Hazara virus infection is lethal for adult type I interferon receptor-knockout mice and may act as a surrogate for infection with the human-pathogenic Crimean–Congo hemorrhagic fever virus

Stuart D. Dowall, Stephen Findlay-Wilson, Emma Rayner, Geoff Pearson, Janice Pickersgill, Antony Rule, Natasha Merredew, Hazel Smith, John Chamberlain and Roger Hewson

Health Protection Agency, Porton Down, Salisbury, Wiltshire SP4 0JG, UK

Hazara virus (HAZV) is closely related to the Crimean–Congo hemorrhagic fever virus (CCHFV). HAZV has not been reported to cause human disease; work with infectious material can be carried out at containment level (CL)-2. By contrast, CCHFV causes a haemorrhagic fever in humans and requires CL-4 facilities. A disease model of HAZV infection in mice deficient in the type I interferon receptor is reported in this study. Dose–response effects were seen with higher doses, resulting in a shorter time to death and earlier detection of viral loads in organs. The lowest dose of 10 p.f.u. was still lethal in over 50% of the mice. Histopathological findings were identified in the liver, spleen and lymph nodes, with changes similar to a recent mouse model of CCHFV infection. The findings demonstrate that inoculation of mice with HAZV may act as a useful surrogate model for the testing of antiviral agents against CCHFV.

Hazara virus (HAZV) is a member of the genus Nairovirus of the family Bunyaviridae. Currently there are 35 tick-borne viruses assigned to the genus, which is further arranged into seven related serogroups: (i) Crimean–Congo hemorrhagic fever (CCHF); (ii) Dera Ghazi Khan; (iii) Hughes; (iv) Nairobi sheep disease; (v) Qalyub; (vi) Sakhalin and (vii) Thialfara on the basis of complement fixation, haemagglutination inhibition and infectivity neutralization studies (Berge et al., 1971; Bishop et al., 1980). HAZV is closely related to the Crimean–Congo hemorrhagic fever virus (CCHFV) and these viruses share the same serogroup (Casals & Tignor, 1974, 1980; Foulke et al., 1981). CCHF is the most widespread tick-borne viral haemorrhagic fever affecting humans; it is endemic in many areas of Africa, the Middle East, Asia and southern Europe (Appannanavar & Mishra, 2011). Endemic foci exist in the Balkans, Russia and the former Soviet Union (Maltezou et al., 2010), whilst other countries such as Turkey, Greece and South Africa have high annual caseloads (Leblebicioğlu, 2010). The extensive distribution of CCHFV, potential for nosocomial transmission, severity of the disease and the high fatality rate, make CCHF a major public health concern (Ergönil, 2006). CCHFV is a hazard group 4 agent and work with infectious CCHFV requires containment level (CL)-4 laboratory facilities. In consequence, research into this pathogen is restricted.

HAZV is non-pathogenic in humans and, as a hazard group 2 pathogen, can be handled in low containment (CL)-2 facilities. It was isolated from Ixodes redikorzevi ticks collected from the Hazara District of Western Pakistan in 1964 using intracerebral inoculation of suckling mice (Begum et al., 1970; Begum & Wissesman, 1970). Studies of CCHF infections have been limited due to the lack of a suitable animal model. Despite many species being tested, only newborn mice have shown signs of disease (Smirnova et al., 1977). However, due to their undeveloped immune system the use of newborn mice is restricted to isolation protocols. The natural route of infection is via percutaneous tick bite, but in these earlier studies the virus needed to be delivered intracranially or intraperitoneally to cause the disease (Begum & Wissesman, 1970; Smirnova et al., 1977). Recently, transgenic mice with knockouts in the STAT-1 signalling molecule and type-I interferon receptor (IFN-α/βR−/−) have been shown to be susceptible to infection with CCHFV (Bente et al., 2010; Berezky et al., 2010). However, these models have yet to be assessed in vaccine efficacy studies, with progress being restricted by the need to work at a high containment level (CL-4). Recently, in vitro-based assays of HAZV infection have been used to show therapeutic effects of antiviral agents, including small interfering RNAs and ribavirin as a model for CCHFV infection (Flusin et al., 2011). Similar surrogate models have been used for other viral haemorrhagic fever viruses, such as Lymphocytic choriomeningitis virus (Zapata et al., 2011) and Pichinde virus as models for Lassa fever (Lan et al., 2009).

The purpose of this study was to determine whether IFN-α/βR−/− mice were sensitive to HAZV infection via intradermal
inoculation similar to the natural mode of transmission. Forty-two adult (6–10-week-old) IFN-α/βR⁻/⁻ (strain A129) and six wild-type control (strain 129Sv/Ev) mice (B&K Universals) were used. Throughout the course of the study animals were housed in flexible-film isolators under climate-controlled conditions. Food and sterile water were available ad libitum. All procedures were undertaken according to the UK Animals (Scientific Procedures) Act 1986.

Mice were injected intradermally with 25 μl HAZV (strain JC280 obtained from J. Cassals in 1980, passaged once in suckling mice at Porton Down and sequenced, GenBank accession nos: L-DQ076419.1, M-DQ813514.1 and S-M86624.1) into each of the left and right hind legs. Dilutions were made with PBS solution so that IFN-α/βR⁻/⁻ mice were infected with 4 × 10⁴, 10³ or 10 p.f.u. virus per mouse (n=12 per dose). Wild-type mice were infected with the higher dose only, 4 × 10⁴ p.f.u. (n=6). IFN-α/βR⁻/⁻ mice were injected with PBS alone as a control (n=6). Back titrations were performed to confirm virus concentrations of the different doses. Animals were weighed and temperatures taken once daily, with twice daily health status checks. Clinical

![Graph showing survival, weight, temperature, and health scores over time](http://vir.sgmjournals.org)
findings were scored according to the following: ruffled fur, 1; lethargy, 2; arched back, 2; inactive, 3; and death, 5. Mice that showed signs of severe disease were euthanized when they reached a predefined, humane clinical end-point.

At days 1 and 3 post-infection, three mice from each of the HAZV-infected IFN-α/βR−/− groups and one from each of the control groups were euthanized. On day 5, three mice from the lowest dose HAZV-infected IFN-α/βR−/− group (10 p.f.u.), and one from each control group were euthanized. All surviving mice were culled on day 14. From all euthanized animals, tissues were perfused with sterile PBS solution and samples of spleen, liver, local lymph node and skin were placed into pre-weighed tubes for virological analysis, and reweighed to determine the mass. Tissues were mechanically disrupted using ceramic beads and a homogenizer to break up cellular structures. Samples were centrifuged to remove cell debris and the supernatant collected to determine viral load by plaque assay on SW13 cells (obtained from the European Collection of Cell Cultures). Viral RNA was also sequenced. The plaque assay results were then calculated to determine the p.f.u. per gram of tissue. The remainder of the organs and the whole animal were stored in 10 % neutral buffered formalin. Samples of liver, spleen, heart, lung, skin (inoculation site), draining lymph node, kidney and brain were processed routinely to paraffin wax. Sections were cut 5–6 μm thick, stained with haematoxylin and eosin (H&E) and examined microscopically.

The genomic sequence of the virus remained unchanged. All the IFN-α/βR−/− mice infected with 4 × 10⁴ and 10⁵ p.f.u. HAZV died (Fig. 1a). However, in the low dose group (10 p.f.u.) one animal survived the length of the experiment (14 days). The median time to death for the 4 × 10⁴ and 10³ p.f.u. doses was 5 days, and for the 10 p.f.u. dose was 7 days. The 50 % lethal dose (LD₅₀) was determined to be 4 p.f.u., using the method of Miller and Tainter (Randhawa, 2009).

Deaths were not observed in the mock-infected IFN-α/βR−/− mice or wild-type mice given 4 × 10⁴ p.f.u. HAZV-infected IFN-α/βR−/− mice showed a dose-specific decrease in weight on days 2, 3 and 4 with the 4 × 10⁴, 10³ and 10 p.f.u. infection doses, respectively (Fig. 1b). Increases in temperature of up to 2 °C were observed in the IFN-α/βR−/− mice infected with 4 × 10⁴ and 10³ p.f.u. HAZV, but not with the group infected with 10 p.f.u. (Fig. 1c). As infection progressed the temperatures in all groups declined rapidly. Weights and temperatures of the HAZV-infected wild-type mice and mock-infected IFN-α/βR−/− mice remained similar throughout the course of the study. Clinical scores indicated that the HAZV-infected IFN-α/βR−/− mice showed clinical signs in a dose-dependent manner compared with the control groups (Fig. 1d).

Virus was detected in the liver, spleen and draining lymph node of all the HAZV-infected IFN-α/βR−/− mice (Fig. 2). Viral antigen was not detected in the skin samples taken from the site of virus inoculation (data not shown). The highest titres seen day 1 post-infection were in the group infected with the highest dose, 4 × 10⁴ p.f.u., in the liver and lymph node. By day 3, all groups had virus detectable in the liver, spleen and lymph node. However, at this time point the group with the lowest dose (10 p.f.u.) had only small concentrations in the spleen. By day 5, all of the animals in the 4 × 10⁴ and 10³ p.f.u. infected groups had died. Samples taken from animals infected with 10 p.f.u. showed high titres of HAZV in the liver and spleen, but lower titres in the lymph node. No virus was seen in the wild-type mice infected with 4 × 10⁴ p.f.u. HAZV- or the mock-infected IFN-α/βR−/− mice (data not shown).

Histopathological changes were observed in the liver, spleen and draining lymph node of HAZV-infected IFN-α/βR−/− mice. In the liver, scattered foci of coagulative necrosis, associated with a mixed polymorphonuclear neutrophil (PMN) and mononuclear cell infiltrate, were observed in two mice infected with 4 × 10⁴ p.f.u. HAZV on day 3 post-infection (Fig. 3a); one mouse in the 10³ p.f.u. group at day 3 post-infection and two mice in the 10 p.f.u. group at day 5 post-infection. Furthermore, a single focus of

**Fig. 2.** Viral loads in liver, spleen and lymph node tissues of IFN-α/βR−/− mice infected with HAZV at doses of 4 × 10⁴ (plain bars), 10³ (striped bars) and 10 (dotted bars) p.f.u. Bars show mean level of a group of three mice, with error bars denoting SE. n=3 per group.
hepatocyte vacuolation associated with a PMN infiltration was observed in one animal given 10 p.f.u. at day 14. In addition, scattered mononuclear cell foci, often associated with PMNs, were seen in some animals in all infected groups and in one animal in the saline control group (Fig. 3b). In the spleen, changes were not seen on day 1 post-infection in any group. On day 3 post-infection, in all HAZV-infected IFN-α/β R−/− mice there appeared to be some lymphocyte depletion of the white pulp which appeared irregular (Fig. 3c), along with a histiocytic and variable PMN cell infiltration (Fig. 3d), consistent with splenitis. This was more prominent in the groups given the highest doses (4 × 10⁴ and 10⁵ p.f.u.). By day 5, changes in all animals in the 10 p.f.u. group were similar to those observed with the higher doses at day 3. At day 14, in the one animal examined in the 10 p.f.u. group, the spleen appeared normal. In all control animals the spleen was normal (Fig. 3e). In the draining lymph nodes, due to difficulties of collection, it was not always possible to identify the lymph node grossly. Changes were not seen on day 1 post-infection in any group. On day 3, in 2/2 animals examined in the 4 × 10⁴ and 10⁵ p.f.u. infected groups, focal areas of subcapsular and cortical necrosis characterized by pyknotic and karyorrhectic debris, decreased numbers of lymphocytes and a local infiltration of macrophages and occasional PMNs were seen (Fig. 3f). On day 5, in the 10 p.f.u. infected animals, similar changes were observed in 2/2 animals examined. In the remaining animals infected with HAZV, and both control groups, the lymph nodes were normal. In the skin, focal inflammation was observed at the injection site in the subcutis of only one animal in the highest dose, 4 × 10⁴ p.f.u. HAZV (day 1 post-infection), and one animal in the 10 p.f.u. group (day 5 post-infection). Remaining samples in infected and control groups were normal. In all HAZV-infected and saline control animals, changes were not observed in the heart, lung, kidney or brain.

Inoculation of transgenic mice via a natural, intradermal route of infection with HAZV resulted in clinical signs, mortalities and pathological changes in the liver, spleen and lymph nodes. The model of HAZV infection described here is similar to that reported in STAT-1 and IFN-α/β R−/− mice infected with CCHFV (Bente et al., 2010; Bereczky et al., 2010). With CCHFV infection, the IFN-α/β R−/− mice exhibited 100% mortality when 10 f.f.u. of virus was administered, whereas wild-type mice survived doses as high as 10⁶ f.f.u. (Bereczky et al., 2010). Similarly, wild-type mice infected with HAZV in the present study survived, confirming the resistance of normal mice to infection with these viruses. The highest dose of HAZV used in this study was
4 × 10^4 p.f.u., due to restrictions with the concentration of the stock virus. The time to death post-infection in IFN-α/βR−/− mice infected with CCHFV was 2–4 days (Bereczky et al., 2010), and in STAT-1-knockout mice 3–5 days (Bente et al., 2010). This is similar to the 5 days for HAZV infection reported here, when most mice reached humane clinical endpoints. However, the former infection was achieved via the intraperitoneal route for CCHFV, whereas HAZV was administered via the intradermal route in the present study. High viral loads identified the liver and spleen as the target organs for HAZV infection in IFN-α/βR−/− mice, similar to that of the CCHFV model (Bente et al., 2010; Bereczky et al., 2010). In people, varying levels of liver lesions are seen in CCHFV-infected patients (Swanepoel et al., 1989). The finding of virus in the spleen is also consistent with CCHFV-infected IFN-α/βR−/− mice (Bereczky et al., 2010).

The histological findings in the liver comprised scattered foci of necrosis. However, scattered foci of mononuclear cell infiltrates were also seen in some wild-type and an un inoculated control. Thus, some of these latter lesions may represent a background change. Future immunological studies might resolve this point. In the spleen, a loss of lymphocytes was observed similar to those reported in a CCHFV model in STAT-1-knockout mice (Bente et al., 2010). Inflammation was also recorded. In the present study changes in the draining lymph nodes at the site of inoculation, comprising foci of necrosis, were also observed and appear to be a novel finding. In a previous report, lesions were also seen in the brain (Smirnova et al., 1977). However, the virus was intracranially delivered in this study and therefore the finding of brain involvement is not surprising. Lesions were not observed in the brain in the present study, where the virus was administered intradermally.

In conclusion, the present study confirms that HAZV infection of IFN-α/βR−/− mice via the intradermal route results in a dose-dependent lethal disease similar to that observed with CCHFV infection. It also demonstrates that HAZV infection of transgenic IFN-α/βR−/− mice may be used as a model with the aim of rapidly assessing potential antiviral agents and vaccines against the CCHFV infection.

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References


