Pathogenesis of Dugbe virus infection in wild-type and interferon-deficient mice

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In 129 mice, infection with the nairovirus Dugbe virus (DUGV) was lethal following intracerebral but not intraperitoneal inoculation. Following both routes of inoculation, immunostaining of tissue sections demonstrated virus-positive cells in the brain, indicating that DUGV is neuroinvasive in mice. Many brain areas were affected and neurones were the main cell type infected. Infected cells showed punctate accumulations of viral nucleoprotein in the cytoplasm, indicative of virus replication sites. Immunostaining for activated caspase 3 demonstrated no evidence of apoptosis. The type I interferon (IFN) system plays a significant role in defence against DUGV, as 129 IFN-α/β R−/− mice died rapidly following both intraperitoneal and intracerebral inoculations. Studies were undertaken to determine whether the IFN-inducible proteins, protein kinase R (PKR) and MxA, were important for protection; neither PKR nor constitutively expressed human MxA played significant roles.

Dugbe virus (DUGV) was the first member of the genus Nairovirus of the family Bunyaviridae to be characterized fully. Other genera in the family are Orthobunyavirus, Hantavirus, Phlebovirus and Tospovirus (Elliott et al., 2000). Nairoviruses are tick-borne viruses; the ticks are often carried by birds and this has resulted in a worldwide distribution of nairoviruses. Nairoviruses have larger genomes than members of other Bunyaviridae genera and more complex glycoprotein processing (Marriott & Nuttall, 1996; Sanchez et al., 2002, 2006). There are 34 recognized nairovirus species within seven serotypes. DUGV is a member of the Nairobi sheep disease group, which also contains Nairobi sheep disease virus, a pathogen of sheep and goats. The most serious human pathogen of the genus is Crimean-Congo hemorrhagic fever virus (CCHFV), which can induce fatal haemorrhagic disease in up to 50% of patients (Swanepoel et al., 1987; Papa et al., 2002). The incidence of CCHFV infection is increasing, with recent outbreaks in regions as diverse as southern and eastern Europe, China, Africa (particularly Mauritania), Pakistan and Russia (reviewed by Whitehouse, 2004; Burt & Swanepoel, 2005). In contrast, DUGV itself is a relatively apathogenic virus of African origin (David-West & Porterfield, 1974; Bridgen et al., 2002), which can induce thrombocytopenia in man (Burt et al., 1996).

There have been two independent studies of the pathogenicity of DUGV in mice (Buckley et al., 1990; Coates &Sweet, 1990). Other murine studies have been performed with the related Hazara virus (Smirnova et al., 1977) and with CCHFV (early experiments are summarized in Hoogstraal, 1979; see also Tignor & Hanham, 1993; Gonzalez et al., 1995). Buckley et al. (1990) demonstrated that DUGV was neurotropic. Both newborn and 3-week-old outbred Swiss white mice inoculated intraperitoneally (i.p.) with DUGV strains including IbAr 1792 and KT 281/75 died from viral infection and virus was isolated from the brains of these mice. Neurovirulence differed among strains, with strains IbH 11480 and IbAr 1792 being more neurovirulent; other strains such as ArD 44313 killed newborn but not adult mice when inoculated i.p. Coates & Sweet (1990), using Dugbe strain KT 281/75, demonstrated lethal disease by all routes of inoculation in neonatal mice, but only by inoculation intracerebrally (i.c.) in adult mice. In infected neonatal mice, the highest viral titres were observed in the upper respiratory tract, spleen, liver, heart and brain.

In this study, we used genetically modified mice to investigate the effect of the host interferon (IFN) system and IFN-related genes on the outcome of infection. Specifically, we used mice in which the IFN-α/β receptor gene was deleted (IFN-α/β R−/− mice; Müller et al., 1994). Cells infected with virus in these animals produce IFN-β but cannot induce an antiviral state. IFN-α and -β (type I IFNs) induce the activation of >300 genes (Stark et al., 1998). Of these, the most thoroughly characterized antiviral factors are the Mx proteins (Haller et al., 1998), the 2′-5′-oligoadenylate synthetase (2′-5′-OAS)/RNase L system (Silverman, 1994) and protein kinase R (PKR; Williams, 1999). Laboratory
mice do not normally express Mx1 (reviewed by Pavlovic et al., 1995). To study the effect of the IFN-inducible genes PKR and human MxA, PKR knockout mice (Yang et al., 1995) and transgenic IFN-α/β R−/− MxA+/+ mice that constitutively expressed the human MxA protein (Hefti et al., 1999) were used. The genotypes of all mice were verified by PCR.

In order to study mortality, groups of at least six 3–4-week-old mice were inoculated either i.c. or i.p. using 1000 p.f.u. DUGV strain IbAr 1792 (kindly provided by Dr Porterfield, Oxford, UK). Virus was grown and titrated in Xenopus laevis XTC-2 cells (Watret et al., 1985; Bridgen et al., 2004). All animal studies were carried out under the authority of a UK Home Office licence. Mice were kept under specific-pathogen-free conditions in environmentally enriched housing with food and water ad libitum. Animals were monitored twice daily and euthanized on reaching clinically defined terminal endpoints. The mortality following infection of wild-type 129 and IFN-α/β R−/− mice with DUGV can be seen in Fig. 1. In contrast to wild-type 129 mice, 129 IFN-α/β R−/− mice died rapidly (within 4 days) after inoculation i.p. with DUGV. This indicated that an intact type I IFN system plays an important role in defence against DUGV. However, neither the presence of MxA nor the absence of PKR appeared to have a major impact on mortality (Fig. 1).

MxA is a 76 kDa GTPase, which is expressed in the cytoplasm in response to IFN-α/β and has been shown to inhibit a wide range of RNA viruses (reviewed by Haller et al., 1998; Kochs et al., 2002). It is particularly effective against the orthobunyavirus La Crosse virus (LACV) both in vitro and in vivo (Frese et al., 1996; Hefti et al., 1999). Previous studies have demonstrated very effective inhibition of nairovirus replication in vitro with MxA (Andersson et al., 2004, 2006; Bridgen et al., 2004). We have observed previously that MxA causes DUGV titres to drop by 400–600-fold, while Andersson et al. (2004, 2006) showed inhibition of CCHFV of up to 1000-fold. The latter authors also demonstrated co-immunoprecipitation of MxA and CCHFV nucleocapsids, as well as co-localization of these two proteins in the perinuclear area. Therefore, we studied the effects of MxA in an in vivo context. To our surprise, there was no significant difference in mortality in the 129 IFN-α/β R−/− mice compared to the 129 IFN-α/β R−/− MxA+/+ mice (Fig. 1). In the presence of MxA, blood DUGV titres showed no consistent reduction. These in vivo results were in contrast to the in vitro results discussed above. One factor that might contribute to the discrepancy between the in vitro and in vivo results is the expression level of MxA. Pavlovic et al. (1995) examined the MxA+/+ transgenic mice that were subsequently used to generate the 129 IFN-α/β R−/− MxA+/+ animals and found that MxA was expressed at low levels in all organs in which DUGV replicates except the liver. The MxA level in the brain was 450 ng (100 mg tissue)−1, substantially lower than the 20 mg (100 mg soluble protein)−1 in the transfected cells. Hefti et al. (1999) extended these observations by demonstrating MxA expression in brain cells including neurones of 129 IFN-α/β R−/− MxA+/+ mice. MxA expression in these animals was sufficient to inhibit low-dose infections with LACV, the alphavirus Semliki Forest virus and the orthomyxovirus Thogoto virus in vivo.

Another important mediator of IFN-induced viral inhibition is PKR. This is activated by the presence of dsRNA (an obligatory product of RNA virus replication). PKR plays a significant role in a number of viral infections, but there has been only one specific study to date of its role in bunyavirus infection (Streitenfeld et al., 2003). There have also been several reports of hantavirus microarray experiments in which raised levels of IFN-induced proteins including Mx...
and 2'-5'-OAS have been observed, but not PKR (Nam et al., 2003; Khaiboullina et al., 2004). Activation of PKR leads to phosphorylation of the translation initiation factor eIF-2α, potentially inhibiting both host and viral protein synthesis. The orthobunyavirus Bunyamwera virus (BUNV) additionally affects host protein synthesis indirectly by altering the activity of host RNA polymerase II, thus reducing host transcription, and by cap snatching of host transcripts.

Streitenfeld et al. (2003) showed that PKR was activated in cultured cells, but that this activation did not lead to inhibition of BUNV replication. However, they observed an in vivo effect of PKR: PKR-deficient mice died on average 1 day earlier than wild-type mice when infected with BUNV. Our mortality results indicated that PKR does not play a significant role in defence against DUGV infection (Fig. 1). DUGV replicates more slowly than BUNV and hence may not activate PKR as readily; this is suggested by the inability of DUGV to shut off host-cell protein expression in vitro (Watret et al., 1985).

For studies of pathogenesis, groups of six mice were infected i.p. or i.c. with DUGV and killed at specific time points post-infection. Samples of whole blood were diluted 1:10 in PBS plus 0.75% BSA and stored at −70°C for subsequent virus titration on XTC-2 cells. Brains were removed and bisected sagittally down the midline. Half was immersion-fixed in 10% neutral buffered formalin (Surgipath) and processed into 5 μm paraffin sections and half was cryo-preserved in sucrose and cut into 15 μm frozen sections. Paraffin sections were assessed first and gave excellent staining results; they were deparaffinized with Clearene, rehydrated and processed for antigen retrieval and then incubated with primary rabbit polyclonal antiserum directed against DUGV proteins (1:100 dilution of antibody; kindly provided by Dr E. Gould, Oxford, UK) or cleaved caspase 3 (PC679, diluted 1:10; Oncogene Research Products). This was followed by incubation with a biotinylated goat anti-rabbit antibody (BA-1000, diluted 1:500; Vector Laboratories). Antibody staining was visualized using Vectastain ABC solution, followed by diaminobenzidine (DAB). Sections were counterstained with haematoxylin, dehydrated, cleared in Clearene and mounted.

Immunostaining for DUGV nucleocapsid protein in brain sections of infected mice revealed virus replication in many brain areas including the cerebral cortex, thalamus, brain stem, cerebellum and olfactory bulb (Fig. 2). Two days after

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**Fig. 2.** Immunostaining for DUGV (brown) in 5 μm paraffin-processed brain sections using a rabbit polyclonal antibody that primarily recognises the viral nucleocapsid protein (Bridgen et al., 2004). Punctate cytoplasmic staining (arrows show examples), putative virus replication centres, was evident in neurons in many brain areas. (a) Diagrammatic representation of a sagittal section of the mouse brain. cb, Cerebellum; cc, corpus callosum; cb, cortex; ob, olfactory bulb; h, hippocampus/dentate gyrus; p, pons; t, thalamus. DUGV-infected cells were observed in all brain areas. (b) Olfactory bulb from an IFN-α/β R−/− MxA+/+ mouse inoculated i.c. with DUGV, at 2 days post-inoculation. Infected cells were apparent predominantly in the neuronal mitral cell layer (m), but also in the external plexiform layer (epl). (c) Higher power magnification showing punctate staining in the cytoplasm of mitral cells, from the mouse shown in (b). (d) Infected mitral layer neurons from a 129 mouse inoculated i.p. with DUGV, at 4 days post-inoculation. (e) DUGV-infected cells (brown) in the dentate gyrus of a 129 mouse infected i.p. with DUGV, at 4 days post-inoculation. (f) Infected cortical neurons in an IFN-α/β R−/− MxA+/+ mouse inoculated i.c. with DUGV, at 2 days post-inoculation. Bars, 40 μm (b); 10 μm (c–f).
inoculation i.p., extensive viral staining was seen, indicating
that this virus is efficiently neuroinvasive. Brain virus dis-
tribution and tropism were similar regardless of the route
of infection or immune status of the mice (Fig. 2). DUGV
was able to enter and replicate in the brain following i.p.
inoculation, even in the presence of an intact IFN system,
although this system provided sufficient protection to avoid
mortality in wild-type 129 mice.

Immunostaining also demonstrated the specific cell types
infected by DUGV within the brain. In all regions, DUGV
staining was observed primarily in the cytoplasm of cells
and predominantly in cells with a neuronal morphology
(Fig. 2). Staining was punctate, a distribution consistent with virus
replication sites. In the olfactory bulb, neurones of the mitral
cell layer were infected. In the cerebellum, viral staining was
observed in neuronal Purkinje cells and in adjacent cells,
probably Bergman glia cells, as well as in cells of the mole-
cular and granule cell layers. In the cortex and thalamus,
large cells consistent with a neuronal morphology were
infected. There was no evidence of extensive infection of
glia, ependymal or meningeal cells. This specificity of
DUGV infection for neuronal cells was striking, particularly
in the light of the normally wide cell tropism of nairoviruses
(unpublished results) and may indicate that there is a specific
viral receptor in these cells.

The immunostaining for active caspase 3 revealed no signs
of apoptosis in infected brain sections. This equates well to
observations in cell culture; there is little cytopathic effect in
most cell lines, with the exception of the X. laevis XTC-2 line
used for viral plaque assays and the human adrenal gland
carcinoma cell line SW13 (unpublished data; it is possible
that this cell line supports high levels of DUGV replication
due to deficiencies in the innate immune response). There
have been mixed reports of the relevance of apoptosis in
infections by members of the family Bunyaviridae; for example some but not other members of the genus Hanta-
virus induce apoptosis (Li et al., 2004; Hardestam et al.,
2005). The orthobunyavirus BUNV is not strongly apoptotic
unless the non-structural NSs gene is deleted (Kohl et al.,
2003), whereas, surprisingly, another orthobunyavirus,
LACV, is strongly pro-apoptotic when NSs is present but
not when it is deleted (Blakqori & Weber, 2005). Murine
infections with LACV show pronounced brain apoptosis
(Pekosz et al., 1996). Homology has been observed between
the NSs gene product and the pro-apoptotic Drosophila
reaper protein (Colo´ n-Ramos, 2003), but this seems
to apply only to the NSs proteins of some bunyaviruses,
especially those of the California serogroup that includes
LACV.

In conclusion, we have confirmed that DUGV is neuro-
invasive in mice. In addition, we have demonstrated that
the main cell type infected in the brain is the neurone, and
indeed that infection in this organ appears to be largely
confined to neuronal cells. We have also confirmed that
the type I IFN system plays a major role in controlling DUGV
replication, but that PKR is not required for this protective
response and that the constitutively expressed MxA is not
sufficient for protection. We are therefore left to speculate
which protein or, more likely, combination of proteins
mediates the IFN response to DUGV in the wild-type 129
mice. Many of the > 300 genes activated by IFN-α/β (Stark
et al., 1998) are only poorly characterized; there are clearly
many other candidates.

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