

has been isolated from cattle in Indonesia (Bartz, pers. comm.).

The alphavirus group also contains viruses that cause fever and neurological disease. The group is represented in Indonesia by Chikungunya virus, confirmed by viral isolation. Infections have been identified serologically in cattle (Bartz, pers. comm.). Its effect in cattle is not known. Other members of the group identified on serological grounds in Indonesia are Ross River virus and Geta.

Of the bunyaviruses, there is serological evidence that Batai virus and bunyavirus or an antigenically similar virus infect cattle and buffalo (Olson et al. 1983). Infections by Akabane (AKA), a Simbu group virus, have been identified by serum neutralisation tests in Bali and East Java (Sudana and Miura 1982), and in southern Sumatra and Lampung by HI tests (Marfiatiningsih 1983). AKA is noted for teratogenic effects, but can cause a nonsuppurative encephalitis in calves (Beveridge 1986). Its effect in adult buffalo and Bali cattle is not known. Another group member, Ingwavuma (ING), has been isolated in Indonesia and antibodies found in 35 of 79 (44%) buffalo sera sampled (Converse et al. 1985). Its clinical significance is unknown.

Of the orbiviruses, there is serological evidence for the presence of several serotypes of bluetongue (BLU) and epizootic haemorrhagic disease virus (EHD) (Sendow et al. 1986). BLU may cause inapparent infections in cattle and also mild fever with salivation, nasal discharge and anorexia.

Ephemeral fever, well known as a transient fever in cattle, has been suspected clinically by the authors on Java and serological evidence for its presence has been presented (Soeharsono et al. 1982).

Hence, among the arboviruses it is possible to list several known pathogens of cattle, as well as some of unknown clinical significance that are present in Indonesia and that have infected cattle and buffalo, as demonstrated either serologically or by viral isolation. The capacity to diagnose infections with these viruses should be established by laboratories conducting investigations of febrile diseases of unknown aetiology. It cannot be assumed without reservation that an animal with a fever in an experiment is showing a mild form of the disease under study.

Other Bovine Viral Diseases

Trypanosomiasis and arboviral infections are considered of special interest in Indonesia, and possibly constitute the main potential problems in differential diagnosis. However, other bovine diseases usually considered in the differential diagnosis of MCF should not be ignored, especially rinderpest, bovine virus diarrhoea-mucosal disease (BVD-MD) and infectious bovine rhinotracheitis (Barker and Van Dreumel 1985). Rinderpest is not present in Indonesia and from a general description (Barker and Van Dreumel 1985) it seems that rinderpest should be easily diagnosed. It would differ epidemiologically from MCF by its high morbidity and mortality rates. Syncytial giant cells with characteristic cytoplasmic inclusions are visible in histological sections and the virus can be isolated readily.

BVD infections have been identified serologically in East Java (Putra 1985), and may be widespread. These present a more difficult problem, for not only can the clinical syndrome and gross pathology be similar to that of MCF, but a fibrinoid necrotising vasculitis may be present in blood vessels of the gastrointestinal tract and the heart, brain and other organs. Massive lymphoid proliferation is not a feature, however. Animals with inapparent persistent infections may have mild perivascular cuffing and hypertrophic endothelial cells in the vessels of the brain, and show satellitosis. Although it should be possible to histologically differentiate BVD infection from MCF when associated with acute disease, it could possibly be associated with chronic infections or inapparent infections.

Other Agents

Protozoa such as *Theilaria* spp. and bacteria such as the rickettsias can cause diseases similar in some respects to MCF. As well as careful comparison of the histopathology described in association with such agents with that of MCF, careful examination of blood smears and histological sections should be conducted to ensure that the presence of agents with visible tissue forms is not overlooked. The presence in Indonesia of disease agents from these groups has not been confirmed, although an *Ehrlichia* sp. is suspected of being involved in Jembrana

disease (Hardjosworo and Budiarmo 1973). This syndrome is not associated with clinical respiratory signs but may show fever, lymphadenopathy due to lymphoproliferative changes, and diarrhoea. Histologically, widespread lymphoreticular infiltration occurs in the liver, kidneys and other organs. Apparently, however, the central nervous system is spared.

Case Reports

Case 1. Spontaneous MCF in a *Bos indicus* (Ongole) from Banyuwangi

The animal was an adult male showing clinical signs of the head and eye form of MCF. It was sent for emergency slaughter, photographs taken of gross pathology by field staff, and tissues submitted in formalin to RIVS. The case showed typical clinical signs of moderate severity and is of local interest in Indonesia where MCF is rare in *Bos indicus* breeds.

There was profuse ocular discharge staining the cheeks, a mucoid nasal discharge and salivation. The corneas showed a peripheral rim of opacity. Prescapular and prefemoral lymph nodes were visible under the skin. At postmortem examination the lymph nodes were enlarged, there were epicardial haemorrhages along the coronary groove, but no other gross changes were recorded.

In the CNS were multiple lesions of vasculitis, perivasculitis and gliosis in proximity to vessels, progressing in places to lesions of granulomatous encephalitis.

In the lymph nodes, the cortex was prominent and there were numerous germinal centres showing depletion of cells and protein deposition. Paracortical areas were moderately expanded, and medullary cords were prominent and contained lymphoblasts. Sinuses contained prominent lymphoblasts, macrophages with haemosiderin, and histiocytes with prominent eosinophilic cytoplasm. There was marked mononuclear cell invasion of the capsule and trabeculae, and perivasculitis of medium-sized arteries. There was marked vasculitis of the vessels of the hilus. The splenic capsule was invaded by mononuclear cells and the red pulp was highly cellular.

Vasculitis and perivasculitis affected the vessels of the corticomedullary junction in the kidney, which also had an interstitial nephritis and a mononuclear cell infiltrate with plasma cells adjacent to the pelvis. The lung showed obvious vasculitis and perivasculitis, interstitial thickening and a subpleural mononuclear cell infiltrate. There was a periportal mononuclear cell infiltrate in the liver. The heart had an interstitial mononuclear cell infiltrate with blast cells. There was perivasculitis and mononuclear cell infiltration at the angle of the cornea.

The skin of the muzzle showed perivascular and papillary mononuclear cell infiltrates, and focal necrosis of the epithelium. Lesions of perivasculitis were present in the submucosa of the abomasum, and vasculitis and perivasculitis in the serosa of the intestine. Mononuclear cell infiltration of the mucosal epithelium of the bladder had occurred, and also of the smooth muscle. In the submucosa vessels showed perivasculitis, and vasculitis as evidenced by mononuclear cell infiltration of the vessel walls.

Case 2. Buffalo from an MCF transmission experiment

This buffalo was a young male inoculated with more than 1 litre of blood from a buffalo with spontaneous MCF. The animal developed a fever several weeks postinoculation, suffered loss of appetite and became progressively emaciated over a 12-month period. It became weak, was reluctant to stand, and died. There were no other marked clinical signs.

The case is presented because of the unusual clinical course. The diagnosis is important, for the case may be an indication of a cause of a wasting disease in buffalo in the field, and hence have epidemiological and disease control implications.

The brain showed perivascular cuffing and vasculitis with mixed mononuclear cells, and foci of gliosis. The vessels of the rete mirabile were mostly unaffected, some showed perivascular foci of mononuclear cells and a few a segmental vasculitis with mononuclear cell infiltration of the vessel wall.

Lymphoid tissues were reduced in size but contained active follicles. The paracortical area was usually not prominent. Subcapsular, trabecular and medullary sinuses were

comparatively empty, containing a few histiocytes and a small number of lymphocytes. Lymphoblastic cells were infrequent.

The heart showed an interstitial mononuclear cell infiltrate and periarteritis and arteritis, particularly of larger vessels. The liver had periportal infiltrates of mononuclear cells with blast cells showing occasional mitoses. The kidney showed a mixed mononuclear cell infiltrate with vasculitis and perivasculitis, particularly of vessels at the corticomedullary junction. The lung had an interstitial infiltrate of mononuclear cells including blast cells that was more marked perivascularly.

There was vesicle formation in the epithelium of the rumen. Necrotic papillae showed vascular congestion and hyaline necrosis while vessels of the submucosa showed a mild vasculitis and perivasculitis. In the serosa was a mild segmental vasculitis. Giant cells were present in the muscle. In the abomasum there was a mixed mononuclear infiltrate of the mucosa and submucosa, perivasculitis with lymphocytes, macrophages and blast cells, and marked lesions of vasculitis. One affected vessel showed a segmental necrotising vasculitis, while another was completely infiltrated by mononuclear cells with partial occlusion of the lumen.

Case 3. Bali breed male, experimental contact with sheep

The calf was 6–12 months old when placed in contact with sheep associated previously with MCF in deer. After a contact period of 100 days, the calf developed nasal discharge and diarrhoea. After 11 months of contact it was again ill for 4 days with fever of 41.2°C. Twenty-one days after the onset of this incident it was killed for postmortem examination. Its temperature was then 39.1°C.

Lymph nodes were slightly enlarged, there was ulceration of the abomasal mucosa on the folds, and there were foci of mild haemorrhage in the small and large intestines.

Histological changes were mild but present in several organs. In the CNS there were mononuclear cells adjacent to blood vessels without definite cuff formation, and distinct foci of gliosis.

Lymph nodes showed prominent germinal centres. Paracortical areas showed tingible body

macrophages, but were not greatly expanded. Medullary cords showed proliferation of lymphoid cells with lymphoblasts in some foci. Subcapsular sinuses were filled with a mixed population of mononuclear cells, but the medullary sinuses were not highly cellular although containing significant numbers of lymphoblasts in some areas.

The heart had a mixed mononuclear cell infiltrate, and the kidney a mild interstitial nephritis with infiltrates of mixed mononuclear cells adjacent to blood vessels and glomeruli. In the lung there was a marked mononuclear cell infiltrate particularly around blood vessels. In the eye, a focus of perivascular infiltration at the angle of the cornea contained mitotic figures.

Sections of intestine showed lymphoid infiltrates in the serosa and submucosa, with some lymphoblastic cells. Foci with erosions or ulceration contained polymorphs also. The abomasum contained mucosal and submucosal infiltrates of mononuclear cells.

Cases 4 and 5. Clinically normal buffalo

These animals were involved in a trypanosomiasis experiment in which the effects of high and low planes of nutrition on immunological responses during *T. evansi* infections were compared (Partoutomo, unpublished data). At necropsy, both animals, which had been kept on a high plane of nutrition, were clinically normal and showed no gross pathological changes.

Case 4 had been experimentally infected with *T. evansi*.

In the brain, mild vasculitis and perivasculitis were evident, with mononuclear cells in the lumen of small vessels and also foci of mild nonsuppurative meningitis. In other tissues there were also mild to moderate changes based on infiltration of mononuclear cells. Interstitial nephritis, periportal involvement in the liver, and an interstitial pneumonia were evident. Germinal centres were active in the lymph nodes which also showed mononuclear infiltration of the capsule and trabeculae and extension of lymphoid cells beyond the capsule. Subcapsular and trabecular sinuses were highly cellular, and the medullary sinuses were becoming progressively affected. Cells in sinuses were

predominantly histiocytic, giving an appearance of early sclerotic change. Small lymphocytes were plentiful, and plasma cells and some lymphoblasts were present. The red pulp of the spleen was highly cellular, showing histiocytosis.

Case 5 was a *T. evansi*-free control

In the brain were vasculitis, perivasculitis and foci of gliosis with granuloma formation with syncytial giant cells. One such cell contained basophilic-staining rod-shaped material. There were severe foci of interstitial nephritis, a mild periportal mononuclear cell infiltrate in the liver, perivascular mononuclear cell infiltrates in the lung and focal lymphoid accumulations in the mucosa, submucosa and serosa of the abomasum. Lymph node germinal centres were active. The trabeculae were infiltrated with mononuclear cells, and the sinuses showed a marked histiocytosis progressing to sclerosis, as described in the previous case.

Discussion

This series of cases included an animal that died of spontaneous MCF, one that showed fever and recovery, another that died in an MCF transmission experiment, and others that were clinically normal during other experiments. Of the cattle and buffalo that died, the first mentioned showed a normal clinicopathological course for MCF and the other a chronic course without the characteristic clinical signs. Hence, the cases cover a wide spectrum of possibilities.

All the animals except one showed variably severe vasculitis in the CNS and infiltration of several tissues by lymphoid cells. On the basis of these changes, a diagnosis of MCF could initially have been considered. However, the full clinicopathological spectrum must be taken into account in each case, and consideration given in the differential diagnosis to other diseases as reviewed.

Case 1 was characterised by clear lesions of vasculitis involving mononuclear cell infiltration of vessel walls, and by lymphoproliferative changes, and so was diagnosed as MCF. However, there was no fibrinising vasculitis, and haemorrhagic and necrotic changes at postmortem were minimal. Corneal opacity was restricted to the periphery, and nasal discharge was mucoid rather than

mucopurulent. Hence, the case more closely resembled the syndrome described by Hoffmann et al. (1984) in buffalo. Lymph node changes were not in exact conformity with the picture usually seen in MCF.

In the authors' experience, the capsule and trabeculae are frequently infiltrated by mixed lymphoid cells during MCF. Germinal centres are few or absent, and the cortex is expanded with pronounced paracortical areas due to lymphoid proliferation that histologically extends into the medullary cords. The sinuses are filled with a mixed population of mononuclear cells in which pleomorphic lymphoblasts with a high mitotic index are prominent, and also macrophages with a clearly delineated cytoplasm that may contain phagocytosed material.

The sections of node examined in the current case differed from this description in having active germinal centres and a greater proportion of histiocytic cells in the sinuses. The possibility that the lymphoproliferative changes were superimposed on another disease process may be considered, or note taken of the variability.

Case 2 also showed lesions of vasculitis and lymphoid infiltration of organs, but contraction of the lymphoid organs and an unusual clinical course involving a progressive wasting disease over a 12-month period. MCF could be suspected on the basis of the history of infection by blood transfusion followed by lesions of severe, necrotising vasculitis in several organs, but Barker and Van Dreumel (1985) have warned that such vascular lesions with an organ distribution as in the present case and unaccompanied by lymphoproliferative changes may occur in mucosal disease.

Laboratory examination of such cases should check their virological and serological status for evidence of superinfection with cytopathic BVD virus in an immunotolerant animal, as described by Brownlie et al. (1984). BVD antigen should be detectable in tissue sections by immunohistochemistry.

CNS changes and progressive emaciation have also been reported associated with a retrovirus infection of cattle, now designated bovine immunodeficiency virus (BIV) (Gonda et al. 1987). The full range of pathological changes associated with BIV infections has not been described. Suspect retroviruses have been

isolated from buffalo in Indonesia (Sudarisman et al. 1986), and serological results indicate infections may be reasonably common. Wasting disease is seen frequently in buffalo in that country (Unruh, pers. comm.). Firm identification of the agent and a study of its effects in buffalo are urgently needed. Investigations of wasting diseases in buffalo should consider not only this virus but also BVD, MCF and trypanosomiasis in the differential diagnosis.

Case 3, on the basis of its exposure to sheep implicated in a previous outbreak and clinical signs at various times of fever, nasal discharge and diarrhoea, invites a diagnosis of mild MCF. Gross pathological changes of some lymph node enlargement, abomasal lesions and mild haemorrhagic lesions in the intestines could be consistent with this hypothesis, but the histological changes were not sufficient to support the diagnosis. CNS lesions were of focal gliosis rather than primary vascular changes. There were interstitial and perivascular infiltrates in the lung, heart, kidney and eye, but these were mild. Lymph node histology, although not conforming to that expected in fulminating MCF, did show proliferative changes in the medullary cords and an increased number of lymphoblasts in the sinuses. These early lymphoproliferative changes were not accompanied by evidence of vasculitis.

In the first instance, alternative diagnoses should be sought. A range of infectious agents known to be present in Indonesia has been reviewed in the introduction. Pre-exposure and convalescent sera are available from this animal, and the immediate task is to develop the serological capacity to investigate infections with the agents listed. It is probable that episodes of fever and diarrhoea in Bali cattle have varied aetiologies.

Cases 4 and 5 involved clinically normal buffalo that showed essentially similar histopathological changes, although infected and uninfected with *T. evansi*, respectively. Although the cerebral granuloma associated with rod-shaped material may indicate a specific cause of the CNS changes in the control animal, the changes in other organs, particularly the lung, kidney, spleen, and lymph nodes were similar in each animal. Similar changes were present in other buffalo in this experiment, although the

CNS changes were the least pronounced (Damayanti, unpublished data).

The changes were not sufficiently severe to indicate MCF, but it should be remembered that the animals were clinically healthy. The question must be raised whether subclinical infections with SA-MCF agent occur, with clinical disease being precipitated in only a few instances by presently undefined factors. This would be consistent with epidemiological observations that, although many animals of susceptible species are exposed to the reservoir host, only sporadic cases of MCF are seen. However, in the case of WA-MCF, serological surveys have shown that infections by AHV-1 are not widespread in normal cattle populations exposed to wildebeest (Rossiter et al. 1980). It has not yet been shown whether it is appropriate to extrapolate from such observations of WA-MCF and AHV-1 infections to SA-MCF.

Lymph node changes showing germinal centre activity and sinus histiocytosis leading to sclerosis were not consistent with those normally seen in MCF, a lymphoproliferative disease. Rather, the lymph node changes were as described in trypanosomiasis (Ladds 1986). Changes in other organs were also consistent with *T. evansi* infections as reviewed above. Trypanosoma-free animals for the experiment were obtained by chemotherapy of animals with Naganol (Suramin, Bayer), and so there is the possibility that the lymph nodes continued to receive antigenic stimulation from phagocytosed material, and that the cerebral granuloma was in response to dead parasites in the tissue rather than to an undiagnosed bacterial infection.

Other possible viral aetiologies can also be considered. The changes resemble a little those reported in persistent BVD infections (Barker and Van Dreumel 1985). In that case, future immunohistochemical studies should identify the presence of the virus. As also discussed in the previous case, possible effects of retrovirus infection must also be investigated.

The discussion of the pathological changes recorded in this paper has not yet been supported by broadly based serological studies or attempted identification of a range of viruses to clarify the status of the animals under review, and so is somewhat speculative. However, such a discussion constitutes a necessary step in the

process of differential diagnosis. A number of avenues for future investigations have been identified.

Laboratory Submissions in Suspected Cases of MCF

A prerequisite for the diagnosis of any disease is a thorough investigation and analysis of all the circumstances surrounding its occurrence, the history, including observations of epidemiological importance, the clinical signs and the findings at necropsy, the histopathological changes and the results of other appropriate laboratory investigations. These notes are not intended as a substitute for normal procedures, but are intended to highlight some aspects to which particular attention should be paid.

Epidemiological Factors

In all suspected cases of MCF, note should be made of whether contact with sheep or wildebeest was possible in the year preceding the onset of disease. It is of value to note movements of groups of livestock at the site, either suspect reservoir species or the affected species, and any births. Notes may be made of the seasonal conditions, and whether the animals involved were under any stress, such as nutritional, climatic, or associated with work or production.

Clinical Signs

Full descriptions of clinical signs may be of help not only in reaching a provisional diagnosis, but also in helping others to subsequently analyse the significance of the disease event in relation to other similar events. The progression of signs during the course of the disease should be noted. Where MCF is common or where reliance must be placed on paraveterinary field staff, it may be helpful to have a checklist of necessary observations.

The Postmortem Examination

Again a detailed and systematic protocol of observing and recording is necessary. Different diagnostic laboratories may vary in their requirements for specimens from necropsies

conducted in the field, but the following recommendations can be offered, bearing in mind that a diagnosis can be made on consideration of lymphoproliferative changes and vasculitis.

A consideration of bovine lymph node drainage patterns (Ladds 1986) shows that the prescapular (superficial cervical) node and the prefemoral (subiliac) node do not drain visceral organs either directly or indirectly, and may therefore contain primary changes of the lymphoid tissue rather than changes secondary to inflammation elsewhere. An added advantage of these nodes is their superficial location making them easy to collect early in the course of the postmortem examination. For routine examination, a transverse section 1 cm wide through the whole node at its mid-point has been found a useful specimen for formalin fixation.

The brain must always be sampled, for the vascular lesions in this organ can be of diagnostic importance. If the whole brain cannot be fixed in formalin for submission to the diagnostic laboratory, then sections through the whole organ at various levels should be made. Sections through the anterior cord, the cerebellum and brain stem, the posterior calliculus (the swelling anterior to the cerebellum lying beneath the posterior aspects of the cerebral hemispheres), and the cerebrum give a representative sample.

Experience has shown that the vascular plexus on either side of the pituitary gland—the rete mirabile—is an extremely useful tissue in which to examine vascular lesions (Liggitt and De Martini 1980a). The tissue is very easy to collect, requiring only a pair of forceps and a scalpel, and is easy to locate, lying on the floor of the cranial cavity immediately beneath and attached to the fascia that contains the pituitary foramen, immediately posterior to the optic chiasma. It can be dissected free from the floor of the cranial cavity by making incisions 1 cm laterally to the foramen, and anteriorly and posteriorly to it, followed by separation of the vascular plexus from the underlying bone (Fig. 1). The plexus is removed attached to the pituitary gland, with both supported by the fascia, and placed in fixative in this fashion.

Fixed tissue is also needed from the parenchymal organs including lung, heart, liver,

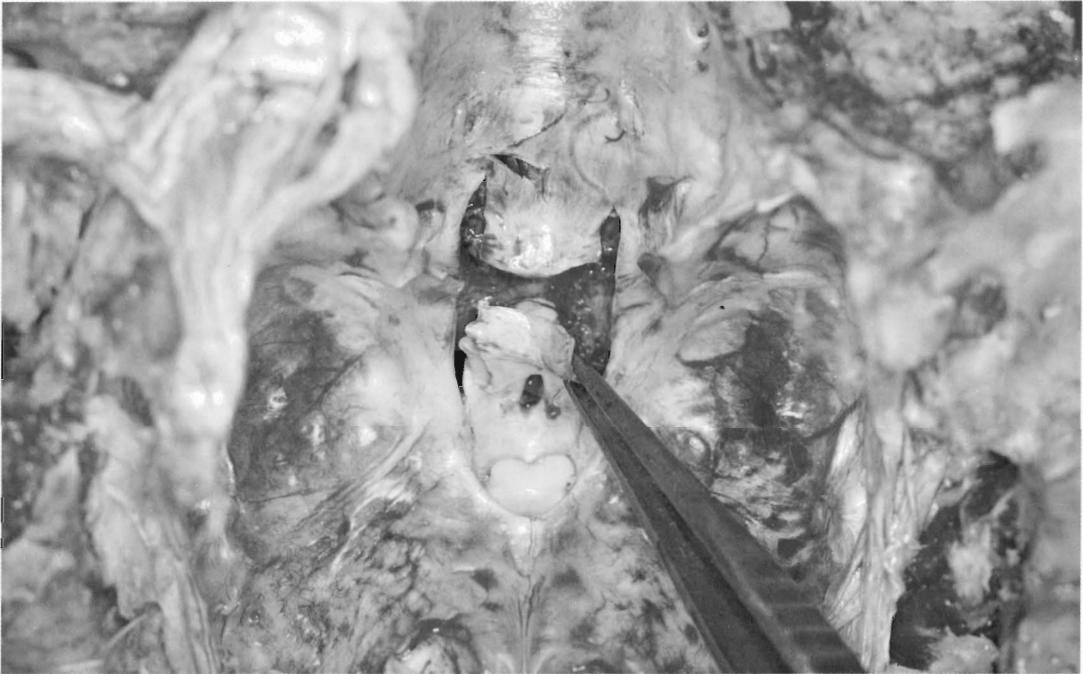


Fig. 1. Removal of the pituitary gland and associated vascular plexus from beneath the fascia of the floor of the cranial cavity.

adrenal and kidney, even if gross changes are not evident. The section of kidney should include a wedge of tissue from the capsule through to the pelvis. This is considered important as in some species the vascular changes are not distributed evenly throughout the organ. Following examination of abomasum, intestines and urinary bladder, specimens should be collected where appropriate, and from other tissues showing gross lesions. Within the constraints as discussed for certain organs, small pieces of tissue that the fixative will penetrate quickly are best, and make for the most economical submission.

Histological Examination

For diagnostic purposes tissues will be examined, noting particularly evidence of vasculitis. The vascular plexus removed with the pituitary gland is very useful, for here the vessels can be observed supported only by a fine connective tissue stroma, facilitating the examination of primary vessel changes free of the influence of other tissue changes (Fig. 2).

Note must also be made of lymphoproliferative changes. The patterns of infiltration in various organs can be examined, and of the cellular composition of the infiltrates which in MCF are normally described as containing lymphoblastic cells.

The range of pathological changes that can occur in bovine lymph nodes under various influences has been extensively described by Ladds (1986). In examining nodes histologically, the following routine has been found helpful. Using alternatively low and high power magnification, the features of the node are examined in turn, beginning with the supporting structures, the capsule, trabeculae and major blood vessels. The features of the lymphoid component are noted, including germinal centre activity and features of the paracortex. Finally, the subcapsular, trabecular and medullary sinuses are examined for cellular content, and the cell types described.

Serology

The agent of SA-MCF has not yet been identified or isolated, and so there is not yet a serological test that can aid in diagnosis. However, whenever possible it is recommended

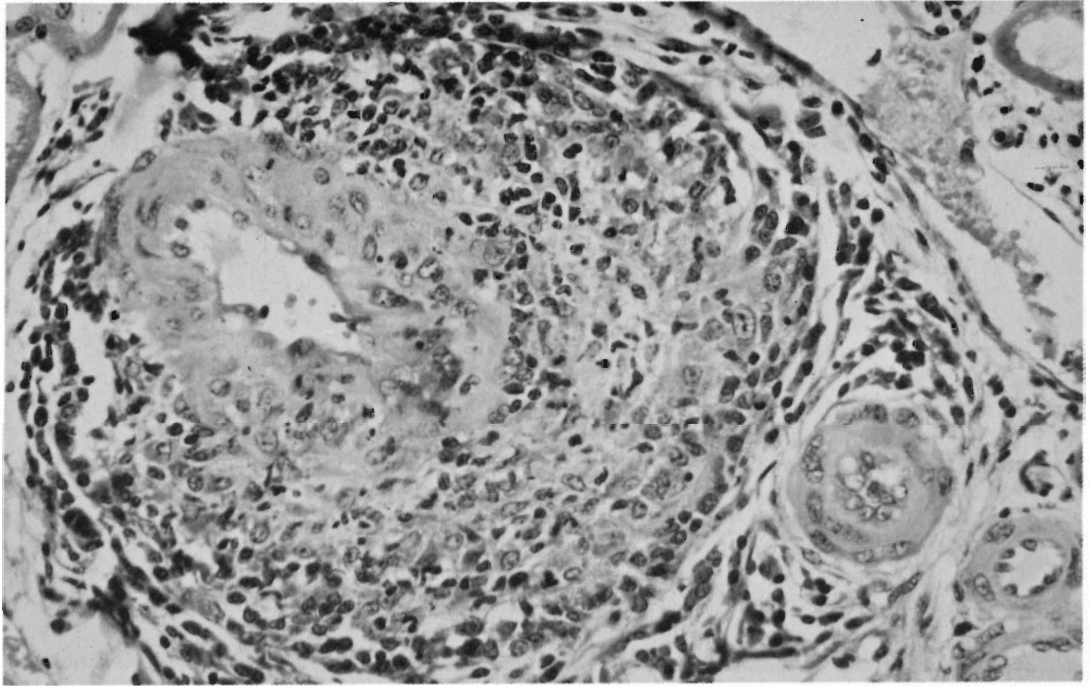


Fig. 2. Histological section through the vascular plexus removed with the pituitary gland: this tissue is useful for determining the presence of MCF-associated vasculitis.

that in areas where MCF is recognised frequently sera from clinically affected animals be submitted to the regional laboratory. Depending on laboratory policy such sera can be stored or forwarded to a reference laboratory for research purposes involving the seeking of serological evidence for the involvement of suspected infectious agents. Field staff should not feel that the collection of such sera is a waste of time, for when collected in some numbers over a period of time they constitute a valuable resource in the task of unravelling the mystery of MCF.

Acknowledgments

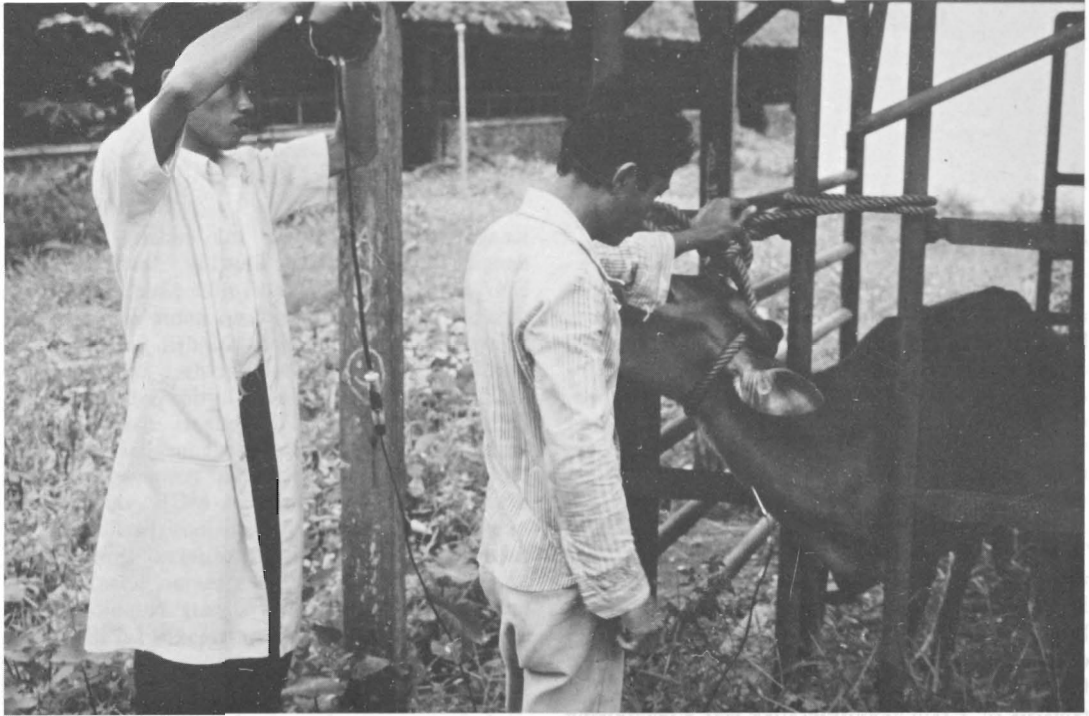
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Aetiology

gastrointestinal tract and the respiratory system. The pathogenesis of the disease is not clear. It is thought that the disease is caused by a virus. The disease is common in the tropics and subtropics. It is caused by a virus which is transmitted by the blood-sucking insects. The disease is characterized by a high fever, headache, and a rash. The disease is self-limiting and usually resolves within a few days. The disease is not fatal.

Current Malignant Catarrhal Fever Research in the United Kingdom

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Abstract

Malignant catarrhal fever (MCF) has a worldwide distribution. Research in the United Kingdom has been directed towards defining the similarities and differences of the wildebeest associated (WA-MCF) and sheep-associated (SA-MCF) forms. Transmission of both forms to laboratory animals has been achieved. Certain aspects of WA-MCF infections in these animals resemble SA-MCF in cattle. Sheep and goats have some antibodies to the WA-MCF agent, as do some cattle infected with SA-MCF. These serological reactions are being analysed using the Western Blotting technique. The virus DNA of the WA-MCF agent has been cloned, and is being used in hybridisation studies to demonstrate cross-reacting DNA in SA-MCF infected cells and tissues. The pathology of both forms of MCF consists of a lymphoproliferative and a necrotising component. The pathogenesis of these effects may be explained by the properties of large granular lymphocytes isolated from cases of SA-MCF, which can transmit the disease. These cells produce a factor that stimulates lymphoid proliferation, and also show marked cytotoxic activity. These cells appear to contain DNA homologous to the WA-MCF agent, and are probably the key to understanding this difficult disease.

Abstrak

Malignant catarrhal fever (MCF) tersebar luas di dunia. Penelitian penyakit ini di Inggris yang ditujukan untuk penentuan persamaan dan perbedaan antara wildebeest associated (WA-MCF) dengan sheep associated (SA-MCF) telah dilaksanakan. Penularan kedua bentuk penyakit tadi ke hewan percobaan telah berhasil. Beberapa aspek dari infeksi WA-MCF pada hewan-hewan tadi mirip dengan SA-MCF pada sapi. Domba dan kambing mempunyai zat kebal terhadap WA-MCF, demikian juga beberapa sapi terhadap SA-MCF.

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Reaksi-reaksi serologis ini sedang dianalisa dengan tehnik 'Western Blotting'. Demikian juga DNA virus WA-MCF telah diklonkan dan sedang dipakai dalam studi hibridisasi untuk menunjukkan infeksi silang DNA dalam sel dan jaringan yang terinfeksi SA-MCF. Patologi dari kedua bentuk MCF tersebut terdiri atas limfo proliferasi dan komponen nekrosis. Patogenesis dari efek ini mungkin dapat dijelaskan dengan sifat-sifat yang terdapat pada 'large granular lymphocytes' yang diasingkan dari kasus SA-MCF yang dapat menularkan penyakit. Sel ini menghasilkan suatu faktor yang merangsang proliferasi limfoid dan juga aktivitas sitotoksitas secara jelas. Sel-sel tersebut mengandung DNA yang homolog untuk agen WA-MCF, karenanya mungkin sekali hal ini merupakan kunci penjelasan penyakit yang sulit ini.

Introduction

This contribution concentrates on laboratory aspects, for until the aetiology is identified diagnosis cannot be precise and optimal control measures cannot be found. After an overview of the malignant catarrhal fever (MCF) situation worldwide, serological data will be examined for information they may yield on the possible aetiology. Viruses isolated from cases of MCF will be considered and an attempt made to construct a unifying hypothesis that could account for some of the the enigmas of this disease.

The World Situation

MCF has a worldwide distribution, and is encountered wherever cattle husbandry is practiced. In some countries, logistical problems in the veterinary diagnostic services may have led to the disease not being widely reported. Through most of its distribution it is a sporadic disease of cattle affecting only a few animals,

but in most countries so affected, such as Australia, the United Kingdom (UK) and the United States (USA), outbreaks involving many animals have been reported. Up to 200 animals have been involved on a single farm in Australia, and there have been several outbreaks in the UK involving over 10 animals.

MCF has a peculiar importance in East Africa, in the great grasslands where wildebeest share grazing with cattle. In this situation, large numbers of cattle die. The wildebeest have been shown to harbour a herpesvirus which can be transmitted to cattle and cause MCF. Throughout most of the world, however, wildebeest are an uncommon species, and epidemiological data indicate that sheep is the host.

This sheep-associated form of the disease is a particular problem in New Zealand where deer farming is important. Deer have an extreme susceptibility and MCF has been recognised as the most serious infectious disease of these animals. From the information presented in this volume, it seems that the Bali cattle of Indonesia have a similar susceptibility.

Aspects of the Natural Disease

The wildebeest is the natural host of one form of the disease caused by Alcelaphine herpesvirus 1 (AHV-1). The sheep-associated agent has not yet been identified. AHV-1 may cause disease naturally in a wide range of ruminants, as can the sheep-associated agent. The wildebeest-associated form (WA-MCF) occurs not only in Africa but also in zoological gardens keeping wildebeest, and the sheep-associated form (SA-MCF) occurs throughout the world, including areas of Africa where the former occurs.

Transmission

Experimentally, WA-MCF can be transmitted from affected cattle and from clinically normal wildebeest to cattle, deer, rabbits, hamsters, guinea pigs and rats. Virus may be recovered from diseased animals other than hamsters or rats.

Transmission of SA-MCF is difficult. Cattle to cattle transmission is usually not successful, although work in Indonesia shows Bali cattle are

more readily infected, as are deer. Using blood inoculation, SA-MCF can be transmitted from deer to rabbits, and from rabbit to rabbit. Only rarely can the disease be transmitted from cattle to rabbits although, once achieved, rabbit to rabbit passage is readily maintained.

Once WA-MCF experimental infections in guinea pigs, hamsters and rats, established from rabbits, are adapted to these rodent species, disease can be passaged from animal to animal, although it is not possible to passage back to the rabbit or to recover virus. Thus, in these cases the virus has adapted itself to the new host species, possibly in an incomplete form. The presence of the virus can be demonstrated only by passage of disease from animal to animal, with histopathological confirmation.

This may be considered analogous to the situation seen with SA-MCF. From cattle, disease can be passaged only irregularly, but from deer it can be passaged readily to other deer and to rabbits, and from rabbits to hamsters, but not to other rodent species. SA-MCF is not readily transmitted from rabbits back to deer. Transmission from cattle to rabbits is relatively unusual, presumably because the virus in cattle is incomplete.

In experiments in the UK, transmission of SA-MCF was achieved three times from 16 bovine field cases. In the first case, transmission was to a bovine but not to rabbits. Attempted second passages to cattle, rabbits and deer were not successful. In the second case, transmission to cattle and deer was unsuccessful, but successful to rabbits. Further passages to rabbits and hamsters were achieved and once the disease was established it could be readily passaged through rabbits but not back from the rabbit to cattle. On the third occasion, passage was to a second and then a third bovine, from which it was passaged to rabbits but not to deer.

These experiences are interpreted as indicating that the virus may be in a form that does not represent the whole virus; perhaps the portion of the virus retained determines whether transmission can be achieved or not. No infectious virus can be identified in these situations.

Serology

All wildebeest have neutralising antibodies to

AHV-1. From this information, certain deductions can be made. The virus must be readily transmitted in this species. Wildebeest never show disease associated with this infection, either in captivity or in open grazing, so there is a very efficient and stable host-parasite relationship.

Other species of large antelope also have neutralising antibodies to AHV-1, indicating that they also harbour this or a closely related agent. In confirmation, herpesviruses similar to the WA-MCF virus have been isolated from some of these animals, and these isolates also cause MCF on inoculation into cattle. However, there is no evidence that transmission of these viruses from other antelope species to cattle occurs naturally. MCF has never been observed to occur naturally where cattle are grazed with these other antelope species.

There is a large body of other African species of ruminants that have no neutralising antibody to AHV-1 but are positive using the indirect immunofluorescent (IIF) test, a test known to be highly cross-reactive. In cattle, a certain amount of nonspecific cross-reactivity has been found, but most sheep appear to have antibody (Rossiter 1981). The sheep that were negative in Rossiter's test were from specific pathogen-free sheep produced at the Moredun Institute by caesarian delivery and reared in isolation.

Subsequent work at Moredun has confirmed Rossiter's observation. Sera from many countries, including Iceland, Peru, USA, Greece, Africa and the UK, have shown low levels of antibody to the WA-MCF virus. This has been interpreted as evidence that there may be an antigen infecting sheep which is antigenically related to AHV-1. Not only sheep have this antibody, but also goats, oryx and various other species related to sheep, indicating that these other species may also be infected with related herpesviruses.

The observations have been further augmented using the Western Blotting technique. Wildebeest sera clearly detect a number of AHV-1 antigens, while sheep sera detect a proportion of antigens detected by wildebeest sera. This is powerful evidence that sheep are infected with a herpesvirus related to AHV-1. If serum from a bovine vaccinated with an inactivated form of AHV-1 and then

experimentally infected with AHV-1 is similarly examined, after having suffered clinical MCF, only a few of the antigens detected by the wildebeest sera react with this bovine serum.

A large number of bovine sera from clinical cases of SA-MCF have been tested in the Western Blotting Test. Most give no reaction, but a few detect one of the AHV-1 antigens. Deer with SA-MCF in the UK have not shown any such antibody. One deer in New Zealand that apparently recovered from clinical MCF and suffered a fulminating relapse 3 months later did develop some antibody against the same antigen as detected by the bovine with SA-MCF.

These observations demonstrate an important point in analysing the antibody response of animals with SA-MCF, particularly those that are extremely susceptible. They may not be reacting with an antibody response to any of the antigens present on the AHV-1 virus. They may, however, be reacting with antibody to other antigens on the sheep-associated agent not shared by AHV-1.

More evidence pointing to the nature of the agent has been found in serological examinations of experimentally infected rodents. All rabbits reacting to AHV-1 infection produce antibody to the virus, but no antibody to AHV-1 has been found in rabbits successfully infected with SA-MCF.

However, hamsters reacting with SA-MCF develop IIF antibody to AHV-1. Confidence can be placed in this observation because the fluorescence appears quite specific, with antibody attaching to the nucleus of infected cells. So there is evidence that a proportion of rodents infected with SA-MCF react to an antigen common to the WA-MCF agent. This further confirms that, in the UK at least, the SA-MCF agent is antigenically related to the wildebeest virus.

Possible Nature of the Sheep-associated Agent

Based on the observations as outlined it can be hypothesised that the SA-MCF agent belongs to the gamma group of herpesviruses. These include Epstein-Barr virus, the cause of infectious mononucleosis in humans as well as Burkitt's lymphoma in Africa and

nasopharyngeal carcinoma in Asia. Other viruses in this group are Marek's disease virus, a virus of rabbits, simian herpesvirus and AHV-1. A consideration of the histopathology suggests that SA-MCF is lymphotropic, which supports inclusion of the agent in this group.

Work is progressing on proving this association by studies of the viral DNA. Analysis of AHV-1 DNA shows it to be very similar in nucleotide patterns to other gamma herpesviruses. Most of the AHV-1 viral DNA has been cloned. When some of these clones were used in hybridisation studies with tissue of animals dying of MCF, preliminary results indicated the presence of DNA homologous to that of AHV-1. Development of these studies is necessary to prove that a similar herpesvirus is present in sheep. In the process, a very potent diagnostic tool, able to detect the presence of virus in field samples, will be developed.

Pathogenesis and Pathology

When animals are infected with AHV-1 there is lymphoproliferation from the time of inoculation. There is no evidence of the necrotising lesions until the animal reacts clinically, with a febrile response. However, if animals are treated with Cyclosporin A, a potent T-cell suppressor, from the time of inoculation the lymphoproliferative response is prevented, but the animal still develops MCF with the necrotising lesions after the same incubation period.

Hence, it seems that the lymphoproliferative component of WA-MCF is separate from the necrotising component, and is essentially benign, while the necrotising lesions are the important part of the disease.

Virus Isolations

A number of viruses have been isolated around the world from cases of SA-MCF but it must be stressed that none of these has been able to reproduce disease. It will probably turn out that these agents have been isolated gratuitously, and that they are not aetiologically associated with the disease. At Moredun, no viruses considered to be important in the study of the disease have been isolated.

Cell Cultures

There has been considerable success in the study of SA-MCF in the culturing of cells from infected laboratory animals, deer and cattle. The cells, which cannot be isolated from control animals, have the morphology of large granular lymphocytes, and have been recovered from a large range of tissues including cerebrospinal fluid. In the UK, this has emerged as a consistent phenomenon of SA-MCF, that large granular lymphocytes can be isolated in culture from the tissues of affected animals.

In many cases, these cells can transmit the disease, and there is evidence of DNA homologous with AHV-1 in these cells. It is considered that granular lymphocytes may play an important role in the development of MCF.

Large granular lymphocytes are also known as natural killer (NK) cells, and are now thought by immunologists to be a primitive type of T-cell active in the early stages of immune responses, being cytotoxic to virus-infected cells and transformed cells. The activity of NK cells is regulated by interleukin 2 (IL-2), one of the substances recognised as having a vital role in the control of the immune system. Activated T-cells and NK cells generate IL-2, which then acts on T-cells to give T-cell proliferation. It also acts back on the NK cell further activating it to become cytotoxic for transformed cells. The supernatant from cultures of cells isolated from animals with MCF causes a blastogenic response in normal bovine lymphocytes, indicating that the isolated cells are secreting a substance with activity similar to IL-2. Also, the cultures have been demonstrated to have natural killing activity against cultures of red deer testes cells and lamb kidney cells. Chromium release assays show effective killing in a short time at relatively low effector to target cell ratios. So these presumed virus-infected NK cells are killing normal cells, which is an unusual situation.

NK activity in the lymphoid tissues of normal and SA-MCF infected rabbits has been examined. Cells from a normal adult show no such activity, but cytotoxic cells can be found in animals starting to react clinically. If it is remembered that the onset of clinical disease coincides with the development of necrotising lesions, there is therefore an association with the

development of such lesions and the onset of NK activity.

A Hypothesis

In developing a hypothesis for the pathogenesis of MCF, the wildebeest- and sheep-associated forms have been considered as being essentially the same. It seems that the virus preferentially infects NK cells, which then secrete a lymphoproliferative factor, presumably IL-2. This drives the proliferative component of the disease from the start of the incubation period. As stated, this is an essentially benign response that can be controlled with cyclosporin A. However, the infection goes on to affect the NK cells in such a way that they become deregulated and kill normal cells. Experimentally, high levels of IL-2 have induced this effect in NK cells, and it may be that this also occurs in the diseased animal.

Discussion

Does such a hypothesis answer the enigmas found in MCF? Although virus replication can be found in WA-MCF, it cannot be detected in SA-MCF. In terms of the above hypothesis, this may be acceptable, if the situation is comparable to that seen with other herpes-

viruses. *Herpesvirus saimiri* also affects large granular lymphocytes, and as little as 30% of the virus genome has been shown to be retained in *H. saimiri* transformed cells.

There is no lateral transmission from affected cattle to other cattle and no infective virus can be demonstrated.

Only with lymphoid cells of affected animals can the disease be transmitted, and here only irregularly, suggesting that only a portion of the virus is present in infected cattle. In spite of the lack of virus, there is widespread cell proliferation which is similar in some respects to an oncogenic response, but can be seen to be a hyperplastic normal immune reaction probably driven by IL-2. We should therefore be seeking to identify a small piece of viral genome within only a proportion of cells if we are to resolve MCF.

This summarises the direction MCF research is taking at the Moredun Institute, and the direction that is most likely to be profitable in defining the SA-MCF agent. It is hoped that these thoughts will be of benefit to the research currently being conducted in Indonesia.

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Virological Investigations of Malignant Catarrhal Fever in Indonesia

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Abstract

As malignant catarrhal fever (MCF) in Indonesia is a disease with serious economic consequences, it is important to isolate and identify the causative agent to allow improved diagnosis, control and prevention. Transmission experiments have proven the disease to be infectious. Viral isolation has been attempted from clinically affected animals and in-contact sheep. Syncytia-forming viruses have been isolated from buffalo with MCF, and subsequently also from healthy buffalo. Their significance is not yet determined. Many buffalo have antibody to this virus, but usually not Bali cattle, including those with MCF. Antibody to the agent of wildebeest-associated MCF has not been detected in animals with MCF in Indonesia.

Abstrak

Malignant catarrhal fever (MCF) di Indonesia mempunyai arti ekonomi yang sangat besar. Karenanya usaha isolasi dan identifikasi agen penyakitnya untuk pengembangan diagnosis, pengendalian dan pencegahan perlu diusahakan. Dari hasil percobaan penularan telah terbukti bahwa penyakit ini infeksius. Isolasi virus penyebab penyakit telah dicoba dari hewan yang secara klinis sakit MCF dan hewan yang kontak dengan domba. Percobaan isolasi, baik dari kerbau sakit MCF atau kerbau sehat, telah berhasil menemukan virus sinsisial. Namun sampai saat ini pentingnya virus tadi dalam MCF belum ditentukan. Banyak kerbau yang dalam tubuhnya mengandung zat kebal terhadap virus tersebut, tetapi sebegitu jauh tidak dalam tubuh sapi Bali, walaupun sapi itu terserang MCF. Sampai saat ini, hewan-hewan MCF di Indonesia, sebegitu jauh belum ada yang mengandung zat kebal terhadap virus WA-MCF.

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Introduction

Malignant catarrhal fever (MCF) is an economically important disease in Indonesia, and in epidemiological terms is the sheep-associated form (SA-MCF) (Plowright 1984). The agent of SA-MCF has not yet been isolated and identified anywhere in the world, although Plowright (1984) has discussed the serological evidence that an agent similar to alcelaphine herpesvirus 1 (AHV-1) that causes wildebeest-associated MCF (WA-MCF) in Africa may be involved.

Various viruses have been isolated from cases of SA-MCF, including bovine syncytial virus (BSV) (Clarke et al. 1973; Storz et al. 1976), enteroviruses and a parvovirus (Storz et al. 1976), morbilliviruses (Coulter and Storz 1979), bovine herpesviruses different from bovine rhinotracheitis virus (BHV-1) (Liebermann et al. 1967; Storz et al. 1976; Storz 1978), and a herpesvirus believed to be closely related to AHV-1 (Hamdy et al. 1978). Another herpesvirus was isolated from a bison with MCF, and this isolate as well as others reported previously (Liebermann et al. 1967; Storz et al. 1976) was considered to have the properties of cytomegaloviruses (Todd and Storz 1983). No isolated virus from cases of SA-MCF has produced the disease on inoculation into susceptible animals. Togavirus-like particles have been seen by electron microscopy in MCF-affected deer tissues (Clark and Adams 1976), and reoviruses, adenoviruses and syncytiogenic viruses have been isolated from sheep associated with cases of MCF in cattle (Snowdon and French 1975; Snowdon 1985).

Adequate control measures for infectious diseases are usually based on a thorough knowledge of the aetiological agent, its

epidemiology and pathogenesis. In some cases, isolation of the agent opens the possibility of developing a vaccine. Hence, isolation of the agent of SA-MCF in Indonesia has been attempted from both natural and experimental cases of MCF, and associated serological studies conducted. The intention in this paper is to give an overview of the techniques used, and a preview of results, some of which are being used by the senior author for a PhD program at Institut Pertanian Bogor.

Transmission Experiments

The transmission experiments have been outlined in a earlier paper in this volume. MCF occurred spontaneously in Bali cattle (*Bos javanicus*) penned in close proximity with lambing small ruminants. The disease was passaged by blood transfusion to other Bali cattle, and to a smaller number of swamp buffalo (*Bubalus bubalis*) and ongole cattle (*Bos indicus*). In each case, sera were collected prior to infection, weekly postinfection and at regular intervals during the course of the disease, and stored at -22°C . Buffy coat cells were also collected and stored in a viable state in a freezing medium containing 20% foetal calf serum and 10% DMSO.

The experiments have yielded valuable information regarding the aetiology by confirming the involvement of an infectious agent, confirming the disease under examination to be MCF, and providing material for subsequent laboratory studies.

Viral Isolation

Cell Culture

A prerequisite for viral isolation is a cell culture system in which the agent under study will grow. The agent of WA-MCF can be isolated in primary bovine thyroid cultures, but isolation in conventional cell culture of the agent of SA-MCF has not yet been reported. Hence, the first phase of the investigation was not only to produce primary bovine cells as used successfully for the isolation of other viruses, but also to attempt the development of other culture systems that might be useful.

In cattle, the agent is present in the blood. This is known from the results of the transmission experiments. The pathology in cattle is associated with proliferative changes in mononuclear cells, which are represented in the blood by lymphocytes and monocytes. Hence, it was hypothesised that the agent may be present, and may even replicate in such cells in the affected animal, and may be adaptable to such cells in culture. Monocytes growing as monolayers is a system that is easy to propagate once established (Asagba et al. 1981) and so was considered for isolation attempts.

In sheep, the presumed reservoir host, excretion of the agent is not accompanied by the development of clinical signs. Hence, there is at present no suggestion of a susceptible ovine tissue. Presumed periods of alternating latency and excretion suggest that it is in equilibrium with the immune system of the sheep. A virus may achieve this biological state by infecting cells of the immune system, and so appropriate cell cultures were again sought by developing cultures from lymphoid tissues, in particular foetal sheep thymus cells, sheep macrophages and foetal sheep macrophages.

The slaughter of female cattle is prohibited in Indonesia, and so bovine foetuses are not readily available. This largely precluded the establishment of primary bovine cells, and so most of the viral isolation work was attempted in ovine cells, although blood macrophage cultures from a Bali cross bull were propagated and used. One buffalo foetus became available, and primary cultures including thyroid cultures were established.

Collection and Storage of Samples

WBCs and sera were collected before, during and at the termination of transmission experiments, and from spontaneous cases of MCF. Samples for sera were allowed to clot at room temperature (approximately 27°C) overnight, separated, clarified by centrifugation, and stored in the RIVS serum bank. Blood for WBCs was collected in heparin, and the cells separated by ammonium chloride lysis of erythrocytes, followed by washing in PBS or physiological saline. Cells were suspended in medium (MEM with 5% FCS) for immediate use, or stored in ampoules in MEM containing

10% FCS and 20% DMSO in liquid nitrogen or at -70°C .

Cocultivation and Inoculation

Virus isolations were attempted by cocultivation of white blood cells from MCF cases with monolayers and cell suspensions as described above.

WBCs were those taken from MCF cases during the febrile period when the disease was transmissible. Cell suspensions from lymph node, thyroid and spleen were prepared postmortem and cocultivated with monolayers as above.

On two occasions, from sheep in Kupang, West Timor, and from the sheep involved in the natural transmission experiment, nasal swabs were used in viral isolation attempts.

From five field cases of MCF in buffalo and from the buffalo in the natural contact transmission experiment during its febrile period (Daniels et al. 1988), syncytia-forming agents were isolated from WBCs. The cytopathic effect in each case was of multinucleate syncytia formation with little or no vacuolation of cytoplasm. A range of cell culture types was subsequently infected (Table 1).

No similar isolates have been obtained to date from similar samples taken from Bali cattle with MCF. One strain of syncytial virus has apparently become cell-free after the 13th passage, an observation which has yet to be confirmed. No viruses other than the syncytial viruses were isolated. Viable WBCs from cases are still being stored for subsequent attempts.

Autocultures

Cell culture techniques were also used in

attempts to grow the agent directly, by establishing cultures of cells from MCF cases. No success has been achieved in attempts to grow monocytes from clinical cases in monolayer cultures. Primary cultures of testes, spleen and lymph node cells were established from some Bali cattle, but did not survive.

Electron Microscopy (EM)

EM has been investigated as a means of locating the agent. Suspensions of white blood cells from animals with clinical disease were examined for the presence of viral particles. In one Bali bull, particles resembling retroviruses were seen.

Serology

Cattle with WA-MCF develop IIF antibodies to AHV-1 and antibodies have been detected in nearly all wildebeest examined (Plowright 1984). Hence, there is interest to know whether animals with SA-MCF have serological evidence of infection with this or a related virus, for there would then be a means of confirming diagnoses. Detection of similar antibodies in the suspect reservoir host may give a means of identifying potential disseminators of the disease.

Indirect Immunofluorescence (IIF)

An adaptation of the IIF test for antibody to the WC-11 strain of AHV-1 as described by Rossiter et al. (1977) was used. WC-11 virus (a gift from Dr Neil Edington) was grown in MDBK cells on 12-well teflon-coated spot slides until CPE was observed. Slides were fixed in acetone and stored at -20°C until use.

Table 1. Isolations of syncytiogenic viruses.

Animal	Clinical status	Primary cell inoculated	CPE at passage
Buffalo (Ciawi)	MCF	Sheep foetal thymus	3
Buffalo (Boyolali)	MCF	Sheep foetal thymus	2 (in sheep macrophages)
Buffalo (Ciawi)	MCF	Sheep foetal thymus	2
Buffalo (Ciawi)	MCF	Buffalo foetal thyroid	1
Buffalo (Balitvet)	Conjunctivitis Nasal discharge	Sheep thymus	2

Sera were screened at a dilution of 1:20. After an hour of incubation, slides were washed and spots incubated with a commercial fluorescein-conjugated rabbit anti-IgG (Miles) for 30 min. After washing and mounting in glycerol saline the slides were viewed by fluorescence microscopy. No reactive sera from cases of MCF were found (Sudarisman et al. 1986). Occasional sheep sera and some goat sera showed variably repeatable nonspecific or weak reactions which may indicate a cross-reaction with a similar herpesvirus, or which may be of no significance.

Animals with WA-MCF show seroconversion to AHV-1 detectable by IIF before the onset of clinical signs in most cases (Rossiter et al. 1977). The lack of any reactions in cases of SA-MCF in Indonesia shows AHV-1 to be an unlikely cause of MCF in that country and, in turn, the IIF test based on the WC-11 strain of AHV-1 cannot be considered useful as a diagnostic aid or seroepidemiological tool.

It remains a valid research objective to investigate nonspecific reactions, in order to determine whether they are associated with a viral infection or whether they are artifacts of the test system. Although this may be achieved by absorptions of test sera another serological test such as the Western Blotting technique may offer more specificity. The latter approach is currently being employed.

Western Blotting

This test allows a more detailed analysis of serological reactions. Antigens were prepared by the method of Sculley et al. (1984) by growing viruses in cell cultures and lysing the cells with an SDS buffer when CPE was at a suitable stage of development. Uninfected cell cultures were similarly prepared to serve as controls. Proteins in the preparations were separated on the basis of their molecular weight by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto nitrocellulose paper. Papers were incubated with test antibody and control antisera, and antibody-antigen reactions detected by demonstrating the presence of bound antibody using a commercial biotin-streptavidin horseradish peroxidase detection system (Amersham®). The methodology for the test was developed using bovine herpesvirus 1

(BHV-1) antigen and antiserum, and the technique applied in three different experiments.

In the first study, soluble antigens were prepared from lysates of cells infected with various isolates of the syncytial viruses, and from uninfected control cells. After SDS-PAGE and electroblotting onto nitrocellulose paper, detection of antigens was attempted with antisera to known ruminant viruses, and case sera. Antisera to AHV-1, BHV-1, bovine virus diarrhoea (BVD) and bovine spumavirus (BSV) did not react with antigens in the lysates. Case sera from buffalo detected two antigens in infected cells. Sera from other species of bovine with MCF were unreactive. The molecular weight of one antigen was approximately 45 000 and that of the other 26 000. Both buffalo with MCF and buffalo presumed unexposed to MCF were subsequently found to have reactive antibody. One buffalo with experimental MCF had no detectable antibody (Table 2). Hence, these studies failed to show a clear relationship between the isolates and MCF in Indonesia, and failed to identify the isolates as any of the four viruses for which type antisera were available.

In the second study, lysates of cells infected with the WC-11 strain of AHV-1 were tested. In the standardisation of the test, 5 of 6 sera—a gift from Dr Rossiter, from cases of WA-MCF in Kenya—reacted with virus-associated antigens. Indonesian case sera from Bali cattle in the blood transmission experiments were unreactive. The work will be extended to examine all sera giving low grade or nonspecific reactions in the IIF test.

Table 2. Antibody to buffalo syncytial virus detected by immunoblotting.

Source of Test Sera	No. tested	No. reactive
Uninfected Bali cattle	5	0
Bali cattle dying of MCF	4	0
Buffalo unexposed to MCF	16	10
Buffalo surviving exposure to MCF	3	2
Buffalo dying of MCF	6	5
AHV1 reactive serum	1	0
BHV1 reactive serum	1	0
BVD reactive serum	1	0
BSV reactive serum	1	0

Techniques are also being developed to detect novel antigen and antibody reactions in Indonesian MCF case material. Lysates were made of stored buffy coat cells from before and after infection of the animal with MCF, and antigens sought using type antisera to known viruses and case sera before and after infection. In two experiments to date, antigens in presumed infected cells have not been detected by antisera to AHV-1. No consistent patterns were observed in other test reactions. Strategies to increase antigen presentation in WBCs are to be investigated. Given the valuable stores of WBCs from MCF cases, and sera from before and after infection, there is scope for the development of this approach using various strategies to enhance antigen presentation in infected material.

Agar Gel Precipitation Test (AGPT)

A commercial test (Leukassay B, Pitman-Moore®) has also been used to test for antibody to bovine leucosis virus (BLV) in buffalo with antibody to syncytial virus, in all animals in transmission experiments in Bogor, and in sheep from the natural transmission experiments. Reactive sera were obtained from two animals in the contact transmission experiment, a buffalo and an ongle from South Sulawesi. There was no evidence that BLV is associated with MCF, or that the isolates are BLV.

Partial Characterisation of Isolates

AHV-1 antigens were not detected in acetone-fixed cell cultures of the isolates by antisera to WC-11. Antigenic preparations from isolates did not react with antisera to AHV-1, BHV-1, BVD or BSV in western blot tests.

Discussion

The information presented confirms that the aetiological agent of SA-MCF in Indonesia, as in other countries, will not be isolated and identified easily.

A syncytial virus was isolated from buffalo with MCF, which was the first isolation of this virus in Indonesia (Sudarisman et al. 1985). However, it is noted that Adiwinata (1967) reported that material from Bali cattle with Jembrana disease induced multinucleate giant

cell formation in calf kidney cell cultures 9 days after inoculation. Rinderpest was suspected, but the diagnosis was not confirmed and the isolates were lost. Peranginangin et al. (1986) reported the isolation of an apparently similar virus from buffalo with MCF in North Sumatra.

The isolates have not been fully identified. Retroviruses can produce a cytopathic effect similar to that observed, one of simple syncytia production without intracytoplasmic or intranuclear inclusions. Spumaviruses (BSV) of the retrovirus group, as isolated in northern Australia (Johnson et al. 1983), give syncytia with vacuolated cytoplasm, a feature not seen with the present isolates. A reference serum against BSV from northern Australia did not react with the isolate antigens in the Western Blotting test. Neither do sera with antibody reactive to the isolates react with bovine leucosis antigen in the Leukassay B test (Pitman-Moore®). If the isolates are confirmed as retroviruses, their relationship to other bovine retroviruses remains to be established.

The significance of the isolates is also not resolved. The original isolates were from buffaloes with MCF, but subsequently buffaloes with no history of clinical MCF have also yielded virus. Some have been exposed to MCF either by contact with sheep or by blood inoculation, while others have had no experimental contact with MCF. To date, the virus has been isolated only from buffalo, and is consequently referred to at the present time as buffalo syncytial virus. It is noted that some ovine fibroblast cell cultures may be infected with retroviruses in a latent form (Barban et al. 1984). However, it is considered unlikely that the cytopathic effect was derived from an endogenous virus in the sheep cells, because not all cocultivations produced CPE, induction of CPE could be reproduced by stored original WBCs, and primary isolations were achieved in buffalo foetal thyroid cells as well as sheep foetal thymus cells (Table 1).

Serological testing using the syncytial virus as antigen has shown that none of four Bali cattle with classical MCF developed antibody, but that 10 of 16 buffalo with no history of MCF did have antibody. Therefore, there is no simple serological association of the isolates with clinical MCF. SA-MCF is obviously a complex disease because its aetiology remains a

mystery, and further serological studies involving more cases must be completed before any of a range possible hypotheses is developed.

As none of the MCF cases tested to date developed antibody to AHV-1, as detected in the IIF test to WC-11, the disease appears to have a different aetiology, for cases of WA-MCF routinely develop antibody prior to the onset of clinical signs (Rossiter et al. 1977). There was no evidence of cross-reactivity in the case sera tested to suggest infection with a related virus. The possibility of weak cross-reactions in sera from some sheep and goats must be considered separately until such time as natural infection of these animals with a herpesvirus is proved more conclusively and its ability to infect cattle and buffalo demonstrated.

Work has started on development of a test system in which a presumed infectious tissue—white blood cells—is being used as the antigen in the Western Blotting test. The first experiment did not yield an identifiable pattern of reaction. Strategies to increase the concentration of antigen in such cells must be attempted by culturing cells with various cell and virus growth promoters.

If such investigations fail to identify the antigen of the agent, consideration must be given to the hypotheses that in bovine tissue it does not express itself in antigenic form, or that large ruminants do not produce antibody to the SA-MCF agent. Another avenue of study may be to use sheep tissues and sera in a semi-blind manner, from sheep known to have been associated with cases of MCF in cattle, and this approach is in progress.

Another approach is to use the techniques of biotechnology to identify the nucleic acid of the agent in tissue or WBC samples. Opportunities to develop such an approach are being investigated.

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Walker helped establish the Western Blotting technique.

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Isolation of a Virus from Buffalo Infected with Suspect Malignant Catarrhal Fever

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Abstract

A disease with clinical signs resembling those of MCF occurred in buffalo in Deli Serdang District, North Sumatra, in 1978. The annual incidence was 6.26 per 1000 during the period 1978-84. Isolation of the disease agent was attempted by inoculating white blood cells, lymph node cells and spleen cells onto primary cell cultures of bovine foetal thyroid and bovine foetal kidney. Syncytia were formed 3-4 days after inoculation. The inoculations were carried out by either the feeder layer method or the cocultivation method, and both methods produced the same results. Six isolates grew in cell culture.

Abstrak

Kejadian penyakit yang menunjukkan gejala klinik malignant catarrhal fever (MCF) pada kerbau, telah terjadi di Kabupaten Deli Serdang Sumatra Utara. Dari hidung dan mata keluar cairan mucopurulen, konjungtiva, opasitas kornea, erosi mukosa mulut, suhu badan lebih 41 C dengan angka kematian sangat tinggi; spesimen diambil dari 3 ekor kerbau di lapangan; buffy coat dari hewan hidup lymphoglandula dan limpa dari hewan mati. Virus sinsitiogenis tumbuh pada biakan sel Bovine Embryo Kidney (BEK) pada hari ke 3-9.

Introduction

Malignant catarrhal fever (MCF) is a disease that has frightened the farmers in Indonesia for a long time. Paszotta in 1894 found cases of infected buffaloes in Kediri district, and since then the disease has been found to occur in many places in Indonesia. Sensitive animals have been Bali cattle, Madura cattle and buffalo, while sheep have been suspected as the carrier (Mansjoer 1954). The diagnosis of the disease has been based on the clinical signs and pathological

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changes (Mansjoer 1954; Ginting 1979; Hoffmann et al. 1984), and attempts at isolation of the disease agent have not yet succeeded.

The cases of suspected MCF in the Deli Serdang District, North Sumatra, were observed for the first time in February 1978 in the Lubukpakae subdistrict. The clinical signs were mucopurulent discharge from nostrils and eyes without crust formation as well as production of a particularly bad smell, conjunctivitis, corneal opacity, mucosal erosion of nostrils and the oral cavity, high body temperature (above 40°C), tiredness and, in the end, death. Total cases in that year numbered eight: seven buffaloes and one ongole (*Bos indicus*) cross.

The annual incidence increased each year from 1978 to 1984, except in 1980, when only six cases occurred. The incidence in 1984 was 62 cases. In that year, the disease spread to neighbouring subdistricts, to Tanjungmurawa subdistrict and Perbaungan subdistrict, with one case each (Table 1). The most susceptible animals in the infected areas were buffalo, with a 6.26 in 1000 average annual incidence during the observation period. Ongole crosses showed relative resistance with a 0.03 incidence in 1000 during the same period (Table 1).

There were large populations of sheep and goats within the affected areas (Table 2), allowing a close relationship with the buffalo. This report is part of a thesis concerning the isolation and identification of a virus from buffalo infected with suspect malignant catarrhal fever.

Materials and Methods

Cell Cultures

Primary bovine foetal cell cultures of thyroid (BFT) and kidney (BFK) were prepared by trypsinisation of bovine foetal organs obtained

Table 1. Incidence of MCF within the infected area in Deli Serdang District, North Sumatra Province (1978–84).

	Buffalo			<i>Bos indicus</i> cattle		
	Popula- tion	MCF cases	MCF cases/ 1000	Popula- tion	MCF cases	MCF cases/ 1000
1978	4 501	7	1.56	11859	1	0.08
1979	326	17	5.25	116	-	
1980	4 286	6	1.40	11519	-	
1981	4 581	24	5.24	4977	-	
1982	4 531	22	4.85	7654	-	
1983	4 521	43	9.29	4521	-	
1984	3 308	61	20.08	8224	-	2.12

from a slaughterhouse. The cells were cultivated in 11 oz. (c. 310 mL) flasks containing Eagle's minimum essential medium supplemented with 10% bovine or sheep serum, and incubated at 37°C. The cell concentration was 300 000/mL of medium. In addition, a continuous cell line, Martin-Derby bovine kidney (MDBK) obtained from the National Institute of Animal Health (NIAH), Japan, was used.

Specimens for Viral Isolation

White blood cells (WBC)

Peripheral whole blood (1–2 mL) was collected from clinical cases into sterilised test tubes containing 0.01–0.02 mL of anticoagulant (EDTA, 1%). The WBC were prepared by centrifugation at 400 *g* for 15 min. in a refrigerated centrifuge after being mixed with 1–2 mL of Ficoll-Paque (Pharmacia Fine Chemicals®). The separated WBC were suspended in medium supplemented with 10% bovine or sheep serum and stored at 4°C.

Table 2. Population of sheep and goats in Deli Serdang District, North Sumatra Province (1978–84).

	Sheep	Goats
1978	1 358	12 219
1979	1 394	12 545
1980	1 533	13 799
1981	766	6 889
1982	1 538	13 839
1983	1 560	14 038
1984	1 190	10 714

Lymph node and spleen cells

The cells were prepared as for primary cell culture, and stored at –40°C.

Viral Isolation

Feeder layer method

Samples (0.5 mL) were inoculated onto preformed primary monolayer cultures of BFT and BFK cells and also MDBK cells in 11 oz. flasks containing cover slips, then incubated at 37°C. Three days after inoculation, cover slips were taken, one a day, and stained with Giemsa.

Cocultivation method

Samples (0.5 mL) were cultivated together with suspended cells of BFT, BFK and MDBK in growth medium (4.5 mL) in 11 oz. flasks that were equipped with cover slips.

Results

Source of Specimens

Samples were taken from seven buffalo: one from Tanjungmurawa subdistrict and six from Lubukpakam subdistricts, at different times (Table 3).

Cell Cultures

Six days after seeding the tissue culture flasks of BFT and BFK were ready to be used for virus

isolation by either the feeder layer or cocultivation methods. MDBK cells were used 2 days after being subcultured. The BFT and BFK were used 2–3 days after being subcultured and were used at up to seven subcultures. The MDBK cells in this study were used from the 90th subculture.

Viral Isolations

Six of seven samples studied produced a cytopathic effect characterised by syncytia (Table 4). Most of the syncytia were formed on the third day after inoculation, except for the cells inoculated with K2/AP and K6/AP (fourth day) and K1/AP (ninth day).

Discussion

In this study, seven viruses were isolated from cases of MCF. Syncytia are formed not only by herpesviruses but also by para-influenza (PI-3) infection. Other ruminant herpesviruses besides MCF, such as infectious bovine rhinotracheitis (IBR) virus, could also be involved. For identification and differentiation, it is proposed to employ antiserum to IBR and to the WC-11 strain of alcelaphine herpesvirus 1 that causes wildebeest-associated MCF, in immunofluorescence tests, and also to do haemadsorption tests that will indicate the presence or otherwise of a PI-3-like agent. The isolated viruses were stored and cultivated for further studies of biologic characteristics, and for making diagnostic reagents and vaccine should they be confirmed as the agent of MCF in Indonesia.

Table 3. Derivation of specimens for viral isolation.

Location	No. of buffalo	Code no. of isolation materials	Nature of isolation materials
Tanjungmurawa	1	K1/AP	WBC, Ln, S
Lubukpakam I	1	K2/AP	Ln, S
Lubukpakam I	1	K3/AP	WBC
Lubukpakam III	1	K4/AP	Ln, S
Lubukpakam IV	1	K5/AP	Ln, S
Lubukpakam V	1	K5.1/AP	WBC
Lubukpakam VI	1	K6/AP	Ln, S

WBC = white blood cells, Ln = lymph node, S = spleen.

Table 4. Results of viral isolation attempts in various cell cultures.

Code of isolation materials	FT cells		BFK cells		mDBK cells	
	FL SC	CC SC	FL SC	CC SC	FL SC	CC SC
K1/AP	+	+	+	+	-	-
K2/AP	+	+	+	+	-	-
K3/AP	+	+	+	+	-	-
K4/AP	+	+	+	+	-	-
K5/AP	0	0	+	+	-	-
K5.1/AP	-	-	-	-	-	-
K6/AP	0	0	+	+	-	-

FL = feeder layer method, CC = cocultivation method, SC = syncytia, 0 = not applied

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Problems in Developing a Rabbit Model of Malignant Catarrhal Fever

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Abstract

Experiments to transmit malignant catarrhal fever (MCF) to rabbits are summarised, in which pathological changes induced included vasculitis, perivasculitis and granulomas in the brain, interstitial infiltration of parenchymal organs with lymphoid cells and an accompanying perivasculitis and vasculitis, and lymphoblastic proliferation in lymphoid organs and lymphoid infiltrates. A review of experimental MCF in rabbits revealed that others had reported similar findings. However, the changes observed were also similar to those associated with *Encephalitozoon cuniculi* infections in rabbits and other species. It is suggested that histological criteria may be an unreliable index of successful passage of MCF to rabbits, and that *Encephalitozoon* sp.-infected rabbits should not be used in pathogenesis studies. *Encephalitozoon* sp. is a ubiquitous parasite, and so the status of experimental animals should be confirmed using serological tests, the most sensitive indicator of the parasite infection.

Abstrak

Pada makalah ini, percobaan penularan MCF pada kelinci diulas. Gambaran patologis akibat hasil penularan tadi antara lain ialah vasculitis, perivasculitis dan granuloma dalam otak, infiltrasi interstitialis sel limfoid pada jaringan parenkhim alat tubuh yang diikuti oleh vasculitis dan perivasculitis serta proliferasi lymphoblastik dalam kelenjar limfa dan infiltrasi limfoid. Hasil tersebut tadi sesuai dengan laporan peneliti lain. Namun demikian perubahan-perubahan ini mirip dengan gambaran yang didapat pada kelinci dan hewan lain akibat infeksi *Encephalitozoon cuniculi*. Karena itu untuk studi patogenesis, kriteria histologi pada kelinci MCF dan *Encephalitozoon* sp. sebaiknya jangan dipakai. Sedang untuk *Encephalitozoon* sp. pada hewan

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percobaan sebaiknya diperkuat dengan uji serologi sebagai indikator yang sangat sensitif untuk infeksi parasit tersebut.

Introduction

From early in the study of malignant catarrhal fever (MCF) until the present time, transmission experiments have played an important role in defining the disease, including its pathogenesis and, to some extent, its aetiology. As well as transmission to naturally susceptible species, passage to other animals has been attempted, including rabbits. Daubney and Hudson (1936) reviewed the early work in this field and reported successful transmission to rabbits of wildebeest-associated MCF (WA-MCF) and passage of disease from rabbits back to cattle.

Plowright (1968) emphasised the relative difficulty of transmitting sheep-associated MCF (SA-MCF). Transmissions of SA-MCF from deer to rabbits have since been reported (Buxton and Reid 1980), and from such rabbits cell lines were established capable of transmitting disease to rabbits, but not back to deer (Reid et al. 1983). The ability to routinely establish SA-MCF infections in rabbits would offer a significant advance towards isolation of the SA-MCF agent and demonstrate the value of rabbit models.

WA-MCF in Rabbits

The histopathology associated with experimental WA-MCF and AHV-1 infections in rabbits has been well described (Plowright 1953; Piercy 1955; Edington et al. 1979). The three reports cited are consistent in their descriptions. There was lymphoid necrosis, lymphoblastic proliferation with excess mitoses in all

lymphoid organs, perivascular infiltration by lymphoid cells and accompanying vasculitis with lymphoid infiltration and destruction of the smooth muscle. Similar necrosis was seen in the capsules and trabeculae of the spleen and lymph nodes. There were also interstitial lymphoid cell accumulations in liver, kidney, adrenal, myocardium and lung, and meningoencephalitis with vasculitis, perivascular cuffing and mononuclear cell infiltration of the choroid plexus. The lymphoid infiltrate in the lung was perivascular and peribronchiolar. Ocular structures and bone marrow were also invaded by lymphoblasts. No syncytial giant cells were mentioned in these reports.

SA-MCF in Rabbits

The first comprehensive report of passage of SA-MCF to rabbits was that of Buxton and Reid (1980). Clinical signs included fever, dullness, anorexia and frequently serous or catarrhal ocular and nasal discharges. Lymph nodes showed haemorrhage and necrosis with proliferation of lymphoblasts. Such cells were present in lymphoid infiltrates in the periportal spaces, perivascular and peribronchiolar spaces in the lung, ocular structures, as well as the lamina propria of stomach and intestines and in the kidney and heart to a lesser degree. Focal necrosis of hepatocytes occurred. Brains showed perivascular cuffing, and one had a focus of necrosis in the cerebellar folia.

Similar changes were reported in subsequent pathogenesis experiments (Buxton et al. 1984). Proliferation of lymphoblasts was prominent. There was interstitial thickening of alveolar walls and mild accumulations of cells in the kidneys. Of particular note was focal necrosis in the liver characterised by necrotic cells surrounded by increased numbers of Kupffer cells. Large multinucleate giant cells were noted in some animals. Hepatic granulomas with necrotic centres and accompanied by multinucleated giant cells had been reported previously in attempted transmission of SA-MCF to rabbits (Pattison 1946).

Well-documented responses following inoculation of SA-MCF infected blood and tissues to rabbits have also been reported by another group, in which rabbits developed pyrexia, depression, anorexia, mucopurulent ocular and

nasal discharges and diarrhoea. Microscopic changes were essentially as described above. There was necrosis of lymphoid tissue and lymphoblast proliferation. Necrosis of vessel walls was seen in many tissues as well as perivascular cuffing and vasculitis in the brain. There were lymphoid infiltrations in the liver, kidney and alveolar septae. Multinucleated giant cells were present in the follicles of some intestinal lymphoid tissue. Control rabbits had perivascular cuffing in the brain and a lymphoid infiltrate in the kidney attributed to *Encephalitozoon* sp. (Westbury and Denholm 1982).

Encephalitozoonosis

Encephalitozoonosis is a protozoal infection of rabbits and other laboratory animals. One species is recognised, *Encephalitozoon cuniculi*. Infection is common and is usually chronic or latent, but disease and deaths have been recorded.

Microscopically, it is characterised by granulomas and nonsuppurative inflammation. The typical granuloma consists of an area of central necrosis surrounded by epithelioid and lymphoid cells. There is perivascular cuffing and gliosis in the brain, an interstitial mononuclear cell infiltrate in the kidney, a mild focal nonsuppurative hepatitis, epicarditis and lymphoid hyperplasia especially of the mesenteric node. There may also be lymphoid infiltration of the myocardium, adrenal glands and retina. Interstitial pneumonia and perivascular lymphoid infiltrates have been reported in the lung of affected mice. Organisms can frequently be seen in tissue sections, but not always (Shadduck and Pakes 1971). Multinucleated giant cells have been reported in brain lesions (Jortner and Percy 1978; Koller 1969).

Another susceptible species is the blue fox (*Alopex lagopus*) (Bjerkas and Nesland 1987). In this species, the vessels of the brain and kidney were the main tissues affected, although foci of granulomatous inflammation in the parenchyma were seen. Parasites were observed in endothelial and smooth muscle cells, macrophages and neurones among other cell types. Vascular lesions resembled polyarteritis nodosa in humans, and although variable in severity, comprised lesions including subendothelial

mononuclear cell infiltration with partial occlusion of lumens, necrosis and mononuclear cell infiltration of smooth muscle walls, and infiltration of the adventitia by mononuclear cells. These changes were said to be pathognomonic of *Encephalitozoon cuniculi* infection in blue foxes.

Experimental Studies

From naturally occurring and experimental cases of SA-MCF in Indonesia, transmission of MCF to pairs of rabbits was attempted by intraperitoneal inoculations of blood from Bali cattle and buffalo. Initially, 20 mL volumes were used, but subsequently this was increased to 50 mL. Rabbits were either transported to the case and inoculated immediately after the collection of the blood in heparin, or the blood was held at 37°C for transportation to the animal house and until subsequent use, normally within 1 hour of collection. On one occasion, rabbits were taken by air and road transport to a field case in a distant province, and on another the veterinary field services purchased rabbits locally, inoculated them and forwarded them by air freight to the research laboratory.

The clinical responses, gross pathology and histopathology were highly variable. Microscopically, vasculitis, perivasculitis and focal granulomas were seen frequently in the CNS. Some CNS granulomas had foci of central necrosis which occasionally contained rod-shaped parasitic forms and which were surrounded by epithelioid cells with a large open cytoplasm with other mononuclear cell types peripherally. Interstitial lymphoid infiltrates or fibrous scarring occurred in the kidneys. Livers frequently showed periportal mononuclear infiltrates and occasional multinucleated cells. Lungs showed vasculitis, perivasculitis, subendothelial infiltrates in vessels, and interstitial pneumonia. In some cases, lymphoid tissue showed blast cell proliferation with pleomorphic forms and excess mitotic figures, and in a few instances suspected syncytia formation. There was sometimes interstitial myocarditis with infiltrates of mononuclear cells including lymphoblasts.

Subsequent work was directed at defining the response to blood inoculation more accurately by using groups of 10 rabbits on each occasion

and performing a necropsy examination on pairs at 1, 2, 3 and 12 weeks post inoculation (PI), or at the time of death. One rabbit was necropsied at the beginning of the observation period and one at the end.

This approach in groups treated with blood from two natural cases of SA-MCF or with normal buffalo blood has also shown variable responses. In one series, the central nervous system (CNS) was progressively affected over the period, showing vasculitis and perivasculitis in inoculated animals but not in controls, while both controls and inoculated rabbits showed interstitial nephritis. Only inoculated rabbits showed periportal mononuclear cell infiltrates in the liver and foci of myocarditis.

In the series inoculated with blood from the second case, changes were milder. Control rabbits showed periportal mononuclear cell infiltration of the liver and one died showing pneumonia at 4 weeks. One inoculated rabbit showed interstitial nephritis, two had periportal infiltrates, another two interstitial pneumonia, and a total of three showed some myocarditis. However, similar changes were seen in the series inoculated with uninfected blood.

Discussion

The descriptions of encephalitozoonosis and MCF show considerable similarity. Many of the lymphoid infiltrates and granulomas described in MCF experiments could be attributable to *Encephalitozoon* sp. infections. Westbury and Denholm (1982) were the only authors to report on the *Encephalitozoon* status of their rabbits. The clinical signs associated with a CNS disturbance as described by Pattison (1946) are consistent with those reported for rabbits with severe encephalitozoonosis (Jortner and Percy 1978). Concurrent infections such as 'snuffles' are said to exacerbate the severity of the *Encephalitozoon* lesions (McCartney 1923), as does immunosuppression with corticosteroids or cyclophosphamide (Shaddock and Pakes 1971).

It is therefore conceivable that the stress of inoculations could exacerbate the subclinical encephalitozoonosis in rabbits. It is further possible that a disease such as MCF could be functionally immunosuppressive, either through lymphoid necrosis or some other mechanism. It thus cannot be assumed that mild lesions in

control animals are attributable to the parasite and florid lesions in treated animals are attributable solely to the treatment. Westbury and Denholm (1982) have identified an important aspect of MCF research in laboratory animals.

The interference the infection can cause to experimental work has been described (McCartney 1923; Shadduck and Pakes 1971; Jortner and Percy 1978), but the similarity in the pathology compared with that expected in MCF makes the infection of even greater significance in MCF investigations. However, the strategies available for eliminating the parasite from rabbit colonies in the past were limited. Howell and Edington (1968) described a technique based on the selection of progeny of dams diagnosed as being free of the parasite retrospectively on the basis of histological examination. As late as 1972, there was no reliable method for detecting infection in living rabbits (Pakes et al. 1972). These authors developed a delayed-type hypersensitivity skin test which detected infected animals, while Cox et al. (1972) developed immunofluorescence tests including a serological test. Serological examination of several rabbit colonies and rabbit sera from around the world frequently showed the presence of infected animals, often at high prevalence (Cox and Pye 1975). Serology is a sensitive measure of infection, antibodies being detectable 2 weeks before organisms appear in urine and 5 weeks before histological lesions appear in the kidney (Cox and Gallichio 1978). In that study, CNS lesions were rarely seen, and then only 8 weeks after seroconversion.

Although the pathological changes observed in the current series are similar to those described by others (Buxton and Reid 1980; Westbury and Denholm 1982), it cannot yet be said with certainty whether any of the lesions are attributable to MCF. The syncytia in liver and lymphoid tissue are particularly interesting and have been reported previously in SA-MCF transmission experiments, in the liver (Pattison 1946; Buxton et al. 1984) and in an intestinal lymphoid follicle (Westbury and Denholm 1982). In rabbits with encephalitozoonosis, such cells have been reported in the brain, but it cannot be assumed that in exacerbated encephalitozoono-

sis they would be restricted to that tissue. It is clear that definitive studies are needed in rabbits specifically free of *Encephalitozoon* sp., in order to accurately describe the range of pathology that can be expected in transmitted MCF. Only then will it be possible to attempt interpretation of the results obtained to date.

Clearly, any pathogenesis experiments should be conducted in parasite-free animals. Until the situation is clarified the status of rabbits used in any MCF work should be recorded, based on serology which is the most sensitive measure of the parasite infection (Cox and Gallichio 1978). There is even one report (Hunt et al. 1972) of gnotobiotically derived rabbits harbouring the parasite.

The presence of parasites should not preclude successful infection of a rabbit by the SA-MCF agent. Indeed, some of the pathology reported in the present series and by other authors may be the result of dual infections. Further research may also reveal that some of the pathology associated with SA-MCF and similar diseases in Indonesian ruminants is the result of dual infections involving other agents. A potential problem with *Trypanosoma evansi* and other undiagnosed infections in buffalo has already been noted (Daniels et al. 1988, Unruh et al. 1988).

It is in attempting to use histological criteria for the diagnosis of successful passage of SA-MCF to rabbits, and in attempting to use *Encephalitozoon* sp.-infected rabbit models for MCF pathogenesis studies, that problems arise. Serial transmission studies in which the disease is successfully passed back to susceptible ruminants obviously remain valid as demonstrations of the partial isolation of the agent, the identification of which is the primary goal of current research.

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Malignant Catarrhal Fever Research in Queensland

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Abstract

A small population of sheep in Queensland was identified as a potential carrier of the sheep-associated malignant catarrhal fever (MCF) agent by its association with buffalo dying of MCF. Extensive protocols attempting virus isolation in cell culture and in laboratory animals failed to isolate a disease agent. A Bali cow penned with these sheep and their progeny developed clinical MCF, but attempted blood transmissions and virus isolations were not successful. It was concluded that the techniques of conventional virology may not be adequate to identify the MCF agent.

Abstrak

Sekelompok kecil dari domba di Queensland diduga keras mempunyai peranan penting sebagai pembawa (carrier) agen penyebab penyakit MCF, sehubungan dengan matinya kerbau dengan gejala MCF. Dengan cara kerja yang terus menerus dalam isolasi agen penyebab penyakit pada biakan sel dan pada hewan percobaan ternyata gagal mengisolasi agen penyebabnya. Seekor sapi Bali yang dikandangan bersama domba diatas dan progeninya menunjukkan gejala klinis MCF tetapi dengan transmisi lewat darah dan isolasi virusnya belum berhasil. Disimpulkan bahwa tehnik virologi konvensional (lama) mungkin tidak cukup untuk menyidik agen MCF.

Introduction

Two distinct forms of malignant catarrhal fever (MCF) have been proposed: the wildebeest-associated MCF (WA-MCF) and the

sheep-associated MCF (SA-MCF). Alcelaphine herpesvirus 1 (AHV-1) causes WA-MCF while the cause of SA-MCF has not been identified. Both agents produce no disease in their respective natural hosts, wildebeest and sheep, but cause a fatal lymphoproliferative disease following transmission to certain other species of ruminants. Only with AHV-1 is there any information on the immune response of the natural host to infection. Epidemiological studies support the view that all wildebeest become infected between 3 and 6 months of age and virus can be isolated periodically from some animals, particularly pregnant females and young calves. The presumptive agent of sheep probably follows a similar epidemiological pattern.

The proposition that the sheep-associated agent behaves in sheep similarly to AHV-1 in wildebeest is supported both by circumstantial evidence and by data suggesting that a virus sharing antigenic determinants with AHV-1 is prevalent in sheep. This proposition has been the main basis for the direction of research carried out at the Oonoonba Veterinary Laboratory (OVL). As numerous unsuccessful attempts to isolate the agent from the end host have been reported, it was felt that a concerted effort directed towards the natural host might be more rewarding. Nine sheep known to be associated with SA-MCF transmission to buffalo in southeast Queensland were purchased in 1983 and transported to OVL for intensive studies including a transmission experiment. Australia's quarantine regulations and the lack of wildebeest in this country necessitated overseas study to compare the serology of the two diseases. The opportunity to do this was provided in both Indonesia and the United Kingdom.

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Materials and Methods

Animals

(a) Sheep

One ram and eight ewes associated with SA-MCF in buffalo were purchased for the studies. They arrived at OVL from southeast Queensland in December 1983.

(b) Rabbits

Animals from OVL's colony of laboratory rabbits were used.

(c) Animals for natural transmission experiments

(i) Cattle (*Bos taurus*)

Two 6-month-old castrated males from the OVL herd were used.

(ii) Buffalo (*Bubalus bubalis*)

Two males, 6 months old, were obtained from the Northern Territory Department of Primary Production. They arrived at OVL in April 1985.

(iii) Bali cattle (*Bos javanicus*)

A male and a female, 6 months old, were obtained from the Northern Territory Department of Primary Production. They arrived at OVL in April 1985.

(iv) Rusa deer (*Cervus timorensis*)

A male and a female were obtained as young adults from Hamilton Island, Queensland, in March 1985.

(v) Chital deer (*Axis axis*)

A male and a female were obtained as young adults from a property 120 km northwest of Charters Towers in Queensland in March 1985.

Cell Cultures

(a) Cell dispersal

To establish cultures from organs and tissues, suspensions of single cells were prepared by treating finely cut material with trypsin at 37°C. To passage established cell cultures, an activated trypsin-versene solution (ATV) warmed to 37°C was used to remove cell monolayers from

culture vessel surfaces as suspensions of single cells.

(b) Media

Minimum essential medium (Eagle) with Earl's salts and nonessential amino acids was used for all cell cultures except the attempts to grow lymphocytes. For the lymphocyte culture attempts RPMI 1640 was used.

Foetal calf serum, which was not heat-inactivated, was used as a supplement in all media. For the lymphocyte culture attempts and for macrophage cultures the supplementation rate was 20%. For all other cultures it was 10%.

(c) Incubation

Cultures were incubated at 37°C, except in the bromodeoxyuridine experiment where each culture was duplicated so that two incubation temperatures—37°C and 33°C—could be used.

Cell Fusion

Cell fusions were accomplished by polyethylene glycol as described by Castro et al. (1983).

Leucocyte Separation

A wide range of centrifugation conditions was investigated to develop the following procedure for extracting leucocytes from sheep blood suitable for culturing. The technique effectively removed erythrocytes, and the percentage viability of recovered leucocytes was high. An important feature of this procedure was the washing of the buffy coat three times with large volumes of Dulbecco's phosphate buffered saline solution A (PBSA) before layering it over Ficoll-Paque (Pharmacia). This washing removed most thrombocytes and thus prevented clotting when the leucocytes were suspended in culture medium supplemented with foetal calf serum.

Twenty millilitres of blood were collected by jugular venipuncture into a 1-oz. (c. 30-mL) bottle containing anticoagulant. It was centrifuged at 1000 g for 9 min. The buffy coat was collected and made up to 20 mL with PBSA. Cells were washed three times in PBSA, being pelleted by centrifugation at 250 g for 10 min and being resuspended in 20 mL in PBSA each time. After the last wash, cells were

resuspended in PBSA to 2 mL and layered over 3 mL of Ficoll-Paque in 13 mm diameter centrifuge tubes, and centrifuged at 1000 g for 20 min. The band of cells at the interface was collected, resuspended to 20 mL in 1 oz. universal bottles and washed once in PBSA as had been done prior to use. All centrifugations were performed at 4°C using a swing-out rotor. The g forces quoted are g maximum, as measured at the bottom of the tube.

Serology

An agar gel diffusion precipitin test (AGDPT) was standardised for the spumavirus isolated from a sheep in a cortisone stressing experiment. The antigen was made by disrupting infected cell cultures either by ultrasonication or repeated freezing and thawing, and the agar used was a high salt agar (10N NaCl). Plates were incubated in a humid atmosphere at room temperature for 3 days before reading.

Summary of Investigations and Results

History of Imported Sheep

Two buffalo were introduced to a property in southeastern Queensland in late 1979 and were pastured with sheep. Early in 1981, a bull calf born on the property died without postmortem examination. In February 1983, the original cow died suddenly, again with no necropsy. In June 1983, the original bull and a heifer born on the property died suddenly, and necropsies were performed. The clinical history, gross pathology and histopathology confirmed a diagnosis of SA-MCF. One ram and eight ewes, the total flock, were purchased and transported to OVL in December 1983.

In vitro Methods Used to Investigate Sheep

The following investigations were performed:

- a. Weekly cultivation of macrophages from the sheep.
- b. Polyethylene glycol fusion of leucocytes with a range of cell cultures, namely:

calf testis	lamb testis
rabbit testis	foetal lamb thyroid
rabbit kidney	foetal lamb adrenal
rabbit macrophage	sheep macrophage

c. Cocultivation of leucocytes with a range of cell cultures, namely:

calf testis	rabbit testis	foetal lamb lung
lamb testis	rabbit kidney	

d. Cultivation of lymphocytes.

e. Inoculation of swab material into calf testis cell cultures.

f. Cocultivation of a range of organs and tissues with calf testis cell cultures.

g. Immunoperoxidase staining of lymphocytes using terminal serum from the Bali cow as a possible source of specific antibody.

Results: no virus was isolated or detected.

Cortisone Stressing Experiment

Seven lambs, 6 months old, the progeny of the imported sheep, were intramuscularly inoculated with 2 mg/kg of a corticosteroid (Opticortenol) daily for 5 days, and were slaughtered 2 days later. Eye, nose and throat swabs and blood were collected for virus isolation before, during and after the cortisone treatment. A range of organs and tissues was collected for virus isolation at slaughter.

Results: virus was isolated from only one lamb. It caused multinucleated syncytia, a CPE identical to that of bovine spumavirus.

Spumavirus Investigations

An AGDPT for serological studies was standardised. This AGDPT could not distinguish between the sheep virus and bovine spumavirus. The effect of the isolated spumavirus on seronegative lambs less than 1 week old was studied.

Results:

- a. 3/3 lambs inoculated intravenously seroconverted.
- b. 1/3 lambs inoculated intranasally seroconverted.

c. Virus was recovered 40 and 74 days after inoculation.

5-Bromodeoxyuridine (5-BDU) Experiment

The leucocyte fraction was extracted from blood collected from each of five sheep and divided into three aliquots, one for each of the following treatments:

5 µg/ml 5-BDU for 3 days

25 µg/ml 5-BDU for 3 days

100 µg/ml 5-BDU for 1 day

Results:

a. The higher the 5-BDU concentration, the greater was the damage done to the leucocytes.

b. After 5-BDU treatment, the cells and culture fluids were tested for virus by growing rabbit testis cells with them.

c. No CPE was seen.

In-Contact Transmission Experiment

The cattle, buffalo and deer listed previously were penned with the sheep implicated in carrying the disease.

Result: only the Bali cow died. MCF was diagnosed on the basis of clinical signs, gross pathology and histopathology.

Experimental Transmission from the Bali Cow

The following inoculations were made to cattle and rabbits:

a. 10% spleen suspension into two *Bos taurus*

b. 10% spleen suspension into two rabbits

c. citrated blood into two rabbits

d. 5% thymus suspension into two rabbits.

Result: no transmission was achieved.

Virus Isolation Attempts from the Bali Cow

1. Autocultures of thymus, lymph nodes, spleen and kidney were prepared.

2. Cocultivation of thymus, lymph nodes, spleen and kidney was performed with rabbit testis cells and sheep macrophages.

3. Polyethylene glycol fusion of thymus and lymph node cells with sheep macrophages was attempted.

Result: no virus was isolated.

Attempts to Transmit SA-MCF to Rabbits from Sheep

Rabbits were inoculated with:

a. blood buffy coats

b. leucocytes separated by Ficoll-Paque

c. eye, nose and vaginal swab material

d. lymph node material

e. 3rd passage of cell cultures inoculated with swab material

f. 5th passage of polyethylene glycol fusions of leucocytes with cell cultures.

Result: no transmission was achieved.

Serology—WC-11

Sera from the nine sheep purchased and 49 sera from sheep originating in western Queensland were tested for antibodies to WC-11 in the United Kingdom. The test was conducted blind.

Result: only one serum reacted in the test. It was from a sheep purchased as a suspected carrier of MCF.

The above work was repeated in Indonesia using similar sera with negative results.

Serology—Spumavirus

a. In Indonesia

Selected sera from a serum bank were tested against spumavirus using the AGDPT. The test was done blind.

Result: 2/11 goats, 0/10 buffalo, 0/28 Bali cattle, 0/17 deer, and 2/117 sheep were found positive. One of two *Bos indicus* cattle had a faint reaction. It is interesting that the four positives and one weak positive were from animals at Ciawi, a known focus of SA-MCF infections (Hoffmann et al. 1984). The significance of the finding is unknown.

b. In Australia

Sheep were tested for antibodies to spumavirus using AGDPT.

Result: antibody prevalence of 25% was found in sera of sheep from western Queensland. No positive sera were detected in purchased sheep or their lambs.

Conclusions

The inability to isolate the putative causative agent from either carrier sheep or sick cattle was most frustrating. The reasons could be many.

Sheep may not be carriers of the agent. However, in view of the overwhelming evidence, albeit circumstantial, it is difficult to believe that they do not play a major role in the disease. If sheep are carriers of the agent, failure could be due to the agent not being present in the tissues or excretions examined, or being present only in a form that precluded isolation. The use of many techniques would suggest that the methods used may not be suitable for isolation of this virus. Isolation of MCF virus of wildebeest origin has been possible if only from a limited range of bovine cell cultures. It equally cannot be ruled out that the agent is in a form which defies standard methods of isolation. There are many examples in virology where a successful cell culture system has not been found for agents that can be visualised under the electron microscope. The situation may be even more difficult with the sheep-associated agent since the agent has not yet been so visualised.

Workers at Moredun Institute in Scotland have characterised a lymphocyte cell line from rabbits infected with the tissues of a deer with SA-MCF and found that inoculation of as few as 100 of these cells induced typical MCF (Reid et al. 1983). This work has led to a hypothesis that the lesions of MCF may arise through parasitism by the causal virus of a

subpopulation of T-lymphocytes known as large granular lymphocytes, and that virus may persist in these cells in an incomplete form, possibly as episomal DNA, and cause a profound dysfunction of this cell type. This could result in both benign and polyclonal T-lymphocyte hyperplasia arising through the absence of suppressor cell activity. Subsequent terminal tissue destruction could be caused by indiscriminate natural killer activity.

If the agent in sheep exists only as DNA, it is difficult to see how it could be transferred from sheep to sheep and from sheep to other ruminants. Methods are now available utilising DNA probes to attempt to detect incorporated DNA in specimens. There is, however, the need to have a probe that might show some homology with the unisolated sheep-associated agent. The obvious probe to try would be one prepared from the wildebeest-associated agent. Until the Australian Animal Health Laboratory in Geelong obtains permission to import the wildebeest-associated agent, this work must be conducted outside Australia.

Such a probe would also allow a detailed examination of sheep to determine where the agent resides, how it is excreted and also the proportion of sheep that carry the agent.

These are very exciting times for an increased understanding of MCF.

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