Experimental Adenovirus Infection in Calves

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SUMMARY

Twenty-four dairy calves ranging in age from six to 15 weeks were divided so that 10 were inoculated with bovine adenovirus serotype 1 (BA-1), eight were inoculated with bovine adenovirus serotype 3 (BA-3), and six were inoculated with control tissue culture and fluids.

Post-inoculation serum samples from most of the calves presented evidence of adenovirus infection, but none of the animals exhibited signs of respiratory or enteric disease. Histopathological examination of lung tissue was not considered to be specific for adenovirus pneumonia, but was characterized by peribronchial cuffing and slight aspiration pneumonia. BA-1 was not isolated from any of the inoculated calves, and BA-3 was only recovered from the feces of two animals.

Reasons for the discrepancy between these results and those of other workers are considered, and the etiology of peribronchial cuffing is briefly discussed.

INTRODUCTION

Bovine adenoviruses were first recognized in America where the prototype strains of serotypes 1 and 2 were isolated from the feces of clinically normal cattle (23,24). Subsequently, bovine adenovirus type 3 was isolated from the conjunctiva of an apparently normal cow in England (13), and proposed adenovirus serotypes 4 and 5 (4) were isolated from calves with pneumonia and enteritis in Hungary (1,3). In addition, adenoviruses of stated and unstated serotype have been isolated from cattle on other occasions (10,16,29), and in many instances have been circumstantially associated with the production of respiratory disease in calves.

The association between bovine adenoviruses and calf respiratory disease has also

been strengthened by experimental results. Newborn colostrum deprived calves inoculated with a strain of adenovirus type 1 in England, for example, developed mild signs of respiratory disease and some histological evidence of adenovirus pneumonia (15). Calves similarly inoculated with adenovirus type 3 developed more severe clinical signs and marked histological evidence of adenovirus pneumonia (14). Very young colostrum deprived calves inoculated with adenovirus strains isolated by Hungarian workers exhibited antemortem and post-mortem evidence of pneumonia (1), and calves inoculated with strain 10088 of bovine adenovirus type 1 (30) developed signs of lower respiratory disease (29). On the other hand, attempts to produce clinical evidence of respiratory disease with the prototype strain of adenovirus type 1 were unsuccessful (25), and although very young calves inoculated with the Hungarian adenovirus isolates developed pneumonia, older calves similarly inoculated apparently did not develop significant respiratory signs (1).

Additional evidence for the implication of bovine adenoviruses as respiratory pathogens was presented by the reports (12,20) that serum samples from cattle involved in outbreaks of respiratory disease exhibited rises in titer of adenovirus group precipitating antibody. Furthermore, adenoviruses were incriminated on a histological basis as the etiology of about 17 per cent of 125 cases of pneumonia in calves examined by Omar (31).

The purpose of the experiments recorded in this article was to examine the possibility that bovine adenovirus types 1 and 3 could produce pneumonia in experimental calves whose ages corresponded to those of calves usually involved in natural outbreaks of enzootic pneumonia. The strain of adenovirus type 1 used had been isolated in Canada from a calf with non-fatal pneumonia and enteritis (16), and was of undetermined respiratory pathogenicity. The strain of adenovirus 3 used was kindly supplied by Dr. J. H. Darbyshire (Central Veterinary Laboratory, Weybridge, Surrey, U.K.) and was known to be capable of producing pneumonia in young calves (14).

TABLE I   General Summary of Experiments

Calf No.	Age in Weeks	Inoculum	Dose Administered (a) TCD$_{50}$†	Expressed In (b) ml.	Route	Post Inoculation Day Killed
1	9	BA-1	$10^{4.5}$	1	AER	NK
2	8	,,	,,	,,	1T	,,
3	9	,,	$10^{5.5}$	10	,,	7
4	12	,,	,,	,,	,,	NK
5	14	,,	$2 \times 10^{5.5}$	20	,,	7
6	12	,,	,,	,,	,,	NK
7	12	,,	$10^{5.5}+$	10+	,,	7
8	12	,,	,,	,,	,,	14
9	7	,,	$5 \times 10^{4.5}+$	5	IV	NK
10	6	,,*	$3 \times 10^{5.5}$	5	IT	7
11	8	BA-3	$10^{3.5}+$	10+	IT	7
12	6	,,	,,	,,	,,	14
13	6	,,	$2 \times 10^{3.5}$	20	,,	7
14	12	,,	,,	,,	,,	14
15	6	,,	$5 \times 10^{2.5}+$	5+	IV	NK
16	9	,,*	$3 \times 10^{3.5}$	5	IT	7
17	14	,,*	$3 \times 10^{3.5}+$	5+	,,	7
18	15	,,*	,,	,,	,,	14
19	10	BTtc	—	10+	IT	7
20	10	,,	—	,,	,,	14
21	8	,,	—	20	,,	7
22	7	,,*	—	,,	,,	14
23	10	,,*	—	5+	,,	7
24	10	,,*	—	,,	,,	14

BA-1	= Bovine adenovirus type 1		NK	= Not killed
BA-3	= Bovine adenovirus type 3		*	= Inoculum concentrated by centrigufation
BTtc	= Bovine testicle tissue culture		†	= as established before and after calf
AER	= Aerosol			inoculation
IT	= Intratracheal IV = intravenous		+	= two doses of indicated titer and volume 20 hours apart

MATERIALS AND METHODS

CALVES

Dairy calves of mixed colostral status were obtained from selected farms immediately after birth and raised in isolation units in pairs. With three exceptions, calves were inoculated via the intratracheal route while restrained in a standing position. One calf was administered the virus inoculum as an aerosol, using a number 40 DeVilbiss nebulizer attached to a plastic cone held tightly over the muzzle. Two were inoculated intravenously.

TISSUE CULTURES AND VIRUSES

Bovine testicle (BT) cell cultures, used at their primary or secondary passage, were grown in Hanks Balanced Salt Solution containing 0.5% lactalbumen (HLa) and 10% fetal calf serum (FCS), and maintained in HLa with 5% FCS. Media was supplemented with 250 units of penicillin, 125 micrograms of streptomycin and 100 units of Mycostatin per ml.

Virus-inoculated cultures were harvested at the time of maximum cytopathic effect (CPE) by thrice freezing and thawing, and were stored in sealed containers at –70°C. Infectivity titrations (27) were determined on virus pools at the beginning and at the end of experiments.

For use in certain experiments a tenfold concentration of the virus inoculum was obtained by centrifugation of the pool at 19,620X g for 60 minutes in a refrigerated Spinco model L-2 ultracentrifuge using a number 30 head.

Uninoculated bovine testicle monolayers rapidly frozen and thawed three times were stored at –20°C and used as the control inoculum. A representative portion of each pool of virus and control inoculum was incubated on blood and PPLO (28) agar and examined on the fifth day after inoculation.

COLLECTION OF SPECIMENS AND VIRUS ISOLATION

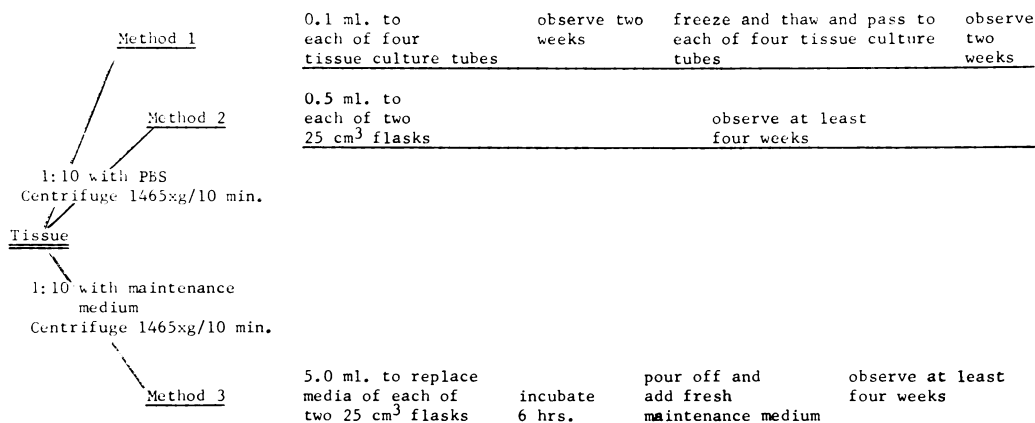With the exception of those inoculated

Method 1
0.1 ml. to each of four tissue culture tubes | observe two weeks | freeze and thaw and pass to each of four tissue culture tubes | observe two weeks

Method 2
0.5 ml. to each of two 25 cm³ flasks | observe at least four weeks

1:10 with PBS
Centrifuge 1465xg/10 min.

Tissue

1:10 with maintenance medium
Centrifuge 1465xg/10 min.

Method 3
5.0 ml. to replace media of each of two 25 cm³ flasks | incubate 6 hrs. | pour off and add fresh maintenance medium | observe at least four weeks

Fig. 1. Methods used for virus isolation from tissue.

intravenously (Table I) virus isolation was routinely attempted on nasal mucus of all calves, on throat mucus and feces of calves 13 to 24 (Table I) and on mediastinal and bronchial nodes, tonsillar tissue and lung tissue from each calf killed. Nasal mucus was collected on preinoculation day one and usually on post-inoculation days four, seven and 14.

Nasal swabs collected from both nostrils were placed together in four ml of antibiotic and Mycostatin fortified phosphate buffered saline (PBS) which was alternately aspirated into and expressed from a 5 ml syringe until the swabs were throughly washed. The expressed fluid was centrifuged to clarify and the supernatant stored at -20° or -70°C until used. Throat swabs were processed in a similar manner.

Fecal samples were prepared as a 10% suspension with PBS, centrifuged to clarify and the top 5 ml of supernatant was re-centrifuged at 4080X g for 30 minutes at 4°C. Two and one-half ml of the resulting supernatant were added to 0.5 ml of a solution previously formulated to contain 25,-000 units of penicillin, 25,000 micrograms of streptomycin and 100 units of Mycostatin per ml, placed at room temperature for 30 minutes and stored at -20°C or -70°C.

Calves were anaesthetized and killed by exsanguination. Two gram (approximate) portions of tonsil, bronchial lymph node, trachea and areas of lungs selected for virus recovery were placed in individual containers and immediately frozen. Lung samples were obtained from the ventral surface of the lungs, at a point approximately in the center of each lobe. Seven samples of lung tissue were thus obtained from each calf, but additional samples were collected from designated areas in those lungs which exhibited macroscopic lesions. Duplicate tissue samples were fixed in Bouin's solution, paraffin embedded, cut at 6 microns and routinely stained with hematoxylin and eosin (11). Selected sections were stained with Lendrums phloxine tartrazine and Gordon and Sweet reticulin stain (11).

Approximate one gram samples of lung, tonsil and bronchial node tissue were ground (in Ten Broeck grinders) in a proportion of 10% with antibiotic-fortified PBS. The resulting suspensions were clarified by centrifugation at 4°C and the supernatant was inoculated onto tissue culture monolayers according to three different methods as shown in Figure 1. Methods 1 and 2 were used for all isolation attempts. Method 3 was used on particularly suspect material when other methods had failed to reveal the presence of cytopathic virus. This method, however, was damaging to most tissue culture monolayers and was seldom of much use when extended observation of inoculated tissue culture was required. Virus isolates were identified by neutralization tests with homologous antibody.

SEROLOGY

Mature New Zealand white rabbits were immunized with Freon (dichlorodifluoromethane) treated virus (18) according to the schedule outlined by Rafajko (33). All serum was heated at 56°C for 30 minutes prior to use in serological procedures.

Complement-fixation (CF) tests were performed according to the method outlined by Lennette (27) using two units of

homologous antigen, two units of complement and overnight fixation at 4°C.

Serum neutralization tests were performed according to "procedure 2" described by Rowe, Huebner, Hartley, Ward and Parrott (35). Serum antibody titers were not determined beyond a 1 in 16 dilution of serum as this was sufficient to determine a fourfold rise in titer between acute and convalescent sera. All positive or suspicious positive serological tests were repeated, and pre-inoculation and post-inoculation serum samples from each calf were subjected to the SN test at the same time.

RESULTS

CLINICAL ILLNESS

None of the calves manifested clinical evidence of respiratory disease or diarrhea at any time during the course of experiments. Several calves exhibited a slight rise in rectal temperature (Table II), sometimes accompanied by partial anorexia. Three calves developed slight pre-inoculation pyrexia, but this was not accompanied by signs of illness.

SEROLOGY

Serum neutralization (SN) titers in virus-inoculated calves were rarely detected by post-inoculation day (pid) 7, but were almost invariably detected by pid 14 (Table III). At the beginning of the experiments pre-inoculation sera were screened for adenovirus antibody by the CF test only, while the pre-inoculation SN titer was determined after virus inoculation. Subsequent to the discovery of one calf with a pre-inoculation SN titer, however, sera were collected on days seven and one prior to virus inoculation and the pre-inoculation SN titer was determined prior to inoculation.

Adenovirus complement fixing antibody (Table III) was only detected in two of the 18 virus-inoculated calves and the antibody titer was very low. These calves had received either BA-1 or BA-3 by the in-

VIRUS ISOLATION

Virus was not recovered from any of the calves inoculated with BA-1, but BA-3 was recovered from the feces of two calves (13 and 14) inoculated with this virus, on post-inoculation day 7 (both calves) and day

14 (calf 14).

LUNG LESIONS

Calves inoculated with unconcentrated BA-1 (Table I): None of the calves developed macroscopic lung lesions, but all exhibited microscopic lesions. The most consistent histological lung lesion was evident

TABLE II Temperature Response of Experimental Calves (Expressed as the Day on which Rectal Temperature was Between 103 and 104°F and Recorded to the Nearest Degree)*

Calf No.	Preinoculation day	Postinoculation day
1		5
2		12
3	4	1 to 7
4		
5		6 and 7
6		6, 7 and 8
7		3, 4, 6 and 7
8		8 and 9**
9		
10		5, 6 and 7
11	5	3 and 4
12		2
13		3 and 4
14		3 and 4
15		5 and 6
16		4
17		2
18		2
19		3
20		
21		
22	3	1
23		3
24		

*Temperatures lower than 103°F are not recorded
**105° on day 9

as varying degrees of lymphoreticular cell accumulation both within and around the walls of bronchioles, a lesion referred to as peribronchial cuffing. Most extensive cuffing was present in the anterior and ventral portions of the lungs but in a few instances was present to a lesser degree in all areas sectioned. Considerable variation occurred in the extent of cuffing between individual calves but in general peribronchial cuffing was more commonly observed in or around the walls of bronchioles larger than 150 microns in diameter. These accumulations of lymphoreticular cells were of varying magnitude and occurred in the lamina propria, peribronchial adventitia, or in both locations (Figures 2 to 6). In some

TABLE III.   Serological Response†

Calf No.	Pre Inoculation Day				Post Inoculation Day							
	7		1		7		14		21		28	
	SN	CF	SN	CF	SN	CF	SN	CF	SN	CF	SN	CF
1			—	—	—	—	—	—	—	—		
2												
3			—	—	—	—*						
4			—	—	—	—	8	—	8	—		
5			—	—	—	—*						
6			—	—	—	—	4	—				
7			16	—	16	—*						
8			—	—	16	—*						
9			—	—	2	—	16	2			16	8
10	—	—	—	—	—	—*						
11	—	—	—	—	—	—*						
12	—	—	—	—	—	—	8	—*				
13	—	—	—	—	—	—*						
14	—	—	—	—	2	—	16	—*				
15	—	—	—	—	—	—	16	—			16	2
16	—	—	—	—	—	—*						
17	—	—	—	—	—	—*						
18	—	—	—	—	2	—	16	—*				
19	—	—	—	—	—	—*						
20	—	—	—	—	—	—	—	—*				
21	—	—	—	—	—	—*						
22	—	—	—	—	—	—	—	—*				
23	—	—	—	—	—	—*						
24	—	—	—	—	—	—	—	—*				

†Dilutions of serum are those at which positive results were obtained and are recorded as the reciprocal of the dilution
—   = antibody titer undetected at serum dilution of 1:2
blank space = serum not tested
*   = day on which calf was killed
SN   Serum neutralization antibody
CF   Complement fixing antibody

areas the epithelium of affected bronchioles was flattened, but in most it was morphologically normal.

Gordon and Sweet's reticulin stain demonstrated that varying degrees of atelectasis had been produced by pressure of peribronchial cuffs on adjacent alveoli. Cuffs which did not appear to contain follicles when subjected to H and E staining were often, in fact, composed of one or more distinct follicles morphologically indistinguishable from those of the tonsil or other normal lymphoid tissue. Follicles with secondary centers or reaction centers (5, 37) were occasionally observed.

Two calves exhibited slight exudate (primarily composed of neutrophils and macrophages in an amorphous proteinaceous material) in a few bronchioles and alveoli of the anterior lobes of the lungs. This reaction was mild, and there was no evidence of alveolar epithelial cell hyperplasia, necrosis, or adenovirus inclusion bodies. Tracheal tissue was normal.

Tonsil and bronchial lymph node from some virus-inoculated and control calves contained scattered cells with large intranuclear bodies which closely resembled virus-induced inclusion bodies. These structures were invariably present only in large lymphoblast-like cells and were considered to be the prominent nucleoli of cells in the active process of multiplication.

Calves inoculated with unconcentrated BA-3 (Table I): Of the four calves administered unconcentrated BA-3 pool one (calf 13) exhibited macroscopic lesions of dark red patchy lobules of atelectasis in both cardiac lobes. These areas were histologically characterized by evidence of aspiration pneumonia. Marked plugging of small bronchioles and alveoli by cellular accumulations in which neutrophils and alveolar macrophages predominated was accompanied by alveolar collapse and the presence within the cellular reaction of apparently "foreign" eosinophilic amorphous debris (Fig. 7). This material was not positively identified but it was thought to be tissue-culture debris. Lung tissue from the other
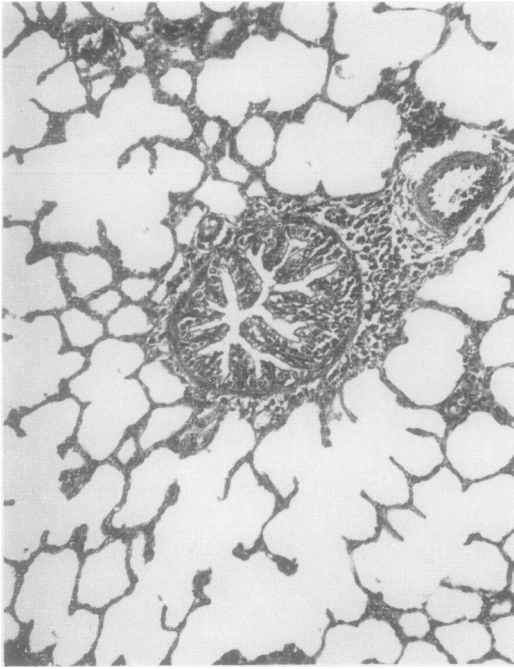
Fig. 2. A small bronchiole with a slight accumulation of lymphoid cells in the adventitia. This represents a very early change.
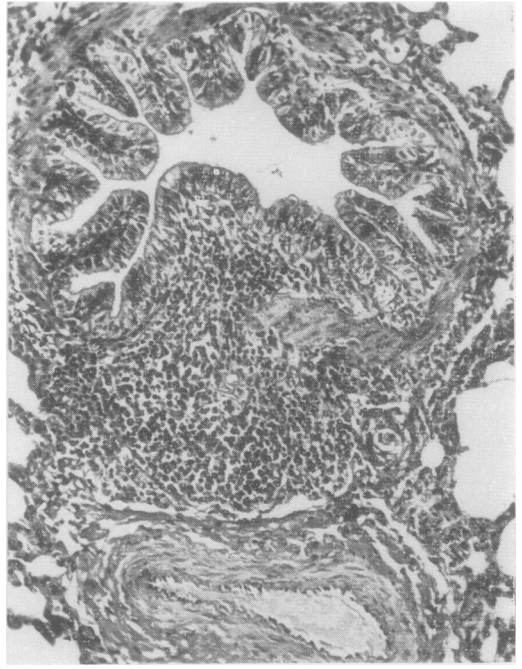

Fig. 3. A lymphoid follicle extends from the lamina propria into the adventitia on one side of a small bronchiole.

calves was not microscopically different from that of calves inoculated with BA-1.

Calves inoculated with concentrated BA-3 or BA-1: Of the three calves which received BA-3 concentrate two (calves 16 and 18) developed macroscopic evidence of scattered lobular atelectasis of the anterior ventral lobes but the lungs of the third (calf 17) remained macroscopically normal. All three exhibited varying degrees of peribronchial cuffing. Calf 16, which had developed the most severe macroscopic lung lesions, presented microscopic lesions suggestive of aspiration pneumonia, and similar to those described above for calf 13. The very small areas of atelectasis observed in lung tissue from calf 18 were histologically characterized by marked peribronchial cuffing and cellular exudate in bronchioles and alveoli. The lungs of the third calf (calf 17) which received BA-3 concentrated inoculum were similar to control calves. Peribronchial cuffing was evident but was unaccompanied by other lung lesions.

The only calf (number 10) which received BA-1 concentrate developed macrospic lung lesions of atelectasis in the ventral aspect of the right cardiac lobe and the microscopic appearance of this area was similar to that described above for calf 13.

Control calves: None of the calves which received uninoculated tissue culture developed macroscopic lung lesions, but all had varying degrees of peribronchial cuffing which was not noticeably less than that of virus-inoculated calves. Three control calves exhibited slight edema and cellular infiltration in some alveoli and bronchioles of the anterior ventral lobes..

DISCUSSION

A major obstacle to the understanding of bovine respiratory disease is the difficulty of determining an experimental challenge dose of an organism equivalent to that contacted by animals under field conditions. Furthermore, the variation of housing, nutrition and climate to which farm calves are naturally subjected are difficult, or impossible, as yet to reproduce in the laboratory and are factors which undoubtedly exert a significant effect on pathogenesis of respiratory disease under field conditions. In addition, virus strains within a serotype may vary in respiratory pathogenicity and the effect of laboratory manipulations on a virus after isolation may affect its virulence. Thus, viruses found incapable of producing respiratory disease under experimental conditions may
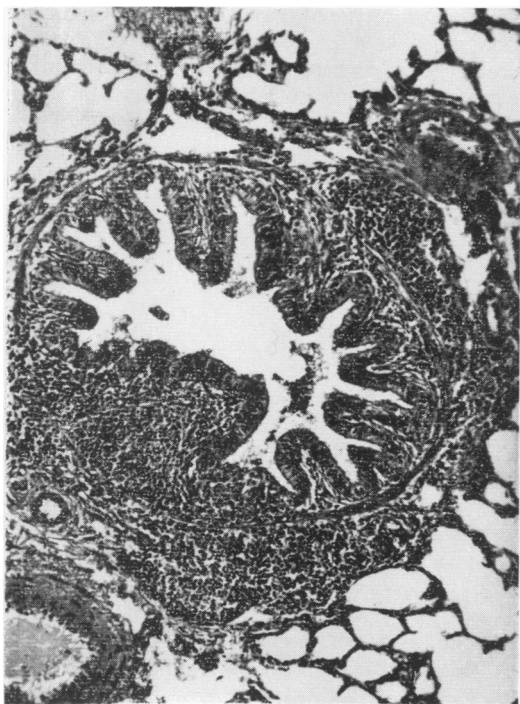
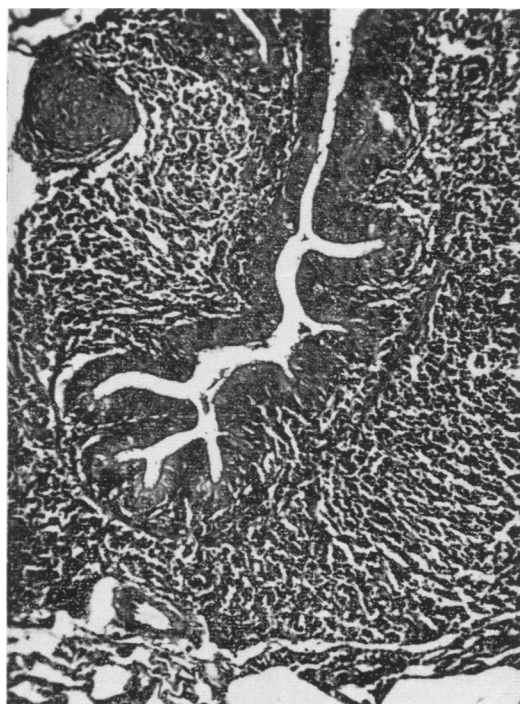Fig. 4. The lymphoid elements are more extensive than in Figure 2.

Fig. 5. Medium sized bronchioles surrounded by lymphocytes. The lesions shown in Figures 5 and 6 would be considered moderate to extensive in degree. More extensive lesions were present.

not be summarily dismissed as non-respiratory pathogens.

Differences between the results of our experiments with BA-1 and those of other workers (15,29) may be explained on the basis that the BA-1 strain used by us was different to strains previously used or the challenge dose was lower (29) or the experimental calves were older (15). Results with BA-3, a strain of known bovine respiratory pathogenicity (14), may be similarly explained because a lower titer of inoculum was administered to calves older than those previously used (14).

It would appear that the correlation between adenovirus infection and the development of adenovirus CF antibody in calves is poor. In all calves except the two which had received the inoculum by the intravenous route, the rise in SN antibody was not accompanied by detectable rise in CF antibody. Perhaps in the absence of active virus multiplication, or when the adenovirus antigen was not administered intravenously a threshold of antigenic stimulation required for CF antibody response was not reached. Complement-fixing antibody developed more slowly than SN antibody, but the persistence of the respective antibody levels was not determined past 28
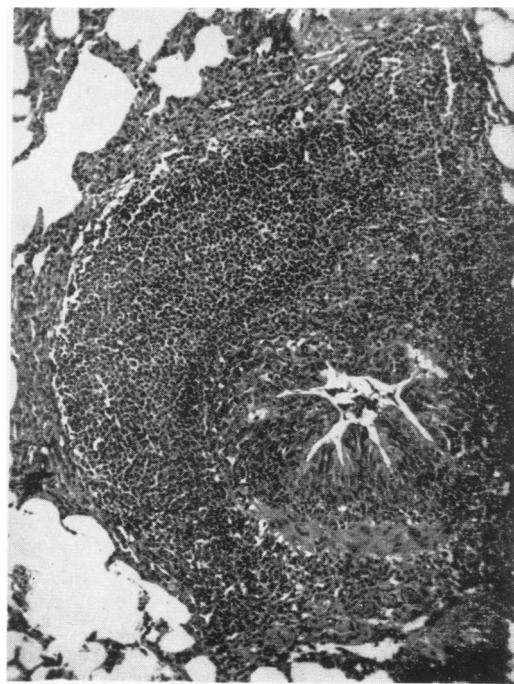

Fig. 6. As in Fig. 5.

days.

It has been reported that several blind passages in tissue culture may be required to isolate bovine adenoviruses from calf tissues (3,10). That this is not true in all cases was shown by Ditchfield (16) who isolated the BA-1 strain used in these experiments on first passage in tissue culture, and by Darbyshire et al (14), who usually recovered BA-3 from experimental calves on first passage. On this basis it was assumed that two blind passages in tissue culture should have uncovered BA-1 or BA-3 in at least some of the tissues examined if these viruses had been present.

The observation that BA-3 was isolated from the feces of the two calves which had received the largest single volume of inoculum (20 ml) suggested that this volume had afforded a greater opportunity for some to be coughed up and swallowed at the time of intratracheal inoculation. Adenoviruses can cause intestinal infection in man (36) and calves (1,14), and there is evidence that the viruses may be isolated from the feces of experimentally infected calves for periods as long as, or longer than, from nasal mucus (1,29). Perhaps during the investigation of pneumonia in calves, virus isolation should routinely be attempted on feces as well as respiratory secretions or tissues.

Macroscopic lung lesions were limited to four calves, of which three had received BA-3. However, the histological lesions in affected lobes were not similar to those previously described (14,15,31) for adenovirus pneumonia in calves. None of the lungs examined presented microscopic evidence of hyperplasia or necrosis of bronchial epithelium, inclusion bodies were not observed, and lesions could usually be attributed to an aspiration pneumonia produced by the virus-containing tissue culture inoculum.

The large basophilic bodies occasionally observed in nuclei of cells in lymphoid tissue were considered to be large nucleoli rather than adenovirus inclusion bodies, on the criteria that developing stages of adenovirus inclusion bodies (6,9,26) were not seen, that virus was not isolated from affected tissue, and that similar structures were seen in tissues from control calves seronegative to both BA-1 and BA-3. However, it is known that adenoviruses may remain latent in lymphoid tissue (34,36) and humans with latent infection may remain adenovirus seronegative (36). The

conclusion, therefore, that the inclusion bodies observed in experimental calves were large nucleoli of cells in the early stages of mitosis may be equivocal, but this point would probably only have been clarified by the application of fluorescent-microscopy techniques to the tissues under question.

Peribronchial cuffing by lymphoreticular cells has been associated with respiratory disease in several species (2,17,19,21,22), but has also been observed in apparently normal animals (7,8). Furthermore, it has been suggested that such cellular accumulations merely reflect a reaction to antigenic stimulation within the lungs (22). All the calves in these experiments exhibited some degree of peribronchial cuffing and the degree of lymphoreticular hyperplasia was of similar intensity in virus inoculated and control calves.

Perhaps a degree of peribronchial cuffing is normal in the immature bovine, but it has been the authors' experience that a large number of calves which have died from causes other than pneumonia do not possess such lung lesions. The possibility that cuffing was induced by an unknown or undetected infectious agent present in
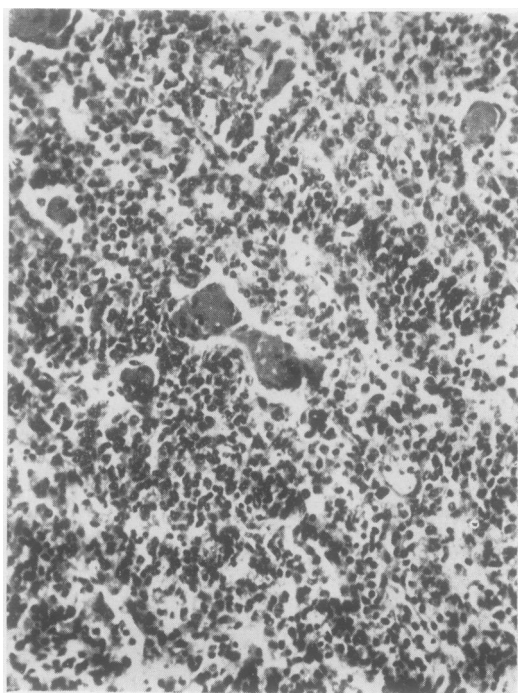

Fig. 7. Atelectasis and debris are shown, apparently caused by the inoculum.

the calves prior to inoculation with adenovirus is of some significance inasmuch as an undetected infection of this sort may have influenced the pathogenesis of administered virus. Equally tenable, however, is the observation that peribronchial cuffing may have been induced by the antigenicity (22) of the tissue culture in which virus inoculum was suspended, a factor unresolved because uninoculated control calves were not included in this study. The exact cause of the cuffing remains problematical but on the basis that all control calves possessed similar lesions, and that cuffing in virus inoculated calves was unaccompanied by virus isolation, it was assumed that peribronchial cuffing was not caused by the activity of replicating BA-1 or BA-3.

It was concluded that under the stated conditions of these experiments neither BA-1 nor BA-3 exhibited respiratory pathogenicity for calves of about 1½ to 4 months of age. That these results may have differed if experimental parameters had included higher titer of inoculum or the use of gnotobiotic calves is recognized.

ACKNOWLEDGEMENTS

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