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Experimental pneumonia in gnotobiotic calves produced by respiratory syncytial virus

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Summary. A bovine isolate of respiratory syncytial virus (RSV), when inoculated intranasally into eight gnotobiotic calves produced significant macroscopic lesions of the lung (2–25% consolidation) but failed to produce any clinical signs of disease. The microscopic lesions comprised proliferative and exudative bronchiolitis with accompanying alveolar collapse and infiltration by mononuclear cells of the peribronchiolar tissue and alveolar walls. Virus was recovered from the nasopharynx between days 2 and 11 after infection with peak titres between days 4 and 7. Demonstration of viral antigen by immunofluorescence in nasopharyngeal cells followed a similar detection pattern. Virus was recovered from lung or detected by immunofluorescence in the bronchiolar epithelium up to 11 days following inoculation. A serological response to RSV was demonstrated both by virus neutralization and single radial haemolysis (SRH) tests, in serum of calves from 11 days following inoculation. Specific anti-RSV IgM was detected from 9 days following infection. It is suggested that the close resemblance between the experimental disease in calves and the pathology of acute bronchiolitis in children make cattle a particularly relevant model for the human disease.

Keywords: pneumonia, gnotobiotic calves, respiratory syncytial virus

Respiratory syncytial virus (RSV) has for some years been accepted as a major cause of lower respiratory disease in young children (Chanock & Parrott 1965). RSV infection has also been associated with bovine respiratory disease in Europe (Paccaud & Jacquier 1970), the USA (Rosenquist 1974) and Japan (Inaba *et al.* 1970) and significantly correlated with outbreaks of disease in Holland (Holzhauer & van Nieuwstadt 1976) and the UK (Stott *et al.* 1980). Because man and cattle are both natural hosts for infection with RSV, a study of the experimental disease in cattle may therefore have particular relevance as a model for the human disease.

Previous experimental work with this virus in calves has indicated that it is capable of reproducing a mild respiratory disease with few if any macroscopic lesions (Jacobs & Edington 1975; Mohanty *et al.* 1975). The numbers of animals used were small and no uninoculated controls were included. Furthermore, evidence for exclusion of bovine virus diarrhoea virus (BVDV) from the experiments was not reported, although this virus is now known to represent a very real hazard when using inocula of bovine origin (Nuttall *et al.* 1977). We have therefore extended the scope of these earlier studies and report in the following paper the successful production of pneumonia in gnotobiotic calves following the intranasal inoculation of RSV.

Materials and methods

Animals. Friesian or Aberdeen Angus cross Friesian, gnotobiotic calves were procured and reared as already described (Dennis *et al.* 1976). Animals were inoculated between 26 and 88 days of age by the intranasal route. All calves received 10 ml of culture fluid on each occasion.

Nasopharyngeal swabs (NS) (Thomas & Stott 1975) were taken immediately before inoculation and then subsequently at 2–4 day intervals. Lung washings were also collected at slaughter (Thomas & Stott 1981). Clotted blood (serum) samples were obtained immediately before inoculation and at time of slaughter.

Daily rectal temperatures were taken from 4–5 days before inoculation until slaughter. Lung lesions were recorded on a standard lung diagram and expressed as a percentage of the surface area affected.

Virus strain. The Snook isolate of RSV was obtained from the lung of a fatal case of bovine pneumonia in December 1976 (Thomas *et al.* 1982) and was passaged twice in gnotobiotic calves, twice in calf testis cells and three or four times in calf kidney cells.

Virus isolation. Cells from lung washings and NS were inoculated into calf testis and secondary calf kidney cells which were maintained and examined as previously described (Stott *et al.* 1980). Plaque assays of NS were performed on secondary calf kidney cells, plaques being mounted after 10 days at 33°C. Cells washed from the lungs were also cultured for at least 6 weeks (Thomas & Stott 1981).

Virus serology. Sera were titrated for neutralizing antibodies to RSV and bovine virus diarrhoea virus (BVDV) in microtitre tests. A four-fold or greater rise in titre was considered significant evidence of infection. Antibodies to RSV and para-influenza virus type 3 (Pi-3) were also measured by single radial haemolysis (SRH) tests (Probert & Russell 1975). An increase in zone area of 12 mm^2 or more was considered significant. Sera were also screened at 1/10 dilution for antibodies to bovine syncytial virus (BSV) by indirect immunofluorescence on coverslips of calf testis cells infected with BSV. Class-specific antibodies to RSV were measured by radioimmunoassay using rabbit anti-bovine IgG1, IgG2, IgA and IgM sera from Miles Laboratories (Taylor 1979). Titres $\geqslant 10^{1.5}$ were considered significant.

Histology. Tissues were taken from all animals at slaughter, fixed in 12% neutral buffered formalin, embedded in paraffin wax, sectioned and routinely stained by haematoxylin and eosin. Lendrum's phloxine/tartrazine, the periodic acid-Schiff and van Gieson's staining methods were also selectively used.

Lung tissue was also taken into 4% paraformaldehyde in a cacodylate sucrose buffer (Patterson *et al.* 1981) embedded in araldite, sectioned at 1 μm and stained by toluidine blue.

Immunofluorescence. Tissues were frozen quickly and stored at −70°C until sectioned. Cryostat sections were cut at 8 μm and fixed in cold acetone at 4°C for 10 min. The direct immunofluorescence technique was used to identify RSV antigen in tissue (Thomas & Stott 1981). Nasopharyngeal specimens were also prepared and stained by direct immunofluorescence (Thomas & Stott 1981).

Bacteriology. One-gram samples of lung tissue were triturated in phosphate-buffered saline. Serial 10-fold dilutions were made up to 10^4 and 0.1 ml of each dilution spread on to half an ox-blood agar plate. Duplicate plates were used, one incubated aerobically and the other anaerobically under H_2 containing 10% CO_2. Colonies were identified by the methods of Cowan (1974).

Table 1. Microbiological and serological findings from gnotobiotic calves inoculated intranasally with the Snook isolate of RSV

Calf no.	Inoculated		Virus detection in nasal swabs			Antibody responses‡						Killed		Virus detection at slaughter			
			Isolation					RIA							IF		
	Age (days)	Total p.f.u. (log_{10})	Day†	Peak titre (log_{10} p.f.u./ml)	IF Day	Neut.	SRH	G_1	G_2	A	M	Day	% pneum.	Isolation Lung	Lung	Trachea	Nose
R190	39	4.2	4,7	0.7	7	<2	0	—	—	—	—	7	25	+	+	−	+
R135	72	3.5	**4,7,9**	2.1	9	2	0	—	—	—	2.7	9	13	−	+	−	+
R117	88	4.7	4,7,9	2.6	7,9	<2	0	—	—	—	3.1	9	5	+	+	−	+
*R121	81	4.4	4,7,9	3.4	7,9	<2	0	—	—	—	—	9	2	+	+	+	−
R140	48	3.5	**2,4,7**	2.5	4,7,9	4	21	—	—	—	3.7	11	10	−	+	−	−
R149	26	4.5	**2,4,7**	1.9	7,9,11	4	21	—	—	—	2.7	11	12	−	+	−	+
*R143	41	4.5	**2,4,7,9,11**	3.3	7,9	2	23	—	—	—	3.4	11	2	−	+	−	−
R192	34	3.9	4,7,9	2.8	7	8	48	—	—	—	3.9	14	18	−	−	−	−

* Inoculated twice daily for 4 days.

† Day of peak titre **bold.**

‡ Slaughter titres shown. Antibody was not detected in preinoculation samples.

Neut, 50% end point titre of virus neutralizing antibody; SRH, Single radial haemolysis zone area in mm^2; RIA, Radioimmune assay end point titre (log_{10}) for indicated Ig class (— = < 1.5); p.f.u., plaque-forming units; IF, immunofluorescence.

Results

Clinical and pathological findings

None of the eight calves developed recognizable clinical signs following inoculation. However, at slaughter, 7–14 days following inoculation, moderate to substantial pneumonic lesions were recorded in all eight calves (Table 1, Fig. 1).

The microscopic lesion comprised an exudative and proliferative bronchiolitis with accompanying alveolar collapse and infiltration of the peribronchiolar tissue and alveolar walls with round mononuclear cells and some polymorphs (Fig. 2).

The exudative bronchiolitis involved predominantly the smaller bronchioles (< 300 μm diameter). The bronchial epithelium was markedly swollen and detached epithelial cells, vacuolated macrophages and polymorphonuclear granulocytes could be seen in the bronchiolar exudate (Fig. 3). Polymorphs could be seen particularly clearly in the

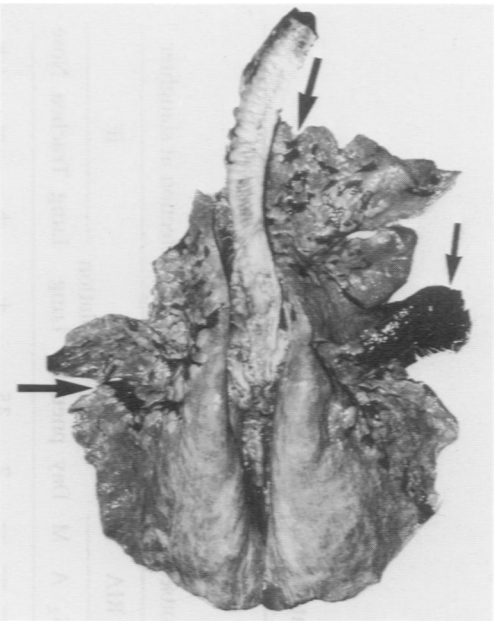

Fig. 1. Pneumonic lesions (arrowed) in calf R192 slaughtered 14 days after intranasal inoculation with RSV.

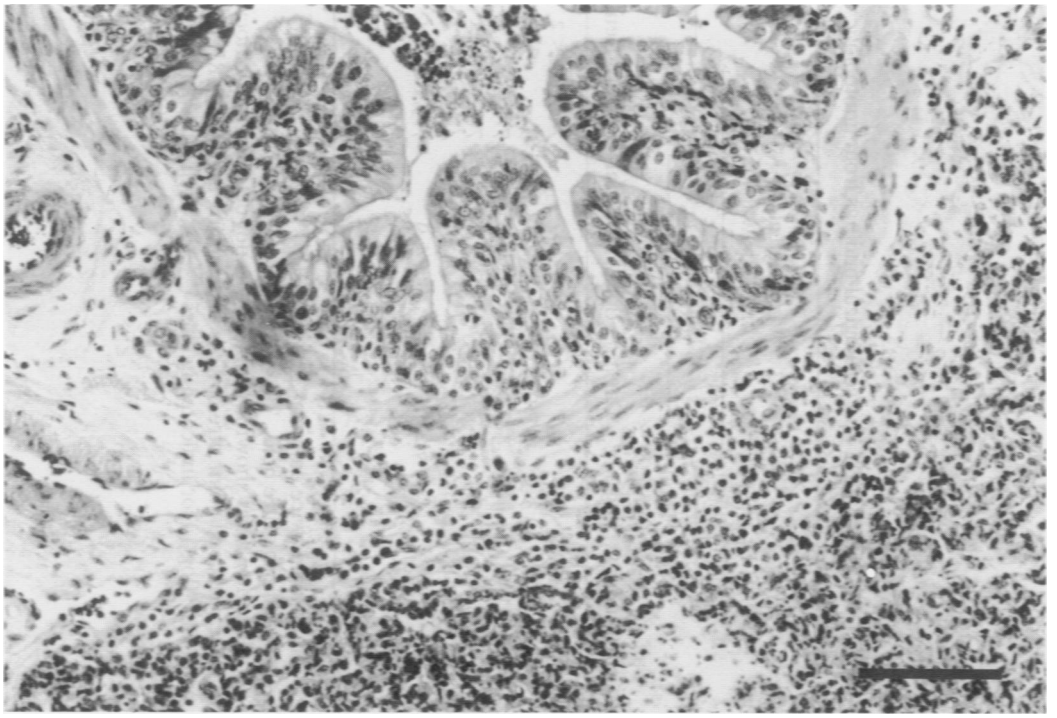

Fig. 2. Acute bronchiolitis, showing bronchiolar exudate, peribronchiolar infiltration by lymphoid cells and collapse of the surrounding alveolae. Calf R149, araldite toluidine blue. Bar represents 100 μm.

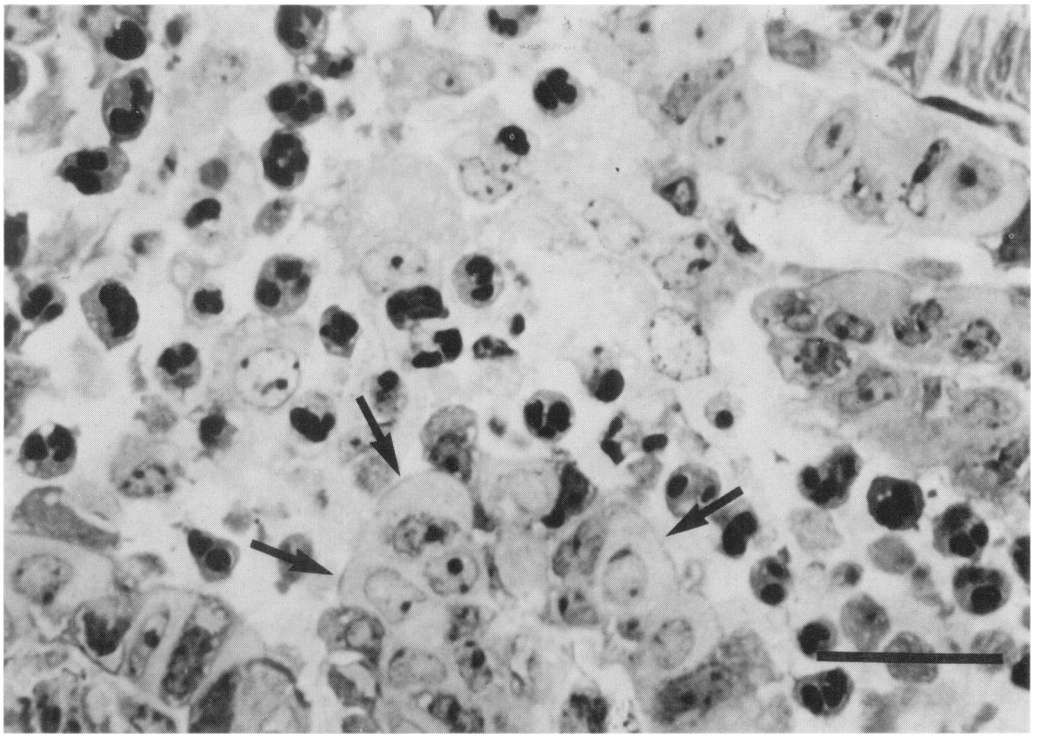

Fig. 3. Detail of the exudative reaction seen in the smaller bronchioles (< 300 μm) showing vacuolated macrophages detached epithelial cells and neutrophil granulocytes. Several swollen, hypertropic cells (arrowed) may be seen in the epithelium. Calf R190, araldite/toluidine blue. Bar represents 20 μm.

tissue surrounding the larger bronchioles (> 400 μm diameter) migrating through the lamina propria and epithelium. Little or no damage was apparent to the epithelium of these larger bronchioles. The cytoplasmic granules of these migrating polymorphs were strongly phloxinophilic and eosinophilic. This predominance of eosinophils was noted in six of the eight calves and particularly in calves R190 and R140 killed on days 7 and 11 respectively following inoculation.

Accumulation of eosinophils in the lamina propria of the nasal and tracheal mucosa was also noted. However, the inflammatory response in the nasal mucosa, which also included focal necrosis and detachment of epithelial cells, may have been attributable to routine swabbing of the nasopharynx.

Infiltration of the peribronchiolar tissue by round mononuclear cells (Fig. 2) was seen as early as day 7 following inoculation (R190), accompanying the exudative lesion described above. By day 11 early organization of these cells into rudimentary lymphoid follicles was noted. This mononuclear response was also responsible for a marked thickening of alveolar walls (Fig. 4). In addition the lesions comprised patchy atelectasis or collapse of whole lobules (Fig. 2), epithelialization of alveolar walls and accumulation of an alveolar exudate. The latter comprised occasional syncytia sometimes with phloxinophilic inclusions, alveolar macrophages, polymorphs and some oedema fluid. Type II alveolar pneumonocytes were particularly prominent in areas of alveolar collapse. Loss of lung function, however, appeared to derive mainly from collapse of alveoli rather than alveolar exudate.

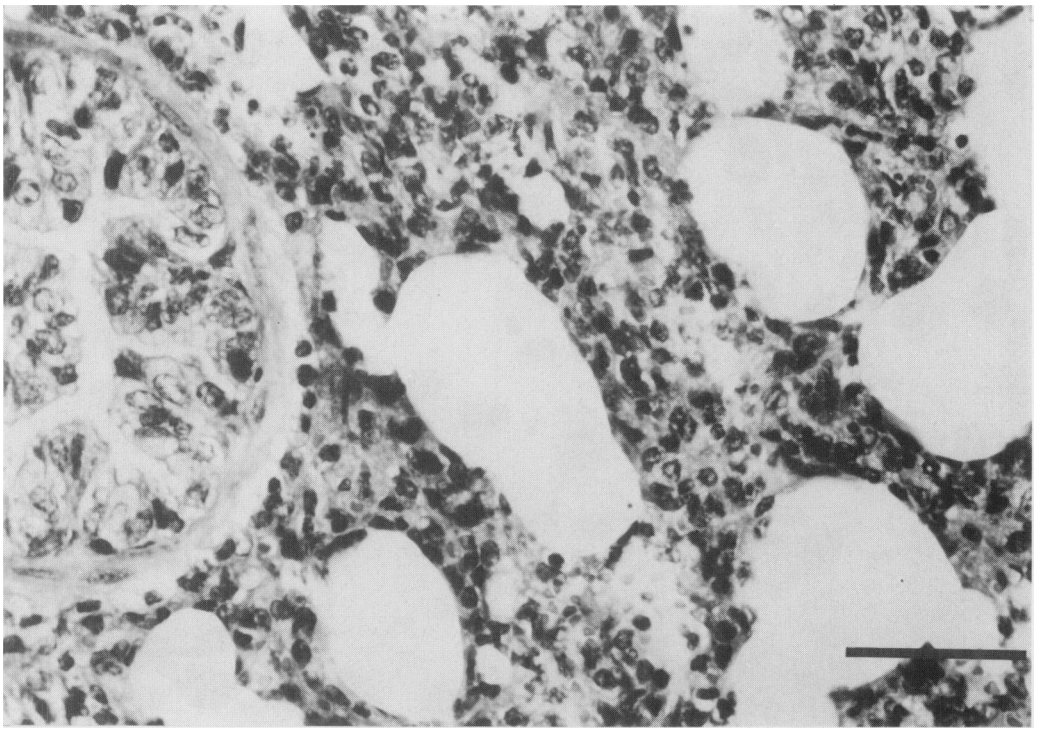

Fig. 4. Thickening of alveolar walls due to infiltration by lymphoid cells and granulocytes. Calf R149, H & E. Bar represents 50 μm.

No microscopic lesions were noted in tissues other than from the respiratory tract.

Immunofluorescence

Detection of viral antigen by imunofluorescence appeared to be dependent on the time of sampling after inoculation (Table 1). In the lungs, antigen was most readily found on day 7 following inoculation (R190), but little was detected on day 9 or 11 and none on day 14. Antigen was located in cells of the bronchiolar epithelium (Fig. 5) and later in cells of the bronchiolar and alveolar exudates (Fig. 6). The latter resembled morphologically those seen detaching from the bronchiolar and alveolar lining, some of which contained phloxinophilic inclusion bodies.

Antigen in the nasal mucosa or in nasopharyngeal smears was seen most frequently and in the greatest quantity on day 7 following inoculation. In sections of nasal mucosa fluorescence was detected in small mononuclear cells located in the lamina propria. Fluorescence was occasionally seen in ciliated, columnar epithelial cells from the nasopharynx, but more often in rounded epithelial cells. Antigen was detected once only and in very small quantity in the tracheal mucosa, on day 9 (R121). RSV fluorescence was bright and largely confined to characteristic, intracytoplasmic aggregates. In the ciliated cells strong fluorescence was also associated with the cilia.

Virological findings

Virus recovery closely paralleled detection of antigen by immunofluorescence. Virus was recovered from the nasopharynx between days 2 and 11 following inoculation, highest titres ($10^{0.7}$–$10^{3.4}$) being detected on days 4

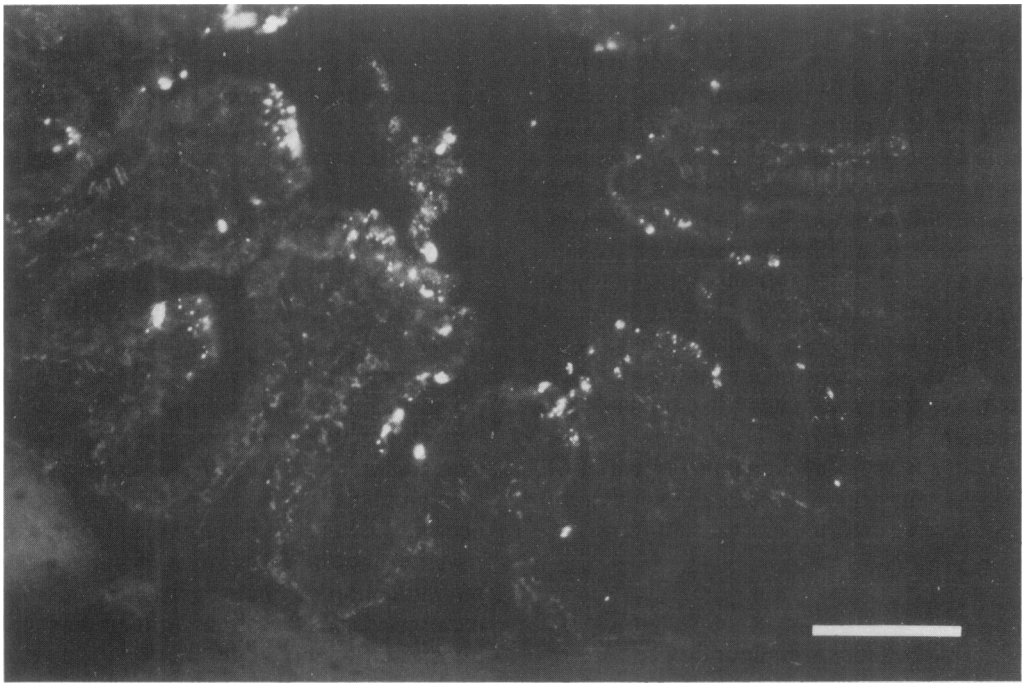

Fig. 5. Immunofluorescence of RSV antigen in cells of the bronchiolar epithelium. Calf R 190 FITC/Evans blue. Bar represents 100 μm.

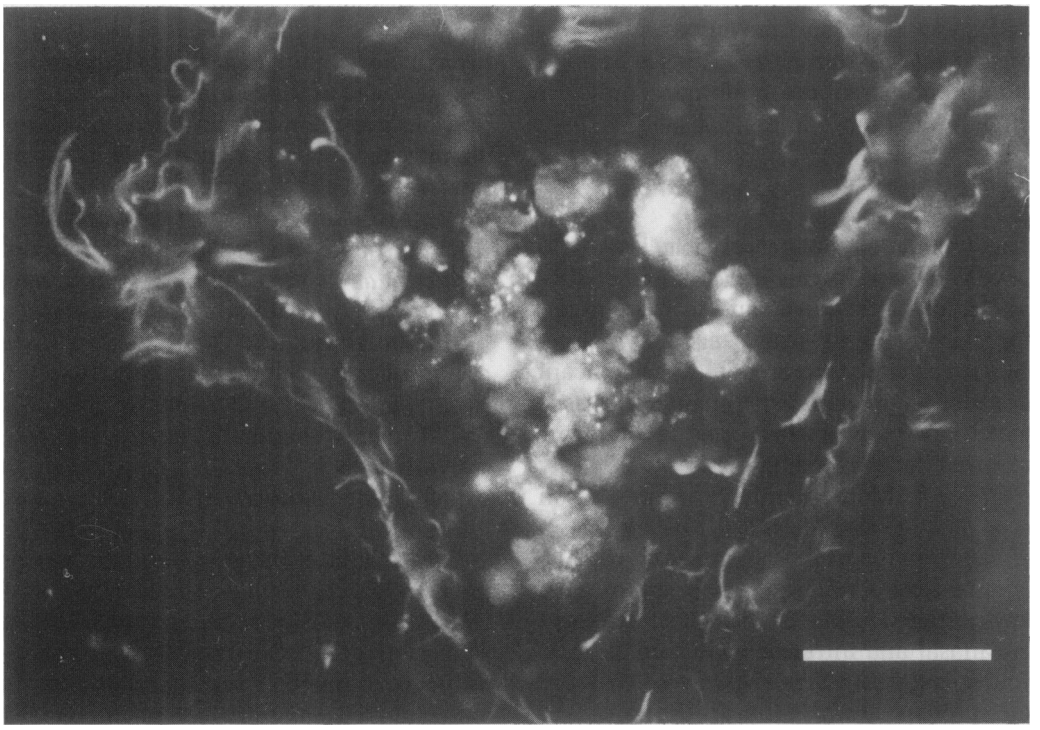

Fig. 6. Immunofluorescence of RSV antigen in cells of the alveolar exudate. Calf R 117, FITC/Evans blue. Bar represents 50 μm.

or 7 (Table 1). One calf (R143) with an apparently longer period of virus shedding (days 2–11 inclusive) received a repeated intranasal inoculum. Virus was recovered from lung up to and including day 9 following inoculation from three calves. A four-fold or greater rise in virus-neutralizing antibody was detected in the serum of three of four calves killed on or after day 11 following inoculation. All four calves showed a response by SRH test and had anti-RSV IgM by RIA. Two of three calves killed on day 9 also had IgM.

There was no evidence of Pi-3 or BVDV infection in any of these calves. BSV antibodies were detected only in calf R192.

Bacteriology

None of the lungs were found to be bacteriologically sterile at the conclusion of the experiments. *Staphylococcus aureus* was isolated from R190, R135, R117, R149 and R143, a *Bacillus* sp. from R192, *Bacillus alvei* and a *Micrococcus* sp. from R140 and *Corynebacterium bovis* from R121. Titres of bacteria did not exceed 10^2 colony-forming units per ml of lung homogenate.

Discussion

The production of macroscopic lesions in bovine lung following inoculation of RSV into gnotobiotic calves and in the absence therefore of mycoplasmas and any significant bacteria represents a significant advance on previous experimental studies in which few or no macroscopic lesions were reported (Jacobs & Edington 1975; Mohanty *et al.* 1975). More recently, however, Bryson *et al.* (1983) and McNulty *et al.* (1983) in joint papers report the experimental production of severe respiratory disease using a low-passaged isolate of RSV given by repeat intranasal and intratracheal inoculation to young colostrum-deprived calves. This and the present study confirm therefore the growing body of circumstantial evidence, accumulated over the last decade, that RSV may be an important pathogen of the bovine respiratory tract (Inaba *et al.* 1970; Paccaud & Jacquier 1970; Rosenquist 1974; Holzhauer & van Nieuwstadt 1976; Stott *et al.* 1980).

The histological lesions, comprising both an exudative and proliferative bronchiolitis, closely resemble in character those described by previous workers on the calf disease (Jacobs & Edington 1975; Mohanty *et al.* 1975; Bryson *et al.* 1983), although in the present study the formation of multinucleate syncytia, with or without eosinophilic inclusion bodies, was a less dominant lesion when compared with the report of Bryson *et al.* (1983). However, these authors also indicate that the lesion is less striking from 7 days after infection. A similar pathology has been described for field cases associated with RSV infection (Bryson *et al.* 1978; Pirie *et al.* 1981; Thomas & Stott 1981; van den Ingh *et al.* 1982) and this further substantiates the important role of RSV in calf pneumonia.

The histological lesion and localization of viral antigen in the epithelium of the small bronchioles in the calf also closely resembles that reported in the human disease with the possible exception of the necrotizing bronchiolitis seen in man (Aherne *et al.* 1970; Gardner & McQuillin 1974). By comparison the experimental disease in Cebus monkeys (Richardson *et al.* 1978) and Cotton rats (Prince *et al.* 1978) does not appear to have the progressive pathology reported for the two natural hosts of RSV. These observations and the fact that a human isolate of RSV will readily infect cattle (Thomas *et al.* 1984) would suggest that cattle are a particularly relevant, albeit large, animal model for studying the pathogenesis of human RSV infection.

The problem of bacterial contamination in gnotobiotic calves has already been discussed by Gourlay *et al.* (1979). We also suggest that their role is not a significant one in the experimental lesions described for the following reasons. Firstly, the numbers of bacteria isolated were small and gnotobiotic calves with comparable numbers of the same

bacteria have in past experiments shown no lesions (L.H. Thomas, unpublished observations). Secondly no particular species of bacteria was related to the pneumonia produced and, thirdly, all the species of bacteria isolated are common in the environment and not those generally considered to be significantly associated with calf pneumonia (Gourlay *et al.* 1970; Bitsch *et al.* 1976).

The narrow period of virus shedding (peak titres between days 4 and 7 after inoculation), the relatively small amount of virus shed and the development of virus-neutralizing antibodies from 11 days after inoculation agrees closely with observations made by other workers (Jacobs & Edington 1975; Mohanty *et al.* 1975; McNulty *et al.* 1983) and suggests that most bovine isolates of RSV are similar in this respect. This also endorses our view (Stott *et al.* 1980; Thomas & Stott 1981) and that of Wellemans & Leunen (1975) that diagnosis of RSV infection should be based primarily on serology and immunofluorescence with less emphasis on virus isolation.

Antibody to RSV was first detected 9 days after inoculation and was primarily IgM. This is to be expected since other classes of specific Ig to RSV do not appear until after 16 days (Thomas *et al.* 1984). RIA, SRH and neutralization tests correlated well, with detection of IgM by RIA being the most sensitive test.

Neither repeated intranasal inoculation nor the age of the calf appeared to influence the extent of the lesions produced, although it must be recognized that the gnotobiotic calves used were in no sense genetically homogeneous. However, the successful production of pneumonia in the current study does contrast with the failure to produce disease using two highly passaged isolates of RSV in another similar study (Thomas *et al.* 1984).

Finally the ability to reproduce substantial lung lesions with RSV now reported both by ourselves and the workers in northern Ireland opens up several avenues of research, e.g. experimental testing of RSV vaccines, experiments with combined infections by other micro-organisms associated with the calf pneumonia complex, and study of the cellular and humoral immune response to RSV infection.

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