Journal of General Virology (2000), **81**, 1439–1446. Printed in Great Britain

Long-term protective immunity to rinderpest in cattle following a single vaccination with a recombinant vaccinia virus expressing the virus haemagglutinin protein

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A recombinant vaccine, produced by using a highly attenuated smallpox vaccine (LC16mO) as a vector and which expresses the rinderpest virus (RPV) haemagglutinin protein, has been developed. The properties of this vaccine, including its heat stability, efficacy in short-term trials, safety and genetic stability, have been confirmed in an earlier report. In the present study, the duration of the protective immunity generated by the vaccine in cattle was examined for up to 3 years following the administration of a single vaccination dose of 10^8 p.f.u. The vaccinated cattle were kept for 2 (group I) or 3 years (group II) and then challenged with a highly virulent strain of RPV. Four of five vaccinated cattle in group I and all six cattle in group II survived the challenge, some showing solid immunity without any clinical signs of rinderpest. Neutralizing antibodies were maintained at a significant level for up to 3 years and they increased rapidly following challenge. Lymphocyte proliferative responses to RPV were examined in group II cattle and were observed in four of the six vaccinated cattle in this group. The long-lasting protective immunity, in addition to the other properties confirmed previously, indicate the practical usefulness of this vaccine for field use.

Introduction

Rinderpest virus (RPV), a member of genus *Morbillivirus* in the family *Paramyxoviridae*, causes an acute and often fatal disease in cattle and other large ruminants and is still prevalent in parts of Africa, the Middle East and south Asia (Wamwayi *et al*., 1995; Rossiter *et al*., 1998). The disease causes severe economic losses in countries where it remains endemic (Barrett & Rossiter, 1999). Outbreaks of rinderpest in wildlife are also observed in some countries, although wildlife do not appear to act as reservoirs for the virus (Barrett *et al*., 1998). More robust, heat-stable vaccines would be an advantage for use in countries where RPV still exists, since many have hot, arid climates.

In recent years, vaccinia virus has been used widely as a vector to deliver immunogenic antigens from other pathogens and, as in the case of rabies virus, these recombinants have been shown to act as novel vaccines to protect against these diseases (Yamanouchi *et al*., 1998). Recombinant rinderpest vaccines based on poxvirus vectors have been shown to protect cattle against RPV infection (Yilma *et al*., 1988; Belsham *et al*., 1989; Giavedoni *et al*., 1991; Romero *et al*., 1994). We have developed a recombinant rinderpest vaccine (rRV) by using a highly attenuated strain of vaccinia virus (LC16mO) as a vector to express the virus haemagglutinin (H) protein, which also protects against RPV infection (Asano *et al*., 1991; Yamanouchi *et al*., 1993). The H protein is responsible for the attachment of the virus to the host cell receptor and neutralizing antibodies generated against this protein are thought to play an important role in protection (Giraudon & Wild, 1985). A single subcutaneous inoculation of the rRV has been shown to give solid protective immunity in cattle for at least a year (Inui *et al*., 1995). The safety of the vaccine has been demonstrated in cattle and laboratory animals and, in addition, its heat stability and genetic stability on passage in cattle have been confirmed in previous studies (Yamanouchi *et al*., 1993; Yamanouchi & Barrett, 1994). In this study, we report further on the duration of immunity to RPV afforded by this vaccine and present the results of 2 and 3 year trials to test its

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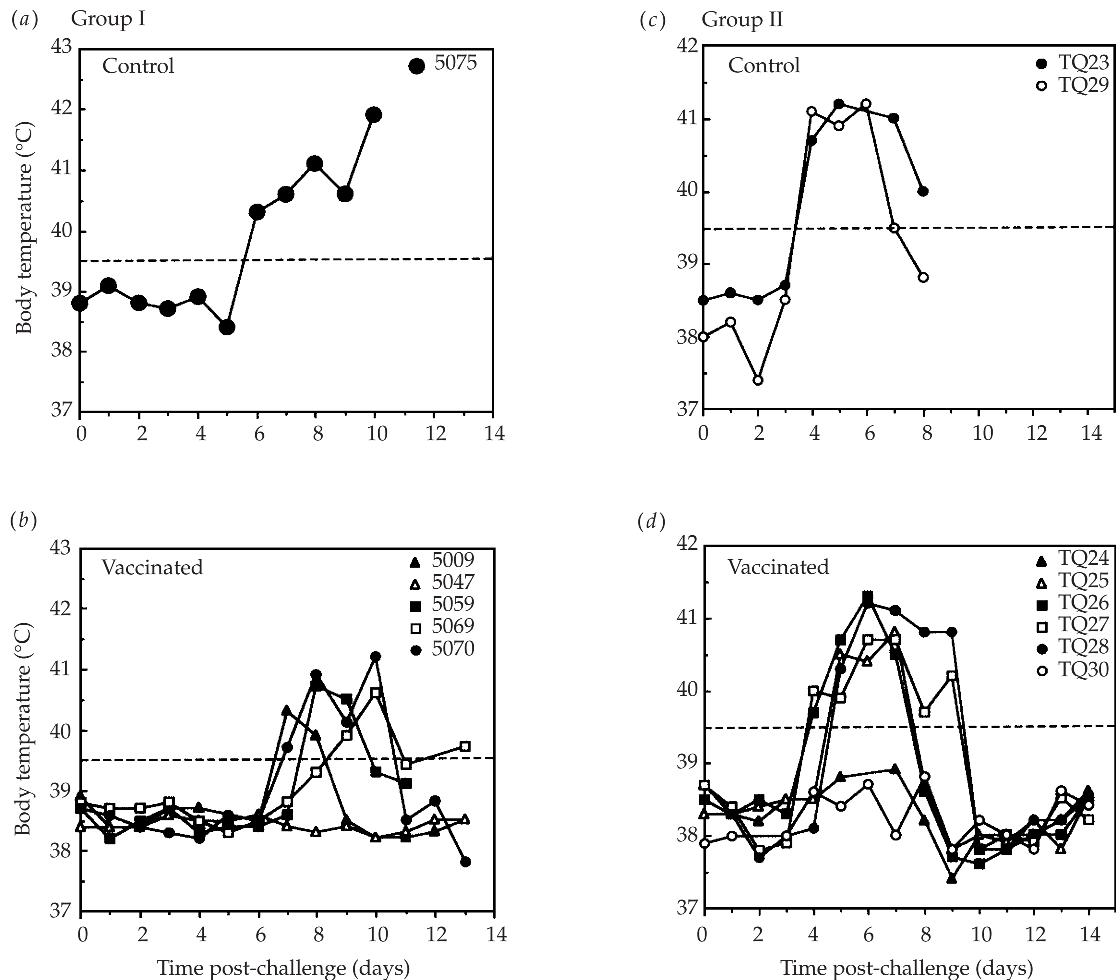

Fig. 1. Rectal temperatures in cattle following challenge with the virulent Saudi 1/81 strain of RPV. Temperatures above 39·5 °C were considered pyrexic. Cattle from group I (*a*, *b*) and group II (*c*, *d*) were either controls (*a*, *c*) or vaccinated (*b*, *d*). Results for individual animals are shown.

long-term efficacy in cattle. We have also examined the immune mechanisms involved in protection.

Methods

■ Viruses and cells. Details of the construction of the rRV have been described previously (Asano *et al*., 1991). In brief, the RPV H gene was inserted into the attenuated LC16mO strain of vaccinia virus (Hashizume *et al*., 1985) under the control of the vaccinia virus p7.5 kD early-late promoter. The highly virulent Saudi 1/81 strain of RPV was used as a challenge virus (Taylor, 1986). Vero cells were maintained in Dulbecco's essential medium supplemented with 5% foetal calf serum, streptomycin (100 μg/ml) and penicillin (100 IU/ml). B95a cells (Kobune *et al*., 1991) were used for virus isolations from lymphocytes. They were grown in RPMI-1640 with the same concentrations of serum and antibiotics.

■ Vaccination and challenge of animals. Friesian cross Aberdeen Angus calves were inoculated subcutaneously with a single dose (10^8 p.f.u.) of rRV in a secure animal facility at the Compton Laboratory of the Institute for Animal Health (IAH), UK. After 6 weeks, they were released to normal pasture and kept for 2 (group I) or 3 years (group II). As controls to monitor for contact transmission of vaccinia virus, unvaccinated cattle were kept together in each group. At the time of challenge, the cattle were moved to the high-containment facility at the IAH Pirbright Laboratory and inoculated subcutaneously with 10^4 $TCID_{50}$ of the virulent Saudi 1/81 strain of RPV (Taylor, 1986). This protocol was agreed with the local genetic manipulation safety committees at each laboratory and approved by the veterinary authorities at the Ministry of Agriculture, Fisheries and Food. All concerned were satisfied that the experiments posed no threat of contamination to the environment or to the health of those involved directly or indirectly in the experiments.

■ Clinical signs and leukocyte abnormalities. After challenge, cattle were examined daily for clinical signs and rectal temperatures were recorded. Blood samples were taken and examined for leukopenia on days 4, 7, 9 and 12 after challenge in group I and on days 5, 8, 11, 14 and 21 in group II. As a measure of immunosuppression, proliferation of purified lymphocytes was assayed in response to the mitogen concanavalin A (ConA), in group II animals only, on days 5, 8, 14 and 21 after challenge according to a method described previously (Ohishi *et al*., 1999). Briefly, peripheral blood leukocytes (PBLs; 2×10^5 cells per well in 96-well microtitre plates) were cultured for 6 days in the presence of

Table 1. Clinical signs and leukocyte abnormalities in cattle vaccinated with rRV following RPV challenge

Numbers in parentheses indicate the day post-challenge of the first appearance of the various signs.

	Clinical sign				Lymphocyte abnormality	
Cattle	Fever*	Mouth erosion	Diarrhoea	Protection	Leukopenia (%)†	Immunosuppression‡
Group I (2 years)						
Control:						
5075	+(6)	+(8)	+(10)	No	71	NT
Vaccinated:						
5009	+(7)	–	–	Partial	69	NT
5047	–	–	–	Complete	3	NT
5059	+(8)	+(11)	–	No	56	NT
5069	+(9)	–	–	Partial	31	NT
5070	+(7)	–	–	Partial	49	NT
Group II (3 years)						
Control:						
TQ23	+(4)	+(8)	+(7)	No	87	Total
TQ29	+(4)	+(8)	+(8)	No	83	Total
Vaccinated:						
TQ24	–	–	–	Complete	15	Transient (5–21)
TQ25	+(5)	–	–	Partial	56	Transient (5–21)
TQ26	+(4)	–	–	Partial	46	Transient (5–14)
TQ27	+(4)	–	–	Partial	66	Transient (5–21)
TQ28	+(5)	–	–	Partial	62	Transient (5–21)
TQ30	–	–	–	Complete	28	No

* Cattle showing temperatures above 39·5 °C were taken as positive.
† Percentage decrease calculated as 100 × (no. of cells before challenge – smallest no. of cells after challenge)/(no. of cells before challenge).
‡ Lymphocyte proliferation to ConA. Numbers in parentheses indicate the days when suppression was observed.
NT, Not tested.

ConA (5 μg/ml). The cells were pulse-labelled with [^{3}H]thymidine for the last 16 h and incorporation of label into cellular DNA was measured in a liquid scintillation counter.

■ **Virus detection following challenge.** Virus isolation from PBLs was attempted by co-cultivation with B95a cells, which are highly sensitive hosts for the replication of RPV (Kobune *et al*., 1991). For this purpose, 10^6 PBLs purified from each blood sample were placed in one well of a 96-well microtitre plate along with 5×10^6 B95a cells, using five wells for each assay. Virus present in the eye secretions (collected by swabbing) was detected by RT–PCR analysis of purified RNA according to a procedure described previously (Forsyth & Barrett, 1995).

■ **Detection of RPV-neutralizing antibodies.** Antibody titres were assayed by microneutralization tests with Vero cells as described previously (Sato *et al*., 1981). The assays were carried out in duplicate for group I sera and in quadruplicate for the group II sera.

■ **RPV-specific lymphocyte proliferative responses.** Lymphocyte proliferation in response to RPV stimulation was assessed by [^{3}H]thymidine incorporation into cellular DNA according to a method described previously (Ohishi *et al*., 1999). In brief, PBLs were cultured (2×10^5 per well) in 96-well microtitre plates for 6 days in the presence of UV-irradiated RPV (pre-UV titre of $10^{3 \cdot 9}$ $TCID_{50}$ per well). All assays were carried out in triplicate. RPV-specific responses were expressed as the stimulation index (SI), which was calculated as the ratio of the mean c.p.m. of lymphocytes cultured in the presence of RPV to the mean c.p.m. in the absence of RPV. Values greater than 2·5 were considered significant.

Results

Vaccination and challenge

In group I, five vaccinated and one control animal were kept for 2 years after vaccination and then challenged with virulent RPV. They were monitored closely for clinical signs typical of rinderpest infection such as fever, erosive stomatitis, ocular and nasal discharges and diarrhoea. The control, unvaccinated animal developed typical rinderpest with high fever, stomatitis and diarrhoea on days 6, 8 and 10 post-challenge, respectively. It was euthanized on day 10, before the disease progressed to the fatal stage. Three of five vaccinated cattle survived the challenge while showing only delayed and transient fever from days 7 to 9, while one (5047) was protected completely with

Table 2. Neutralizing antibody titres against RPV induced by rRV before and after challenge

For group I, assays were performed in duplicate and the results are given as the highest $\log_2$ titre that gave complete neutralization. For group II, the assays were performed in quadruplicate and the results are given as the $\log_2$ titre that gave 50% neutralization.

	Time after vaccination (months)							Time after challenge (days)			
Cattle	0	1	6	12	18	24	36	5	7/8*	12/14	21
Group I (2 years)											
Control:											
5075	< 2	< 2	< 2	< 2	< 2	< 2	NT	NT	< 2	< 2†	Dead
Vaccinated:											
5009	< 2	3	5	4	2	3	NT	NT	6	> 10	NT
5047	< 2	3	3	4	4	4	NT	NT	4	7	NT
5059	< 2	2	3	3	< 2	< 2	NT	NT	< 2	9	NT
5069	< 2	3	4	3	2	2	NT	NT	2	> 10	NT
5070	< 2	< 2	3	3	3	3	NT	NT	3	> 10	NT
Group II (3 years)											
Control:											
TQ23	< 2·0	NT	NT	< 2·0	NT	< 2·0	< 2·0	< 2·0	< 2·0	Dead	Dead
TQ29	< 2·0	NT	NT	2·3	NT	2·7	2·2	2·3	< 2·0	Dead	Dead
Vaccinated:											
TQ24	< 2·0	NT	NT	6·0	NT	5·0	4·7	4·3	6·3	> 10·0	> 10·0
TQ25	< 2·0	NT	NT	5·7	NT	4·5	4·0	4·5	> 10·0	> 10·0	> 10·0
TQ26	< 2·0	NT	NT	3·4	NT	4·0	3·5	4·5	> 10·0	> 10·0	> 10·0
TQ27	< 2·0	NT	NT	3·5	NT	3·5	2·7	3·7	6·7	> 10·0	> 10·0
TQ28	< 2·0	NT	NT	4·7	NT	3·5	2·7	2·5	7·5	> 10·0	> 10·0
TQ30	< 2·0	NT	NT	5·7	NT	5·7	5·7	5·5	> 10·0	> 10·0	> 10·0

* Where two numbers are given, the first refers to group I animals and the second to group II animals.
† Titre on the day the animal was euthanized (day 10).
NT, Not tested.

no evidence of clinical disease throughout the experiment. One vaccinated animal (5059) showed moderate clinical signs of rinderpest consisting of a delayed, transient fever and slight mouth lesions on days 8 and 11. This animal, however, developed a fatal black-leg infection. Symptoms included a swelling of one hind leg with difficulty in standing on day 11, due to the severe bacterial infection, and this animal was euthanized on that day. Severe leukopenia was observed in the control animal from day 7, which was maintained until it was euthanized. Leukopenia was not seen in the completely protected animal (5047), while the four remaining vaccinated cattle showed moderate leukopenia from days 7 to 9. Rectal temperatures recorded post-challenge are shown in Fig. 1 (*a*, *b*) and the appearance of clinical signs is listed in Table 1.

In group II, six vaccinated and two control animals were kept for 3 years after vaccination and then challenged with virulent RPV. The control, unvaccinated animals developed typical rinderpest from day 4, with high fever, stomatitis and diarrhoea, and were euthanized on day 8. In contrast, all the vaccinated animals survived the challenge. Two (TQ24 and TQ30) were solidly protected and showed no clinical disease throughout the experiment. The other four vaccinated cattle suffered a delayed, transient fever from days 4 or 5. They also showed slight nasal and ocular discharges and reddening at the base of the incisors, but the disease did not progress and all clinical signs had disappeared completely by day 22. Severe leukopenia was observed in the two control cattle, but at only a moderate level in the partially protected animals; significant leukopenia was not seen in the completely protected cattle. Rectal temperatures recorded post-challenge are shown in Fig. 1 (*c*, *d*) and the appearance of clinical signs is listed in Table 1. Clinical signs and leukopenia followed the same time-course and their degrees of severity were roughly correlated, as shown in Table 1.

Virus-neutralizing antibody in the vaccinated cattle

A single vaccination with rRV induced a low but significant level of anti-rinderpest neutralizing antibody in all animals. The titres reached maximal levels between 6 and 12 months after vaccination, after which they decreased slightly but then remained at the same levels until the experiment was completed at 3 years. A rapid rise in the level of anti-rinderpest neutralizing antibodies was observed in all the vaccinated cattle 1–2 weeks following challenge. No neutralizing antibody was produced in any of the control animals (Table 2).

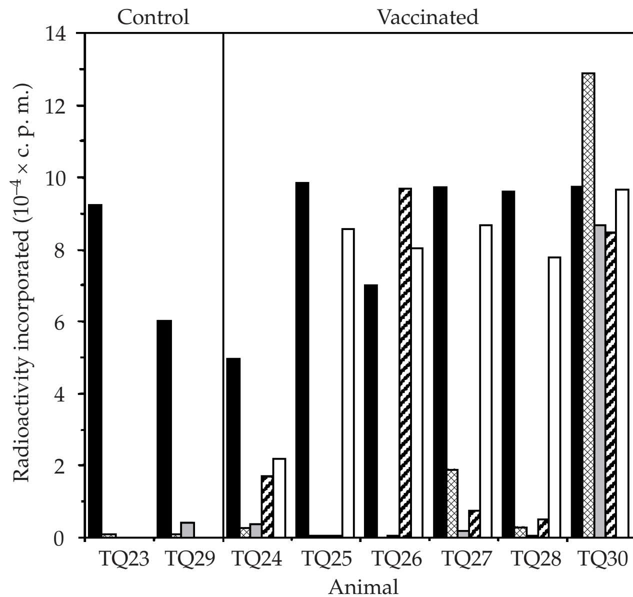

Fig. 2. Lymphocyte proliferation in response to ConA. Proliferation was determined by using PBLs from group II animals on days 0 (filled bars), 5 (cross-hatched bars), 8 (shaded bars), 14 (hatched bars) and 21 (open bars) following challenge. Briefly, PBLs (2×10^5 cells per well in a 96-well microtitre plate) were cultured in the presence of 5 µg/ml ConA. The cells were pulse-labelled with [^{3}H]thymidine and incorporation into cellular DNA was measured in a liquid scintillation counter.

Immunosuppression

Mitogen-induced lymphocyte proliferation was examined as a measure of immunosuppression in group II animals only. All animals except TQ30 showed marked immunosuppression on days 5 and 8 following challenge; their responses decreased to less than 6% of those seen before the challenge. This severe immunosuppression continued in the control animals until they were euthanized. However, recovery was observed in all vaccinated cattle after day 14 (Fig. 2). All assays were performed in triplicate except for TQ24 on day 21 and TQ30 on day 14, which were only duplicate samples. TQ26 was not tested on day 5.

Detection of challenge virus in PBLs and in ocular secretions

Growth of the challenge RPV was monitored by virus isolation from PBLs or by detection of virus-specific RNA sequences by RT–PCR. These results are summarized in Table 3. In group I cattle, the virus was not detected in the PBLs or eye secretions of the one animal that was protected completely (5047) or in one of the partially protected animals (5069). In the remaining vaccinated animals and in the control animal, virus was detected in PBLs on day 7 and in eye secretions on days 7 and 9. The results were essentially the same for group II cattle. In the two cattle that showed solid immunity, virus could not be detected in the PBLs on the days that they were examined. However, virus RNA was detected in the eye of TQ30 on day 7. The virus was detected by both assays in the two controls and in the four vaccinated cattle that showed partial protection. Viraemia also followed the same time-course as the clinical signs mentioned above.

RPV-specific lymphoproliferative responses

Lymphoproliferative responses to UV-irradiated RPV were examined in animals in group II. None of the cattle showed a response to RPV prior to challenge. Following challenge, four of the six vaccinated animals showed significant responses to RPV on days 14 and 21; TQ26 and TQ28 had particularly high proliferative responses on days 14 and 21 (Fig. 3).

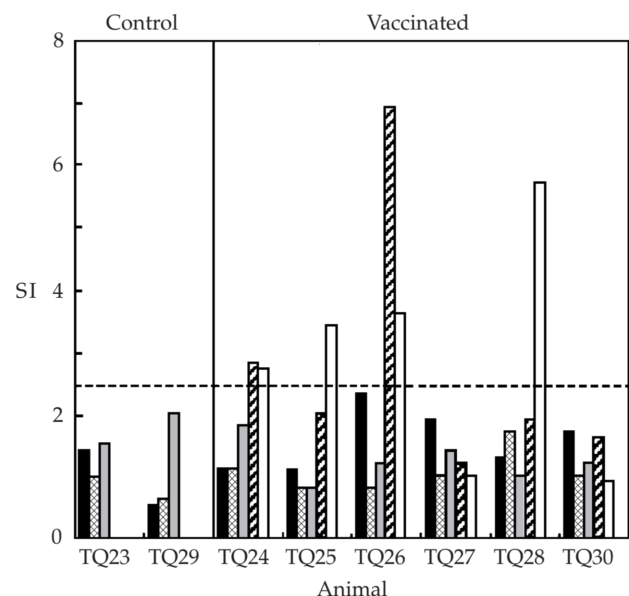

Fig. 3. Lymphoproliferative responses to RPV antigen stimulation in PBLs derived from cattle in group II. Lymphocyte proliferation was assayed on days 0 (filled bars), 5 (cross-hatched bars), 8 (shaded bars), 14 (hatched bars) and 21 (open bars) following challenge. The SI was calculated as described in Methods. Values greater than 2·5 were considered positive.

Discussion

The duration of the immunity induced by an rRV expressing the H protein of RPV was examined by challenging animals, all of which had received only a single subcutaneous dose of the recombinant vaccine, 2 and 3 years later with virulent RPV. When challenged, four of five cattle survived after 2 years and all six cattle after 3 years. One animal at 2 years and two animals at 3 years were protected completely and showed no clinical signs of infection, while all unvaccinated control cattle had to be euthanized in the acute stages of rinderpest. These results revealed that a single inoculation of rRV induced a long-lasting protective immunity against RPV. Virus was detected in PBLs following challenge in six of seven

Table 3. Detection of virus and virus-specific RNA after challenge with RPV

Viraemia indicates isolation of virus from PBLs. PBLs (10^6) from each animal were co-cultivated with B95a cells (5×10^6) in each of five wells of a 96-well microtitre plate. Virus excretion denotes detection of virus-specific RNA in RNA extracted from eye swabs. Virus RNA was detected by RT–PCR.

	Time post-challenge (days)									
	Viraemia					Virus excretion				
Cattle	0	5	7/8*	12/11	14	0	4/5	7/8	9/11	12/14
Group I (2 years)										
Control:										
5075	–	NT	+	Dead	Dead	–	–	+	+	Dead
Vaccinated:										
5009	–	NT	+	–	NT	–	–	+	+	–
5047	–	NT	–	–	NT	–	–	–	–	–
5059	–	NT	+	Dead	Dead	–	–	+	+	Dead
5069	–	NT	–	–	NT	–	–	–	–	–
5070	–	NT	+	–	NT	–	–	+	+	–
Group II (3 years)										
Control:										
TQ23	–	+	+	Dead	Dead	–	+	NT	Dead	Dead
TQ29	–	+	+	Dead	Dead	–	+	+	Dead	Dead
Vaccinated:										
TQ24	–	–	–	–	–	–	–	NT	–	–
TQ25	–	–	+	–	–	–	+	NT	–	–
TQ26	–	+	+	–	–	–	+	NT	–	–
TQ27	–	–	+	–	–	–	+	+	+	+
TQ28	–	–	+	+	–	–	–	+	+	+
TQ30	–	–	–	–	–	–	–	+	–	–

* Where two numbers are given, the first refers to group I animals and the second to group II animals.
NT, Not tested.

partially protected animals, but was not detected in animal 5069 or in any of the solidly protected animals. The levels of virus-neutralizing antibody increased markedly after challenge in all vaccinated cattle, including those where no virus could be detected in the blood. This indicates that the growth of challenge virus occurred locally at the inoculation site and that it was eliminated rapidly by long-lasting immunological memory in these animals. The remaining vaccinated animals, except for one in group I that died from a bacterial superinfection, showed only mild clinical signs, such as a transient fever, slight reddening in the mouth and mild nasal and ocular discharges, following challenge with the same dose of virus that led to severe rinderpest infection in the control animals. In these, the challenge virus spread systemically but was eliminated by a long-lasting immunological memory before it could result in severe clinical disease. The one animal in group I that succumbed to challenge showed only moderate clinical signs of rinderpest but had to be euthanized on day 11 post-challenge as a result of a severe black-leg infection. The infection might have been triggered by a traumatic injury and compounded by the immunosuppression induced by RPV.

The Saudi 1/81 strain of RPV used as a challenge virus is the most virulent strain among a number of well-characterized RPV isolates and can kill cattle within 3 days (Taylor, 1986). We have shown that a single subcutaneous vaccination with rRV provided either complete or partial protection against such a virulent virus for at least 3 years. Vaccinia virus replicates locally in the squamous epidermal cells without causing viraemia, and the optimal route for inoculation is intradermal rather than subcutaneous. However, safety considerations dictate that only the subcutaneous route of inoculation is acceptable when using vaccinia-vectored rinderpest vaccines in the field (Office International des Epizooties, 1989). These results indicate that the rRV would be effective in protecting cattle from RPV infection under these conditions.

In contrast to the local replication of the rRV at the site of inoculation, RPV causes systemic infection with marked growth in the lymphoid tissues as its main targets. It is rather surprising that such limited local growth of the rRV provides long-lasting immunity against systemic infection with RPV, and it raises interesting questions concerning the immune

mechanisms involved. Neutralizing antibodies were maintained at detectable levels for up to 3 years following a single vaccination in these cattle, and they increased rapidly following challenge. Specific lymphoproliferative responses to RPV were not detected before challenge in our assay system, but a marked increase in SI was observed after challenge in four of six animals tested. This indicates that specific immunological memory for both cell-mediated and humoral immunity persisted over this time-period and that they were activated rapidly by the antigenic stimulus provided by the limited growth of the challenge virus.

The relative importance of cell-mediated versus humoral immunity in the protective response remains to be defined clearly in the case of morbillivirus infections. In the present study, the cattle with solid immunity showed a higher neutralizing antibody titre compared with those with only partial protection. Overall, however, the levels of neutralizing antibody induced by rRV were low. This LC16mO-based vaccine system has been shown to be capable of inducing a protective cell-mediated immunity, including the ability to generate CTL responses in some cattle (Ohishi *et al*., 1991, 1999). A different recombinant vaccinia vaccine, expressing the H and F proteins of RPV, protected goats from peste des petits ruminants virus (PPRV) infection in the absence of PPRV-neutralizing antibodies in the vaccinated animals (Jones *et al*., 1993). Furthermore, it was reported that the H antigen alone when expressed in a baculovirus recombinant system could induce antibodies to RPV; however, these did not protect cattle against RPV infection (Bassiri *et al*., 1993). These observations indicate that there is probably a greater role for cell-mediated over humoral immunity in protection from RPV and that such an immunity is induced effectively by vaccinia-based vaccines.

This research was supported by a grant from the British Council, a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan, and a grant to K. O. from the Daiwa Anglo-Japanese Foundation.

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Received 19 November 1999; Accepted 3 March 2000