosive epidemic also swept through several islands of the South Pacific resulting in tens of thousands of cases (Aaskov & Doherty, 1994). The principal symptom of EPA is arthritis/arthralgia, which is often severe and usually lasts for 30 to 40 weeks, with about 25% of EPA patients experiencing symptoms for a year or more (Fraser, 1986). Approximately half of the EPA patients also experience a rash, fever, myalgia and/or lethargy. The current treatment with non-steroidal anti-inflammatory agents can provide relief, but it is often inadequate. EPA is rare in children and overall reports for the ratio of asymptomatic to symptomatic infections (resulting in EPA) range from 50:1 to approximately 2:1 (Aaskov & Doherty, 1994; Fraser, 1986).

The rheumatic synovial exudates from EPA patients are largely devoid of neutrophils and contains a mononuclear cell infiltrate, predominantly composed of monocytes, vacuolated and phagocytic macrophages, T cells and B cells. RRV can be isolated from the peripheral

blood and viral antigens can be detected in synovial monocyte/macrophages during the first week after the onset of symptoms (Fraser, 1986). Elevated interferon- $\gamma$  (IFN- $\gamma$ ) levels were also found in exudates taken during this time (J. R. E. Fraser & A. L. Cunningham, personal communication).

RRV has a large host range and can infect many cell types (Aaskov & Doherty, 1994) including murine muscle cells, and human epidermal and synovial cells (Seay *et al.*, 1981; Fraser, 1986). Using the RRV prototype strain T48, we found that the following human cell types could be productively infected with RRV resulting in overt cytopathic effect (CPE): human primary dermal and synovial fibroblasts, primary endothelial cells, osteogenic sarcoma (ATCC CRL 1547), intestinal smooth muscle (ATCC CRL 1692) and HeLa (ATCC CCL 2). In contrast, B cell blasts, lymphoblastoid cell lines and T cell blasts (Thomson *et al.*, 1995) could not be infected by RRV (data not shown). T48 RRV stock virus was prepared by infecting HeLa cells (m.o.i. = 1) and harvesting supernatants, which contained approximately  $10^7$  TCID<sub>50</sub>/ml, at 60 h. Test cells ( $\approx 10^6$ ) were infected at m.o.i. = 1 for 90 min at 37 °C, then washed four times and cultured in 24-well plates and after 18, 24 and 48 h (i) supernatants were harvested for virus titration (TCID<sub>50</sub>) and (ii) cells were fixed in 100% cold methanol for 2 min for indirect immunofluorescence staining (IFA) using rabbit polyclonal anti-RRV sera and anti-rabbit

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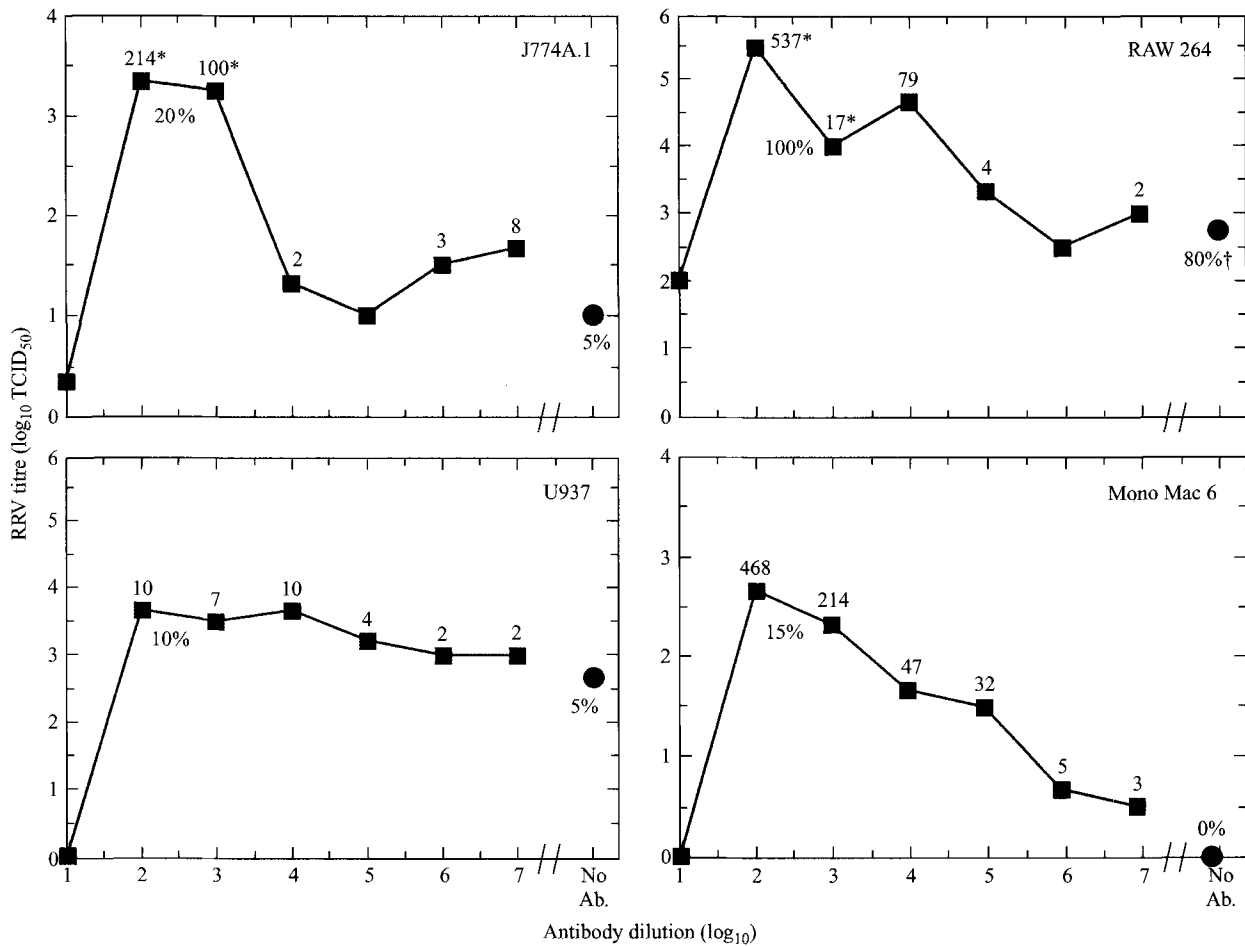


Fig. 1. ADE of RRV infection in four monocyte and macrophage cell lines. Cells were infected in the presence of different dilutions of antisera (from a recovered EPA patient) and RRV titres in the supernatants measured at 18 h p.i. The titre of RRV obtained in the absence of antisera is shown (No Ab; ●). Fold enhancement values (calculated as TCID<sub>50</sub> with antibody divided by TCID<sub>50</sub> without antibody) are given above the ADE curves for each antibody dilution. An estimate of the number of IFA-positive cells (%) is given for the 1:100 antibody dilution and in the absence of antibodies (No Ab). Asterisks indicate CPE observed at 18 h p.i. Dagger indicates CPE observed at 2-3 days p.i. The RRV titres obtained using the same dilutions of non-immune human sera were similar to the titres given for No Ab (data not shown).

FITC-labelled F(ab')<sub>2</sub> (Selenius). The TCID<sub>50</sub> assay was performed using 10-fold serial dilutions of 100 µl supernatant samples in flat-bottomed 96-well plates. Vero cells ( $2 \times 10^4$  in 100 µl) were then added into each well and the plates incubated at 37 °C for 7 days. The plates were stained with 0.05% crystal violet in 10% formaldehyde. Replication of RRV in the wide range of human cell types may correlate with the wide range of symptoms associated with EPA (Fraser, 1986); synovial fibroblasts with arthritis, myocytes with myalgia and epithelial and epidermal cells with the rash often seen in EPA patients. The ability of RRV to infect most adherent cells but not non-adherent cells suggests that the receptor for RRV may be a protein used by cells to adhere to the extracellular matrix, possibly an integrin (White, 1993).

Testing of monocyte and macrophage cells showed for the first time that macrophages but not monocytes could

also be infected with RRV. The murine macrophage lines J774A.1 (ATCC TIB 67) and RAW 264.7 (ATCC TIB 71), the human histiocytic lymphoma line U937 (ATCC CRL 1593) but not the mature human monocyte line Mono Mac 6 (Zeigler-Heitbrock *et al.*, 1988) could be productively infected by RRV. The percentages of cells which were IFA positive and the TCID<sub>50</sub> in the supernatants at 18 h post-infection (p.i.) are given in Fig. 1. Macrophages isolated from human peripheral blood mononuclear cells (PBMC) by adherence and cultured for 24 h could be infected, whereas PBMC could not be infected (data not shown). Cells were infected and washed as above and were grown in RPMI 1640 medium (Gibco BRL) with 2 mM-L-glutamine, supplemented with 10% endotoxin-free heat-inactivated fetal calf serum (FCS; PA Biologicals, Australia), 2 g/l NaHCO<sub>3</sub>, 100 U/ml penicillin and 100 µg/ml streptomycin (Flow).

Table 1. Lack of correlation between development of EPA and pre-existing ADE titres

The paired sera for patients 1 and 2 were taken within 2 weeks of each other. The first serum sample for patient 3 was taken 5 months before the second sample.

Serum samples	ELISA results			Maximum fold enhancement at antibody dilution
	RRV IgM	RRV IgG	BFV IgG	
EPA patient 1				
1st serum sample	+	-	-	No enhancement
2nd serum sample	+	+	-	468 at 10 <sup>-4</sup>
EPA patient 2				
1st serum sample	+	-	-	No enhancement
2nd serum sample	+	+	-	320 at 10 <sup>-4</sup>
EPA patient 3*				
1st serum sample	-	-	ND	No enhancement
2nd serum sample	+	+	-	100 at 10 <sup>-2</sup>
Asymptomatic individual 1*	-	+	-	513 at 10 <sup>-2</sup>
Asymptomatic individual 2*	-	+	ND	1479 at 10 <sup>-2</sup>
Seronegative control*	-	-	-	No enhancement
QML serum sample 15	-	-	+	No enhancement
QML serum sample 17	-	-	+	No enhancement

\* Identified by serological survey of Queensland volunteers.

Murine lines were supplemented with  $1 \times 10^{-5}$  M-2-mercaptoethanol.

The ability of macrophage cell lines to replicate RRV suggested that RRV might infect these cells using antibody and Fc receptor, resulting in the classical antibody-dependent enhancement (ADE) of infection described for other viruses, including related alphaviruses (Porterfield, 1986; Morens & Halstead, 1990). To determine whether ADE of RRV infection would occur, each monocyte and macrophage cell line was infected with RRV complexed with serial dilutions of heat-inactivated anti-RRV serum (neutralizing titre of 1/160) obtained from a patient (JFF) who had recovered from EPA. Each serum dilution (200  $\mu$ l) was first incubated with 200  $\mu$ l of RRV containing 10<sup>6</sup> TCID<sub>50</sub> at room temperature for 1 h. The virus-antibody complex was used to infect 10<sup>6</sup> cells as above and TCID<sub>50</sub> determination and IFA were performed at 18 h p.i. Subneutralizing levels of antibody dramatically increased the level of infection of U937 and the murine macrophage lines, and resulted in infection of the monocyte line, Mono Mac 6 (Fig. 1). Maximum enhancement of infection occurred at an antibody dilution of 1:100 (log<sub>10</sub> antibody dilution of -2) with the 1:10 dilution resulting in virus neutralization. Both the number of cells infected (measured by IFA) and virus production (also indicated as fold enhancement values) were increased in the presence of subneutralizing levels of antibody (Fig. 1). The ADE curves conform to the criteria for viral enhancement described by Morens & Halstead (1990). Control sera containing no RRV-specific IgG failed to give ADE of RRV infection (Table 1). Neither lymphoblastoid cell lines nor B cell blasts (both Fc receptor

positive) could be infected using ADE. ADE via the complement receptor (Cardosa *et al.*, 1983) could not be detected using IgM-positive/IgG-negative sera and Mono Mac 6 in the presence of complement (data not shown).

The ADE phenomenon, clearly demonstrated here for RRV, is implicated in the aetiology of dengue haemorrhagic fever, in which a second infection with a different dengue serotype is exacerbated by cross-reacting subneutralizing IgG raised during the first infection (Morens & Halstead, 1990). Different RRV serotypes or secondary infections with RRV have not been reported, however, pre-existing enhancing IgG titres might arise from previous infections with serologically related alphaviruses, for example Barmah Forest or Sindbis. Paired sera from three EPA patients showed that ADE titres for RRV did not exist prior to the development of RRV IgG, showing that development of EPA was not due to pre-existing enhancing antibodies. Sera from asymptomatic RRV-seropositive individuals and chronic EPA patients also had similar enhancing titres and sera specific for the serologically related Barmah Forest virus failed to enhance RRV infection (Table 1). No simple correlation between ADE titres and EPA could thus be established and the role, if any, of ADE in EPA pathogenesis remains elusive. One might speculate that ADE operates early in infection when rising (but still subneutralizing) IgG titres coincide with the serum viraemia (Seay *et al.*, 1981) giving rise to increased infection of macrophages and monocytes. Such a mechanism may be important for establishing a persistent infection in these cells (Kanno *et al.*, 1993).

The importance of tumour necrosis factor- $\alpha$  (TNF- $\alpha$ )

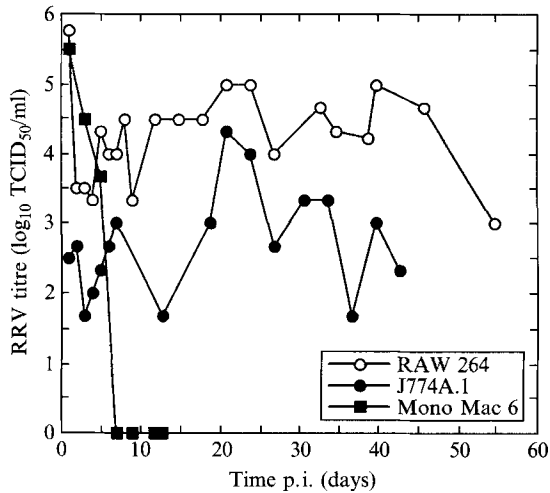


Fig. 2. Persistence of RRV production by RRV-infected J774A.1 and RAW 264 cells and ADE-infected Mono Mac 6 cells. The cells were washed four times after a 2 h infection period and the culture medium was changed completely every day for the first 10 days and every 3 days thereafter. Cells were split and reseeded at  $5 \times 10^5$ /ml every week.

in the immunopathology of rheumatoid arthritis (RA) (Elliott *et al.*, 1993) and the production of TNF- $\alpha$  by monocytes and macrophages following infection with some viruses (Chang & Shaio, 1994) prompted an extensive examination of RRV- and ADE-induced TNF- $\alpha$  production by the cell lines, PBMC and PBMC monocyte-derived macrophages. Cells were infected with RRV (m.o.i. = 1), with and without ADE (antibody dilutions as Fig. 1) and supernatants taken at 3, 6, 12, 24 and 48 h p.i. The standard L929 bioassay was used to quantify TNF- $\alpha$  (Hogan & Vogel, 1994) after exposure of the supernatants to  $960 \mu\text{W}/\text{cm}^2$  of UV-C for 2 h to inactivate the RRV. Lipopolysaccharide-treated cultures ( $5 \mu\text{g}/\text{ml}$ ; positive controls) consistently produced TNF- $\alpha$  levels 10-fold higher than untreated cells (negative controls) and always exceed 2 (usually 4–7) ng/ml TNF- $\alpha$ . In this extensive series of experiments no RRV-induced TNF- $\alpha$  could be detected (data not shown). Although TNF- $\alpha$  has clearly been shown to be an important mediator of RA (Elliott *et al.*, 1993) an involvement of this cytokine has not been reported for viral arthritides. The cellular infiltrate of RA is also polymorphonuclear whereas it is predominantly mononuclear in EPA effusions. The cytokines found in RA joint effusions are also predominantly macrophage-derived (Elliott *et al.*, 1993) whereas the EPA effusions contain elevated levels of the T cell-derived cytokine, IFN- $\gamma$  (J. R. E. Fraser & A. L. Cunningham, personal communication). The cytokines and the pathological mechanisms responsible for RA and EPA, where the arthritides have an autoimmune and infectious (viral)

aetiology respectively, may thus be quite distinct (Simon *et al.*, 1994).

Alphaviruses are classically believed to induce rapid lysis (or CPE) of infected vertebrate cells, however, recently several reports have shown that alphavirus infection can be persistent (Levine *et al.*, 1994). The possible persistence of RRV infection in monocyte/macrophages was analysed by infecting RAW 264, J774A.1 and Mono Mac 6 (the latter by ADE using a 1:100 dilution of anti-RRV antibody) and determining RRV titres in the supernatants at the indicated time points. The RAW 264 and J774A.1 cultures continued to produce virus for over 50 days p.i. Virus yields from Mono Mac 6, however, dropped to zero by day 6 p.i. (Fig. 2). J774A.1 and Mono Mac 6 cultures showed no signs of overt CPE. RAW 264 showed overt CPE by day 3 p.i. but the few remaining cells in this culture divided and reached the original input cell number by day 10 p.i. No overt CPE was observed in these cultures after day 6 p.i. despite continuous production of virus. Virus taken from day 40 p.i. cultures was able to induce overt CPE in fresh RAW 264 cells. About 5–10% of the persistently infected RAW264 cells were IFA-positive at day 40 and 50 p.i. (data not shown). ADE infection of Raw 264 did not change these observations or the pattern of persistent virus production (data not shown). While viral persistence in monocyte/macrophages has previously been reported (Kanno *et al.*, 1993; Kurane *et al.*, 1990; Narayan *et al.*, 1992) this is the first report, to our knowledge, of alphavirus persistence in this cell type.

Recent developments in the understanding of infectious arthritides have highlighted the importance of persistent organisms in synovial macrophages in the pathogenesis of viral (Narayan *et al.*, 1992; May *et al.*, 1994) and bacterial (Keat & Hughes, 1993) arthritides. RRV antigens can be detected in macrophages from EPA effusions. However, the limited early attempts at isolating infectious RRV from the joint fluids were unsuccessful and occurred before the highly sensitive C6/36 mosquito cell line was established (Fraser, 1986; J. R. E. Fraser, personal communication). These experiments are difficult to repeat as joint aspirations are now considered unethical for EPA patients. The detection of viable RRV in EPA effusions would clearly be an important step toward understanding the pathogenesis of EPA.

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