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Exposure of rabbits to ultraviolet light-inactivated rabbit haemorrhagic disease virus (RHDV) and subsequent challenge with virulent virus

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SUMMARY

This study investigated whether exposure to inactivated rabbit haemorrhagic disease virus (RHDV) can produce an antigenic response in rabbits and protect them from a subsequent challenge with virulent virus. The aim was to determine if the spreading of baits containing RHDV, which is a common management practice in New Zealand to reduce rabbit numbers, could result in protective immunity in wild rabbits. RHDV was inactivated by ultraviolet (UV) light using an electronic UV crosslinker with a UV dose of 168·48 W-s/cm^2 and a UV intensity of 0·0078 W/cm^2. Two groups of four rabbits were then inoculated with inactivated virus via oral and intramuscular routes. Rabbits were monitored for 30 days post-inoculation and then challenged orally with virulent virus. No rabbit exposed to inactivated RHDV developed clinical signs of RHD or had antibodies at day 30 post-infection and all animals died within 82 h after challenge with virulent virus. No antibodies were detected at the time of death. These findings suggest that exposure to virus completely inactivated by UV light in the field or on baits will not protect rabbits against challenge with virulent virus.

INTRODUCTION

Introduced European rabbits (*Ortycolagus cuniculus*) are a major vertebrate pest in New Zealand and Australia. The use of rabbit haemorrhagic disease virus (RHDV) for biological control of rabbits is a common practice in New Zealand. In rabbit-infested areas, a commercial RHDV product ('RCD-ZEN', Zenith Technology Corp. Ltd, Dunedin, New Zealand) is typically distributed on baits to initiate RHDV epidemics. However, not all baits are taken up by rabbits, and residual RHDV baits may undergo prolonged exposure to environmental conditions. O'Keefe and colleagues [1] suggested that large-scale use of baits coated with RHDV, which might be inactivated in the environment, could induce protective immunity of rabbits and reduce the effectiveness of biological control programmes.

Environmental factors such as changing temperatures, humidity and sunshine are likely to influence the survival and infectivity of RHDV on baits. In particular, exposure to ultraviolet (UV) radiation from sunlight is likely to be an important determinant of the duration of virus infectivity. In a field study of RHDV survival, dried virus exposed directly to sunlight on cotton tape remained infective for susceptible rabbits for >10 days, but <44 days [2]. In contrast, under identical environmental conditions, RHDV injected into organic tissue (liver) and, therefore, not exposed to direct sunlight, remained infective for at least 3 months [2]. The possibility that environmentally degraded RHDV virus could induce

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protective immunity in wild rabbit populations has considerable implications for the efficacy of biological control using this agent. This is the second of two studies investigating the influence of the environment on RHDV properties. We conducted experimental investigations to determine whether exposure to UV-inactivated RHDV virus would induce seroconversion of rabbits and protect against exposure to virulent virus.

MATERIALS AND METHODS

Animals

New Zealand White rabbits aged between 10 and 11 weeks old were purchased from a commercial laboratory colony and housed in standard rabbit cages ($56 \times 44 \times 45$ cm) in climate-controlled rooms at 17 °C. They had not been vaccinated against RHDV and were tested immediately prior to the study to confirm the absence of RHDV antibodies. Rabbits were fed *ad libitum* with commercial rabbit pellets and had constant access to water.

RHDV inactivation

A commercial product 'RCD-ZEN' (Zenith Technology Corp. Ltd) that was produced from RCD CAPM V-351 (Czechoslovakian strain) Master Seed Virus was used for the study. The batch purchased for this study (Z25) had a rabbit LD_{50} titre of approximately 10^6 per ml (M. Shepherd, personal communication).

The RHDV product was exposed to UV light using an electronic UV crosslinker (CEX-800, Ultralum, Inc., Paramount, California, USA). UV crosslinkers are designed especially to provide uniform irradiation with short-wave UV light (254 nm) for crosslinking DNA and RNA. A high dose of UV exposure was chosen, based on the resilient properties of RHDV [3] and published reports on the use of UV energy to inactivate viruses and sterilize or disinfect effluents. The samples were exposed to a UV dose of 168·48 W-s/cm^2 with a UV intensity of 0·0078 W/cm^2.

Experimental design

Two groups of four rabbits were inoculated with 1 ml of undiluted UV-inactivated virus; one group was inoculated orally and the other group intramuscularly. Thirty-five days post-inoculation (p.i.) with inactivated RHDV, rabbits were challenged orally with approximately 10^4 LD_{50} RHDV (RCD-ZEN) in a volume of 1 ml. A similar concentration of the commercial product was found to be the minimum amount of virus required to produce infection in a companion study [2]. A positive control group of two rabbits, not previously inoculated with inactivated virus, was also challenged with virulent RHDV, and two negative control rabbits were dosed orally with saline solution. Blood samples were collected prior to commencement of the study, at 5, 10, 20 and 30 days p.i. with inactivated virus, and at 5, 10, 20 and 30 days post-challenge (p.c.) with virulent virus, and at the time of euthanasia. If an animal was seronegative at 30 days p.i., samples from 5, 10 and 20 days p.i. were not tested. Blood samples were centrifuged for 15 min at 1800 g to separate the sera. The sera were tested for antibodies to RHDV with the Capucci-competition ELISA [4] by AgResearch (Wallaceville Animal Research Centre, Upper Hutt, New Zealand). Fourfold serial dilutions from 1:10 to 1:640 were assayed. Samples were classified as RHDV positive if inhibition was $\geqslant 50\%$ in serum diluted 1:40.

Assessment of outcomes

Following inoculation with the inactivated virus, rabbits were observed several times daily for clinical signs. Following challenge with virulent virus, rabbits were observed continuously by an observer for the first 7 days and then at 4-h intervals until 10 days p.c., followed by once daily until 30 days p.c. Clinically affected rabbits were anaesthetized with an intramuscular injection of ketamine hydrochloride (100 mg/ml; Phoenix Pharm Distributors Ltd, Auckland, New Zealand) and xylazine (20 mg/ml; Phoenix Pharm Distributors Ltd,) as soon as signs of rabbit haemorrhagic disease (RHD) [2] were observed, and then euthanized by intracardiac injection of sodium pentobarbitone (Pentobarb 300, 300 mg/ml; National Veterinary Supplies Ltd, Auckland, New Zealand). Necropsies were performed on all rabbits and gross pathological observations were recorded. The presence of pathological changes typical of RHD (pale yellow or greyish liver with marked lobular pattern, petechial and echymotic multifocal haemorrhages of the lung, lung oedema, lung congestion, splenomegaly, poor blood coagulation and swollen, dull pale to patchy reddish discolouration of the kidney) was interpreted as confirmation of RHD.

Table 1. *Serology and mortality results after inoculation of rabbits with inactivated rabbit haemorrhagic disease virus (RHDV) followed by challenge with virulent RHDV*

Group	Sex	Antibodies at 30 days p.i.	Time to death p.c.	Antibodies at time of death
1. Inactivated RHDV i.m. route	F	Neg.	53 h	Neg.
	F	Neg.	82 h	Neg.
	M	Neg.	47 h	Neg.
	M	Neg.	45 h	Neg.
2. Inactivated RHDV Oral route	F	Neg.	42 h	Neg.
	F	Neg.	55 h	Neg.
	M	Neg.	55 h	Neg.
	M	Neg.	54 h	Neg
3. Positive control RHDV challenge only	F	n.a.	46 h	Neg.
	F	n.a.	50 h	Neg.
4. Negative control Saline challenge only	F	n.a.	n.a.	n.a.
	M	n.a.	n.a.	n.a.

Rabbits in groups 1 and 2 were inoculated with inactivated RHDV via intramuscular (i.m.) and oral routes respectively. Groups 1–3 were orally challenged with virulent RHDV 35 days later.
p.i., post-inoculation with inactivated RHDV; p.c., post-challenge with virulent RHDV; Neg., ELISA titre $<1:10$; n.a., not applicable.

RESULTS

All animals were seronegative before inoculation. None of the rabbits inoculated with irradiated virus developed clinical signs of RHD or had detectable antibodies to RHDV 30 days p.i. (Table 1). Challenge with virulent virus resulted in clinical signs and pathology typical of RHD in all rabbits, including those previously inoculated with irradiated virus. The mean time to death post-challenge was 52·9 h (S.D. = 11·2 h). No antibodies were detected at the time of death in any rabbit. The two negative control rabbits did not show any signs of disease and were negative for RHD antibodies 30 days p.i.

DISCUSSION

UV light at a wavelength between 100 and 280 nm is considered to be 'germicidal'. It damages the DNA and RNA of bacteria, viruses and other pathogens and thus destroys their ability to multiply and cause disease. It does this by eliciting single photon photochemical effects in nucleic acids through forming covalent bonds between certain adjacent bases [5]. This modification results in incorrect codes being transmitted from the nucleic acids and causes irreversible damage to the microorganisms. Inoculation with UV light-inactivated RHDV did not produce any antibody response in the rabbits, and they were not protected against challenge with virulent virus.

Considering these results, it is unlikely that inactivated RHDV on baits, in rabbit carcasses or excreted into the environment will produce an antibody reaction in naive rabbits. Thus, rabbits are likely to be fully susceptible to further RHDV epidemics in the field. Inactivated RHDV administered by the oral route (which would mimic ingestion of virus) or administered intramuscularly (imitating a stinging or biting insect) will not induce a protective antibody titre. O'Keefe and colleagues [1] proposed that inactivation of virus after large-scale baiting with RHDV may result in seroconversion of rabbits and protective immunity. However, the current experimental study showed no evidence to support this hypothesis. Seroconversion in rabbits following baiting operations is more likely to result from contact of rabbits to sublethal doses of virulent virus.

High titres of RHDV antibodies in surviving rabbits do not necessarily indicate immune protection of rabbits as suggested by O'Keefe and colleagues [1]. Exposure to RHDV in new epidemics can produce re-infection in animals that have survived previous outbreaks [6]. Persistence of RHDV antibodies over longer time periods has not been well documented

Table 2. *UV energy dosages required to inactivate selected organisms* [*13*]

Organism type	Organism	UV energy (mW-s/cm²)
Bacteria	*Clostridium tetani*	23–36
Mould spores	*Aspergillus niger* (black)	333–468
Protozoa	*Paramecium*	200–315
Virus	*Rotavirus*	24–29
	Tobacco mosaic virus	440–720

mW-s/cm², microwatt-seconds per square centimetre.

and is worthy of further investigation in longitudinal studies.

RHDV antibody responses have been documented in experimental studies by feeding foxes RHDV-infected rabbit carcasses [7] and by immunization of rabbits with recombinant virus-like particles and structural virus proteins [8, 9]. In these studies, the ability to induce antibodies (in foxes and rabbits) may be attributable to the large, multiple doses of antigen administered, the use of adjuvants, or the chemical stabilization of the antigen. None of these factors are present in a true field situation. These shortcomings are addressed in the current study, which more closely mimics field conditions.

The rate of inactivation of RHDV in the field will depend on the specific meteorological conditions, but the quantification of these influences is difficult. The death rate of microorganisms after UV light exposure decreases with increasing humidity [10]. In addition, UV radiation has increased over the recent years in New Zealand [11], especially the DNA and plant-damaging UV light. There are regional and seasonal differences in UV radiation even within New Zealand, so that the north of New Zealand receives up to 25% more UV than the south [12]. All of these factors influence how quickly RHDV will be inactivated under field conditions.

The high inactivation dose and intensity chosen in this experiment was appropriate considering the resilient properties of RHDV. The UV dose or exposure is defined as the UV light intensity multiplied by the exposure time [10]. Table 2 shows some inactivation dosages with UV light of 254 nm for a range of organisms. UV inactivation with wavelengths of 254 nm is commonly applied in the wastewater industry. Currently UV dosages of 60–90 mW-s/cm² are necessary to inactivate certain human pathogenic viruses, such as Poliovirus, Rotavirus and Hepatitis A virus, while MS-2 bacteriophage shows more resistance [14]. Ho Chu-Fei and colleagues [15] have shown that a UV exposure dose of 65 mW-s/cm² is required in a water pollution control plant to achieve the target coliform level 95% of the time. A 95% inactivation of human enteric Adenovirus type 40 occurs with UV doses of 103 mW-s/cm² in treated groundwater [16]. It has been shown also that a UV dose of 13 mW-s/cm² is able to reach 99% inactivation of Feline calicivirus (used as a surrogate to monitor the unculturable Norwalk virus) in treated drinking water [16].

This study has shown that exposure of rabbits to inactivated RHDV will not protect them from further challenge with virulent RHDV virus. Partial inactivation of RHDV by UV light resulting in exposure of rabbits to sublethal doses of viable virus might result in seroconversion and immunization. This could be determined with further challenge trials using stepwise decreased UV light exposure and intensity.

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REFERENCES

1. **O'Keefe JS, Tempero JE, Motha MX, Hansen MF, Atkinson PH.** Serology of Rabbit haemorrhagic disease virus in wild rabbits before and after release of the virus in New Zealand. Vet Microbiol 1999; **66**: 29–40.
2. **Henning J, Meers J, Davies PR, Morris RS.** Survival of rabbit haemorrhagic disease virus (RHDV) in the environment. Epidemiol Infect 2005. DOI: 10.1017/S0950268805003766.
3. **Smid B, Valicek L, Rodak L, Stepanek J, Jurak E.** Rabbit haemorrhagic disease: an investigation of some properties of the virus and evaluation of an inactivated vaccine. Vet Microbiol 1991; **26**: 77–85.
4. **Lavazza A, Capucci L.** Viral haemorrhagic disease of rabbits. Office International des Epizooties. Manual of standards for diagnostic tests and vaccines. Paris: OIE, 1996: 589–597.
5. **Jori G, Spikes JD.** Mapping of the three dimensional structure of proteins by phytochemical techniques. In: Smith KC, ed. Photochemical and photobiological reviews. New York: Plenum, 1976: 193.
6. **Cooke BD, Robinson AJ, Merchant JC, Nardin A, Capucci L.** Use of ELISAs in field studies of rabbit

haemorrhagic disease (RHD) in Australia. Epidemiol Infect 2000; **124**: 563–576.

7. **Leighton FA, Artois M, Capucci L, Gavier-Widen D, Morisse JP.** Antibody response to rabbit viral hemorrhagic disease virus in red foxes (*Vulpes vulpes*) consuming livers of infected rabbits (*Oryctolagus cuniculus*). J Wildl Dis 1995; **31**: 541–544.
8. **Plana-Duran J, Bastons M, Rodriguez MJ, et al.** Oral immunisation of rabbits with VP60 particles confers protection against rabbit hemorrhagic disease. Arch Virol 1996; **141**: 1423–1436.
9. **Castanon S, Marin MS, Martin-Alonso JM, et al.** Immunisation with potato plants expressing VP60 protein protects against Rabbit hemorrhagic disease virus. J Virol 1999; **73**: 4452–4455.
10. **Koller LR.** Ultraviolet radiation. New York: Wiley, 1965: 236–245.
11. **McKenzie R, Conner B.** Increased summertime UV radiation in New Zealand in response to ozone loss. Science 1999; **285**: 1709–1712.
12. **McKenzie R, Bodeker G, Conner B.** Increased UV radiation in New Zealand: a cautionary tale. Water Atmosph 1999; **7**: 7–8.
13. **Trojan Technologies.** Ultraviolet doses required for 99·99% inactivation of various microbes (mW-s/cm^2) (www.trojanuvlogic.com/UV_Applications/doses.html). Accessed 3 October 2002.
14. **Malley Jr JP, Margolin AB, Snice GA, Hogan SP.** UV inactivation of viruses in natural waters (project no. 180) (www.awwarf.com/exsums/90773.html). Accessed 3 October 2002.
15. **Ho Chu-Fei H, Pitt P, Mamais D, Chiu C, Jolis D.** Evaluation of UV disinfection systems for large-scale secondary effluent. Water Environ Res 1998; **70**: 1142–1150.
16. **Thurston-Enriquez JA, Haas CN, Jacangelo J, Riley K, Gerba CP.** Inactivation of Feline calicivirus and Adenovirus Type 40 by UV radiation. Appl Environ Microbiol 2003; **69**: 577–582.

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