J. Hyg., Camb. (1984), **93**, 251–261
Printed in Great Britain

A comparison of three vaccines against respiratory syncytial virus in calves

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(*Received* 31 *January* 1984; *accepted* 25 *February* 1984)

SUMMARY

An inactivated vaccine against respiratory syncytial virus (RSV) was compared with two live vaccines. The inactivated (GC) vaccine consisted of glutaraldehyde-fixed bovine nasal mucosa cells persistently infected with RSV and emulsified with oil adjuvant. The live vaccines were a modified virus (MV) derived from a bovine strain of RSV and a temperature-sensitive mutant (ts-1) derived from a human strain. The GC vaccine was inoculated subcutaneously into 12 calves and the live vaccines intramuscularly into eight calves each. Nine unvaccinated calves acted as controls. The vaccines were administered in two doses 3 weeks apart and all calves were challenged intranasally with 2×10^7 p.f.u. of bovine RSV 3 weeks after the second dose.

At the time of challenge calves given GC, MV and ts-1 vaccines had mean serum neutralizing antibody titres of 25, 19 and 2 respectively; mean titres of IgG_1 antibody by radioimmunoassay were $\log_{10}$ 4·5, 1·3 and 2·6 respectively and mean zone areas by single radial haemolysis (SRH) were 107, 27 and 36 mm^2 respectively.

Eleven of 12 calves given GC vaccine were completely protected against challenge but all control animals and those given the two live vaccines were infected. The mean peak titre of virus in nasal swabs of control calves was 3·0 $\log_{10}$ p.f.u./ml and the mean duration of virus shedding was 6·8 days. Both these parameters were significantly reduced in animals given MV and ts-1 vaccines: mean peak titres were 2·1 and 2·4 $\log_{10}$ p.f.u./ml and mean duration of shedding was 3·4 and 3·3 days respectively.

Thus, protection correlated better with RSV antibody detected by radioimmunoassay and SRH than with neutralizing antibody. These results are discussed in relation to the possible mechanism by which protection was mediated.

INTRODUCTION

Annual epidemics of respiratory syncytial virus (RSV) infection are the major cause of severe lower respiratory disease in young children and are significantly associated with economically important disease in cattle (Chanock & Parrott, 1965; Grist, Ross & Stott, 1967; Holzhauer & Van Nieuwstadt, 1976; Scott *et al.* 1980).

Despite the urgent need for an effective vaccine, progress towards this goal has been slow and often disappointing. The first vaccine was formalin-inactivated, but trials of this material in young children showed that vaccinated individuals had more frequent and more severe lower respiratory tract involvement than their unvaccinated counterparts when they were infected with RSV during the subsequent winter (Kapikian *et al.* 1969). This alarming experience has never been satisfactorily explained, but inactivated vaccines were abandoned in favour of live temperature-sensitive mutants. However, despite 10 years of intensive research no satisfactory mutants have yet been isolated. Those tested so far have either been over-attenuated and insufficiently immunogenic or they have been insufficiently attenuated and genetically unstable, resulting in unacceptable reactions when tested in young children (Hall, 1980; Tyeryar, 1983). An alternative approach to live vaccination was developed independently by Wellemans and co-workers (1978) in cattle and Buynak and colleagues (1978) in children. These studies used virus modified by passage in cell culture and inoculated intramuscularly. However, subsequent trials have indicated that maternal antibody severely inhibits the response to this type of vaccine (Belshe, Van Voris & Mufson, 1982).

During the development of an RSV vaccine for cattle we decided to re-examine the possibility of using inactivated antigen. This paper describes a trial in which an inactivated vaccine was compared with two live vaccines given intramuscularly.

MATERIALS AND METHODS

Animals

Forty-five calves were collected into 15 pens 5–10 days after birth and reared in isolation until their maternally derived antibodies to RSV had declined to low or undetectable levels 4–8 months later.

Virus

Strain 127 of RSV was isolated at Compton from a calf with respiratory disease in February 1973. The virus was passaged nine times in calf kidney (CK) cells, once in a calf, twice in organ cultures of bovine foetal trachea and a further 10 times in CK cells including three passages at terminal dilution. Each calf was challenged by spraying intranasally 5 ml of tissue culture fluid containing a total of 2×10^7 p.f.u. of RSV. The 127 strain was used in all antibody assays except plaque reduction tests against the human A2 strain (Lewis *et al.* 1961), which was kindly supplied by Dr C. Pringle (Institute of Virology, Glasgow) and grown in CK cells.

Vaccines

(1) *Glutaraldehyde-fixed cells (GC).* A cell strain (SW129.NM7) persistently infected with RSV was derived from organ cultures of bovine foetal nasal mucosa inoculated with strain 127. The cells carried the bovine diploid karyotype of 60 chromosomes, and between passages 15 and 35, 95–100 % of them expressed RSV antigen on their surfaces as judged by immunofluorescence of unfixed intact cells. Cells were fixed in 0·075 % glutaraldehyde using the method of Zaia & Oxman (1977) and stored at −70 °C. These cells retained viral antigen on their surface but no infectivity could be detected by three blind passages in CK cells. To prepare vaccine, cells were thawed, washed and resuspended in phosphate-buffered saline

Table 1. *Plan of RSV vaccine trial*

Group	No. of pens	No. of calves	Vaccine*	Route	Challenge† (p.f.u.)
1	4	12	GC	Subcutaneous	2×10^7
2	4	8	MV	Intramuscular	2×10^7
		4‡	None	—	2×10^7
3	4	8	ts-1	Intramuscular	2×10^7
		4‡	None	—	2×10^7
4	3	9	None	—	2×10^7

* Two doses are given 3 weeks apart.

† All calves were challenged 3 weeks after the second vaccination.

‡ One calf in each pen acted as an unvaccinated sentinel animal to detect spread of vaccine virus to in-contact susceptible calf.

and emulsified with two parts of Freund's incomplete adjuvant, 0·5 % Tween 80 and 0·01 % thiomersal. Each 2 ml dose of vaccine contained 4×10^6 fixed cells.

(2) *Temperature-sensitive mutant* (*ts*-1). The ts-1 mutant was derived from the human A2 strain of RSV. The vaccine (Code Lot No. F-061) was prepared in bovine embryonic kidney at 30 °C as described previously (Wright, Mills & Chanock, 1971) and was kindly supplied by Dr R. M. Chanock. A 2 ml dose contained 5×10^4 p.f.u. of live virus and no adjuvant.

(3) *Modified virus* (*MV*). A strain of bovine RSV was modified by 96 passages in bovine kidney cell cultures (Wellemans *et al.* 1978). The vaccine (marketed as 'Rispoval') was produced and kindly supplied by Dr N. Zygraich (Recherche et Industrie Therapeutiques S.A., Rixensart, Belgium). A 3 ml dose contained 5×10^5 p.f.u. of live virus and no adjuvant.

Conduct of the trial

The housing, handling and clinical assessment of the calves have been described previously (Thomas *et al.* 1977). The 45 calves were divided into four groups, vaccinated and challenged as outlined in Table 1. Sera and nasopharyngeal swabs (NS) were collected from each calf before vaccination (day 0), before challenge (day 42) and 3 weeks after challenge (day 63). In addition, NS were collected every 2 days between days 43 and 59. Each calf in groups 2 and 3 was also swabbed twice weekly between days 3 and 36.

Nasal washings (NW), collected into 30 ml of PBS by irrigating each nostril twice, were taken from six calves in each of groups 1 and 4 and from four calves in each of groups 2 and 3. Lung washings (LW) were collected into 150 ml of PBS by irrigation via an endobronchial catheter while calves were under general anaesthesia. Two calves in each of groups 1 and 2 and three calves in group 4 were sampled in this way. Both NW and LW were collected on days 0, 42 and 63 and were concentrated tenfold using immersible CX-10 ultrafilters (Millipore Ltd, London) before assay for antibodies.

Virus isolation

All NS were inoculated into coverslip cultures of calf testis cells (CT) and petri dishes of CK cells. The CT cells were maintained and examined microscopically as previously described (Stott *et al.* 1980) except that 7 days after inoculation

coverslips were removed, fixed in acetone and stained by immunofluorescence using monospecific gnotobiotic calf antisera to RSV and bovine virus diarrhoea virus (BVDV). The CK cells were overlaid with Eagle's minimum essential medium containing 5% heat-inactivated foetal calf serum, 0·1% yeast extract, 0·25% agarose, 30 mM magnesium chloride, 25 μg/ml bromodeoxyuridine, 100 units/ml ampicillin, 100 μg/ml streptomycin, 100 μg/ml kanamycin and 50 units/ml fungizone; pH was adjusted to 7·6 with 25 mM HEPES buffer. The dishes were then incubated at 33 °C. After 10 days CK cells were fixed in 1% buffered formol-saline and stained in 0·1% crystal violet. Visible plaques were then counted.

Assays for virus antibodies

Neutralizing antibodies to RSV and BVDV were titrated in microtitre tests (Stott *et al.* 1980). Certain sera were also tested by plaque reduction assay against human and bovine strains of RSV. Antibodies to RSV and parainfluenzavirus type 3 (Pi-3) were also assayed in single radial haemolysis tests (Probert & Russell, 1975). The RSV antigen was prepared in CK cells by infection with the 127 strain of bovine RSV. When cytopathic effects were extensive, cells were shaken from the glass, concentrated to 10^7 cells/ml by centrifugation at 1000 ***g*** for 5 min and stored at −70 °C. After thawing and centrifugation at 1000 ***g*** for 5 min, the supernatant fluid constituted the antigen.

Class specific antibodies to RSV were measured by radioimmunoassay (Taylor, 1979) using rabbit anti-bovine IgG_1, IgG_2, IgA and IgM sera (Miles Laboratories Ltd, Slough). The RSV antigen was prepared as for SRH except that before the final centrifugation cells were solubilized by the addition of 0·5% Nonidet P40. Antigen was diluted to 1 in 400 in PBS before addition to polyvinyl microtitre plates.

RESULTS

Response to vaccines

(*a*) *Spread of vaccine virus.* RSV was not isolated from any calf between days 0 and 42. However, BVDV was isolated on days 3 and 7 from six of eight calves given the ts-1 vaccine. No other viruses were isolated during the trial.

(*b*) *Antibody responses.* Significant neutralizing antibody responses to vaccine were detected in 11 of 12 calves given GC vaccine and in eight of eight given MV vaccine (Table 2). The geometric mean titres of these groups on day 42 were 25 and 19 respectively. There were no responses between days 0 and 42 in any of the control or sentinel calves, nor in any animals given ts-1 vaccine. The mean titres of these groups were between 1·2 and 2·0 and did not change significantly during the period of vaccination.

The SRH assay detected significant responses to vaccine in all 12 calves given GC vaccine and seven of eight given either MV or ts-1 vaccines. Mean zone areas of the groups were 107, 27 and 36 mm^2 respectively. Antibody was not detected by SRH in control or sentinel animals between days 0 and 42.

Failure to detect a response to the ts-1 vaccine by the standard neutralization test might have been caused by antigenic differences between the bovine strain 127 used in the assay and the human ts-1 virus. To test this hypothesis all sera

Table 2. *Neutralizing and SRH antibodies to RSV*

Group	No. of calves	Neutralizing antibody: No. responding to vaccine*	Neutralizing antibody: No. responding to challenge†	Neutralizing antibody: Geometric mean titres (and ranges) Day 0	Day 42	Day 63	SRH antibody: No. responding to vaccine‡	No. responding to challenge§	SRH antibody: Mean zone areas (mm²) (and ranges) Day 0	Day 42	Day 63
(1) GC vaccine	12	11	1	1·5 (<2–4)	25 (2–128)	30 (8–128)	12	0	1 (0–13)	107 (88–126)	78 (46–122)
(2) MV vaccine	8	8	6	1·2 (<2–4)	19 (8–64)	69 (16–>128)	7	6	0	27 (8–48)	48 (25–65)
Sentinel	4	0	4	1·4 (<2–4)	1·2 (<2–2)	7 (4–16)	0	4	0	0	37 (23–48)
(3) ts-1 vaccine	8	0	8	1·5 (<2–4)	2·0 (<2–4)	32 (8–128)	7	7	0	36 (0–59)	74 (62–102)
Sentinel	4	0	4	1·4 (<2–2)	1·2 (<2–2)	9 (4–16)	0	4	0	0	41 (34–57)
(4) Control	9	0	9	1·2 (<2–2)	1·4 (<2–4)	9 (4–32)	0	9	0	0	33 (11–59)

* Fourfold or greater rise in titre between days 0 and 42. † Fourfold or greater rise in titre between days 42 and 63. ‡ Increase of 12 mm² in zone area between days 0 and 42. § Increase of 12 mm² in zone area between days 42 and 63.

Table 4. *Antibody to RSV in sera and nasal and lung washings on day 42* measured by radioimmunoassay*

Group	No. of calves examined	Mean antibody titre (and range) of indicated immunoglobulin class in indicated sample (log_{10}): Serum IgG_1	Serum IgG_2	Serum IgA	Serum IgM	Nasal washing IgG_1	Nasal washing IgG_2	Nasal washing IgA	Nasal washing IgM	Lung washing IgG_1	Lung washing IgG_2	Lung washing IgA	Lung washing IgM
(1) GC Vaccine	6	4·5 (3·9–5·0)	5·3 (4·8–6·2)	1·3 (0–2·3)	2·3 (0–3·0)	0·7 (0–1·8)	0·1 (0–0·4)	0·1 (0–0·5)	0	2·0 (1·8–2·1)	2·8 (2·7–2·8)	0	0
(2) MV Vaccine	4	1·3 (0–2·3)	0·4 (0–1·5)	1·0 (0–2·2)	1·1 (0–1·4)	0·9 (0–1·2)	0·2 (0–0·8)	0·4 (0–1·0)	0	1·2 (1·1–1·3)	0	0·4 (0–0·8)	0
(3) ts-1 Vaccine	4	2·6 (1·4–3·4)	0·6 (0–2·2)	0	1·5 (0·8–2·1)	0·2 (0–0·9)	0	0·2 (0–0·6)	0	ND	ND	ND	ND
(4) Control	6	0	0	0	0·6 (0–1·9)	0·2 (0–1·1)	0	0·3 (0–0·5)	0	0	0	0	0

ND = not done.

* On day 0 mean titres in serum and nasal washings were ⩽0·8 for IgG_1, ⩽0·7 for IgG_2 and ⩽0·4 for IgA and IgM. No antibody was detected in lung washings.

Table 3. *Neutralizing antibody to bovine and human strains of RSV measured by plaque reduction assay*

Group	Mean 50% plaque reduction titre against indicated virus strain (log_{10})		Statistical significance of differences between titres, P*
	Bovine	Human	
(1) GC Vaccine	2·60±0·89	2·18±0·84	>0·5
(2) MV Vaccine	2·35±0·57	1·29±0·67	<0·001
(3) ts-1 Vaccine	1·26±0·77	3·16±0·31	<0·001

* Probability calculated by Student's t test for paired data.

Table 5. *Mean peak titres and duration of RSV shedding in nasopharyngeal swabs after challenge*

Group	No. of calves	No. shedding virus	Mean peak titre (log_{10} p.f.u./ml)	Mean duration (days)
(1) GC Vaccine	12	1	0·2	0·1
(2) MV Vaccine	8	7	2·1±1·1	3·4±2·7
Sentinel	4	4	3·3±0·9	7·5±4·1
(3) ts-1 Vaccine	8	8	2·4±0·9	3·3±2·0
Sentinel	4	4	3·3±0·6	5·5±1·0
(4) Control	9	9	3·0±0·8	6·8±2·1

collected on day 42 from vaccinated calves were titrated by plaque reduction against the 127 strain and the human A2 strain, parent of the ts-1 mutant. The results (Table 3) indicated that in calves given the GC vaccine there was no significant difference between titres obtained with either the bovine or human strain. However, in calves given the two live vaccines there were highly significant differences ($P < 0·001$) between the values obtained with the two viruses. Thus, MV vaccine induced antibodies with a mean titre 11-fold higher against bovine than against human virus. Conversely, ts-1 vaccine stimulated antibodies which had a mean titre almost 80-fold higher against human RSV than against the bovine strain. The mean titres of antibody against the bovine virus subsequently used as a challenge are similar for groups 1 and 2: log_{10} 2·60 and log_{10} 2·35 respectively. However, the titre of log_{10} 1·26 for group 3 is over tenfold lower.

The distribution of RSV antibody within each immunglobulin class and between serum and respiratory secretions was determined by radioimmunoassay (Table 4). On day 42 mean RSV antibody titres of IgG_1 and IgG_2 subclasses in serum were a 100-fold to 10000-fold higher in calves given GC vaccine than in calves given either of the live virus vaccines. Antibody titres in the nasal washings remained low even after vaccination. Antibody of IgG class was detected in lung washings of all vaccinated calves examined but titres were at least eightfold higher in those given GC vaccine. Three weeks after live virus challenge increases of at least tenfold in mean titres of serum IgG_1 and IgG_2 were detected in the control calves and those given live vaccines (data not shown).

Significant antibody responses to BVDV were detected in all calves given ts-1 vaccine. No responses to Pi-3 were detected during the trial.

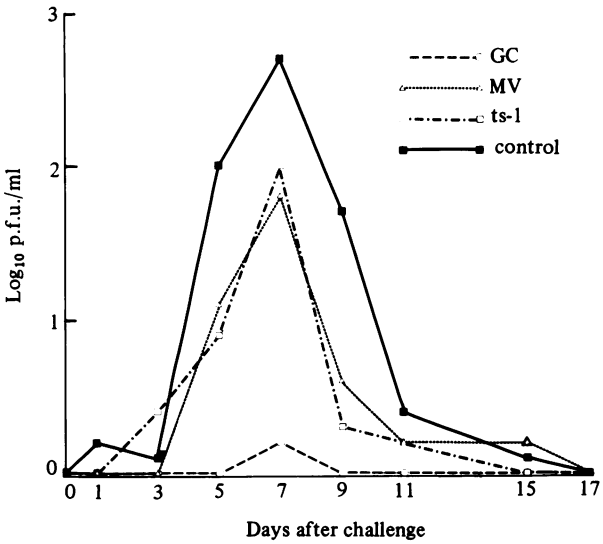

Fig. 1. Shedding of RSV in nasopharyngeal swabs after intranasal challenge. Mean virus titres in nasal swabs.

Response to challenge

(*a*) *Virus shedding*. After intranasal challenge on day 42 RSV was recovered from all unvaccinated calves in control and sentinel groups (Table 5). The mean of the peak titres detected in each calf was between $\log_{10}$ 3·0 and $\log_{10}$ 3·3 and the mean duration by shedding between 5·5 and 7·5 days. In animals given MV or ts-1 vaccines all but one excreted virus but the mean peak titre was reduced ten- and fivefold respectively and the mean duration of shedding was halved when compared with the control group. Only one of the 12 calves given GC vaccine excreted virus and only for 1 day.

The daily mean virus titres of each group are shown in Fig. 1. Excretion was greatest 7 days after inoculation and ceased within 15 days. The quantity and duration of virus excretion was similar in the two groups given live vaccine but less than that in the control group.

An analysis of variance was carried out on all data obtained from NS. Calves given MV or ts-1 vaccine shed significantly less virus than control animals ($P < 0{\cdot}05$) but there was no difference between the two groups given live vaccines. The reduction in virus shed by calves given GC vaccine was highly significant ($P < 0{\cdot}001$).

(*b*) *Antibody response*. All sentinel and control calves showed a significant neutralizing and SRH antibody response to challenge (Table 2). Mean titres of neutralizing antibody after infection were between 7 and 9. Most calves given live vaccine (groups 2 and 3) also responded to challenge. Only one of 12 calves given GC vaccine showed a neutralizing antibody response after challenge and this animal had not developed neutralizing antibody after vaccination. Increases in mean antibody titres after challenge were also detected in groups 2, 3 and 4 by radioimmunoassay.

(*c*) *Clinical response.* Detailed daily examination of each calf began 1 week before challenge and continued for 4 weeks. Significant clinical signs were not observed in any calf.

DISCUSSION

The GC vaccine completely protected 11 of 12 calves against RSV infection. This level of protection is considerably greater than that afforded by natural infection. In both children and cattle reinfections frequently occur often with lower respiratory tract involvement (Beem, 1967; Henderson *et al.* 1979; Mohanty, Lillie & Ingling, 1976; Holzhauer, 1978). Our own unpublished observations also indicate that cattle can be reinfected within three weeks after a primary infection with RSV. The two live vaccines did not confer protection against infection although they did reduce virus shedding. Since the challenge virus did not induce disease in the control animals, the ability of these vaccines to prevent or increase clinical reactions could not be assessed. However, there was certainly no evidence to suggest that these vaccines might enhance RSV infection and it is unlikely that the GC vaccine which prevented infection would not also prevent disease. The final answer to these questions will come from large field trials in which animals are challenged naturally and from experimental trials in which animals are challenged with strains of bovine RSV which cause lower respiratory disease (McNulty, Bryson & Allan, 1983; Thomas *et al.* 1984).

The mechanisms by which the vaccines induced protection were difficult to define. Neutralizing antibody did not correlate with protection. The GC and MV vaccines stimulated similar levels of neutralizing antibodies against the challenge virus (Tables 2 and 3) but very different degrees of protection. Conversely, calves given MV and ts-1 vaccines were protected to the same extent but had very different levels of antibody. Antibodies measured by SRH or radioimmunoassay appeared to correlate better with protection. Passive transfer of RSV monoclonal antibodies (Mab) into mice has indicated that some Mab which neutralized virus strongly protected mice poorly or not at all. In contrast, other Mab to F protein having only weak neutralizing activity protected completely (Taylor *et al.*1983, 1984). These observations could be explained by reference to the antigenic structure of RSV. On the surface of virions and virus-infected cells there are two glycoproteins, a large protein (GP84) equivalent to the haemagglutinin-neuraminidase of other paramyxoviruses and a fusion (F) protein (Fernie & Gerin, 1982). It may be that, as with other paramyxoviruses (McClelland, 1980), antibody to the larger glycoprotein of RSV neutralized most effectively but antibody to fusion glycoprotein protected most efficiently. Thus, neutralization tests primarily measured antibody to GP84 whereas SRH and radioimmunoassay probably measured antibody to F protein. Direct evidence for this latter conclusion was the ability of radioimmunoassay to detect Mab to F protein more readily than Mab to GP84 (Stott and co-workers, unpublished observations).

The importance of antibody to F protein in protection may also account for the ability of the human ts-1 vaccine to protect against a bovine RSV challenge. All human and bovine strains of RSV so far tested carry the same protective epitope on the F protein (Stott *et al.* 1984). In contrast, Mab to GP84 of human RSV do not react with bovine RSV strains.

The mechanism by which circulating antibody protected the upper respiratory tract against infection is not clear. On the day of challenge, antibody was not detectable in nasal secretions of calves given GC vaccine although it was found in the lower respiratory tract. It is possible that damage to the nasal mucosa by initial virus infection released antibody from the underlying capillaries.

Our failure to detect RSV in NS of calves given MV or ts-1 vaccines or their contacts confirms previous observations in cattle, man and cotton rats (Wellemans *et al.* 1978; Buynak *et al.* 1978; Prince *et al.* 1979) that RSV given intramuscularly does not reach the respiratory tract. Indeed, Prince and colleagues (1979) found all trace of virus and its antigens disappeared from the site of inoculation within 5 min and did not reappear indicating only very limited or abortive virus replication. Nevertheless, some form of replication was required since u.v.-inactivated virus was not immunogenic (Prince *et al.* 1979; Zygraich & Wellemans, 1981). Our finding that the ts-1 vaccine reduced virus shedding as effectively as the MV vaccine was remarkable since growth of the ts-1 mutant was restricted at 39 °C, which is the normal bovine body temperature. The mutant was defective in a late function so that some antigen was detected at the restrictive temperature although no mature virions were formed (Gharpure, Wright & Chanock, 1969). It is possible that in the calf this antigen was produced and expressed on cell surfaces and so presented to the immune system. Such an abortive cycle of replication may be common to all live RSV vaccines given intramuscularly.

Evidence for BVD virus infection was found in all eight calves given ts-1 vaccine but not in any other animals, indicating that the vaccine was the source of infection. This was confirmed by direct isolation of non-cytopathic BVD virus from unopened vials of vaccine. Contamination of the vaccine was disturbing since this batch had been 'tested for the presence of adventitious agents in accordance with the standards for experimental vaccines' and subsequently used in human volunteers (Wright *et al.* 1971). The BVD virus was probably present in the embryonic bovine kidney cells in which the vaccine was produced or in the foetal calf serum in which the cells were grown (Nuttall, Luther & Stott, 1977). It was probably not detected because the conventional interference test for BVD virus in use in 1971 is insensitive compared with immunofluorescence (Nuttall *et al.* 1977). Thirteen pairs of sera from volunteers given the vaccine were tested for antibody to BVD virus by neutralization and immunofluorescence tests and were all negative (unpublished observations). This indicated that BVD virus did not replicate in the men and is consistent with the failure of this virus to replicate *in vitro* in cells of human origin. Thus, the BVD virus did not constitute a serious hazard to the volunteers. In cattle acute BVD virus infection is associated with transient pyrexia and leukopaenia (Nuttall, Stott & Thomas, 1980) and must be rigorously excluded from bovine vaccines.

Further work will be necessary to determine whether the superior protection afforded by the GC vaccine is due primarily to the method of presentation of the antigen on fixed whole cells, to the higher antigenic load or to the added oil adjuvant. Although infected cell vaccines have been used against herpesviruses (Powell, 1975), this approach has not previously been tried with paramyxoviruses and may offer a superior method for stimulating antibody to F protein. The role of cell-mediated immunity to these vaccines has also been investigated and will form the basis of a separate communication.

The authors wish to thank Dr W. Plowright, F.R.S., for suggesting glutaraldehyde fixation of RSV-infected cells and for advice during the planning of this vaccine trial. We are also grateful to Drs R. M. Chanock and N. Zygraich for generous gifts of vaccine, and to Dr S. Lucey for statistical analyses.

The cell strain SW129.NM7 is protected by European Patent no. 0043272 and corresponding foreign patents.

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