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A STUDY OF HERPESVIRUS ISOLATION
FROM HEALTHY SHEEP

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DESCRIPTION AND REVIEW OF THE LITERATURESUMMARY

One hundred and fifty healthy lambs were screened for herpesviruses. Nasal swabs were collected from fifty lambs, blood samples from fifty lambs and both nasal swabs and blood from a further fifty lambs. Virus isolation was attempted in foetal lamb kidney cell cultures. No virus was recovered.

A technique, involving hypotonic lysis of erythrocytes, was developed for the separation of ovine leucocytes. White blood cell recovery rates, percentage viability of leucocytes and the degree of red cell contamination compared favourably with other methods of leucocyte separation.

The virus isolation system was tested by propagating equid herpesvirus 2 in the foetal lamb kidney cells. Acridine orange staining of infected monolayers detected degenerative changes not readily discernible by haematoxylin and eosin staining methods.

Indirect virus isolation in cell cultures or of virus recovery during investigations into acute disease outbreaks. The question therefore arises can previously unreported or undescribed herpesviruses be detected in livestock before they assume importance as a consequence of changing environmental factors?

With the exception of the sheep, all species of common domestic livestock, found in Europe, have at least one host-specific, recognised pathogen belonging to the herpesvirus genus. Many of these viruses have been isolated from the blood or shown to be present in the nasal cavities of

INTRODUCTION AND REVIEW OF THE LITERATURE

The herpesvirus group is an expanding genus and at present encompasses over fifty herpes, herpes-like and cytomegaloviruses. A feature of the herpesviruses is the frequency of persistent infections. Obviously, detection of this type of infection depends upon the recovery of virus from apparently healthy individuals. Veterinary virology, for a variety of reasons, has been mainly concerned with acute disease outbreaks and many facets of low grade viraemias, persistent and latent infections have remained unexplored.

As in the field of human medicine, the introduction of efficient vaccines has led to a reduction in some of the markedly acute viral diseases. However, accompanying this there has been an unmasking of other conditions, including virus infections, whose symptoms were previously unnoticed. To some extent the importance of these newly revealed virus infections has been accentuated by the intensification of livestock husbandry practices and the greatly increased stocking densities.

The herpesvirus group continues to enlarge usually as the result of inadvertent virus isolation in cell cultures or of virus recovery during investigations into acute disease outbreaks. The question therefore arises: can previously unimportant or undescribed herpesviruses be detected in livestock before they assume importance as a consequence of changing environmental factors?

With the exception of the sheep, all species of common domestic livestock, found in Europe, have at least one host-specific, recognised pathogen belonging to the herpesvirus genus. Many of these viruses have been isolated from the blood or shown to be present in the nasal cavities of/

of the effected species. This dissertation describes the investigations undertaken, with these considerations in mind, to establish whether herpesviruses were present in the sheep on one farm in eastern Scotland.

Isolation of herpesviruses and cell cultures.

The history of herpes and the development of the concept of the herpesvirus group has been described by Kaplan (1973). Amongst the earlier descriptions of viral diseases was the herpesvirus infection, Aujeszky's disease. Pseudorabies was described over seventy years ago in cattle, dogs, cats and rabbits (Aujeszky, 1902). This virus also became one of the first of the herpesviruses to be grown in mammalian cell cultures when Scherer (1953) grew it in mouse fibroblasts. In the same year, Randall, Ryden, Doll and Schell (1953) cultivated, in foetal horse tissue, the virus presently designated as equid herpesvirus 1 (EHV-1). Listed in Table I are the first isolations, in mammalian cell culture systems, of herpesviruses affecting domestic livestock.

It is generally recognised that all herpesviruses will grow in cell culture (Andrewes and Pereira, 1972; Plummer, 1967) but the range of suitable cultures varies greatly. Roizman (1969) commented on the considerable variation in the biochemical properties of the individual viruses within the group. One manifestation of these differences is the varying abilities of these herpesviruses to grow in cell cultures derived from aberrant host species. Neither Kaplan (1973), nor Plummer (1967) compiled comprehensive lists of the cell cultures recorded as being suitable for the isolation of the viruses mentioned in Table I. Consequently, an attempt has been made in Table II to draw together a summary of the recorded cell systems in which the various herpesviruses can be cultivated. However, the prudent words of Kaplan (1973) that "one should be cautious in/

in making the assumption that if a particular cell type is not listed, the virus will not grow in it", should be borne in mind.

There is a graduation within the herpesvirus group with respect to host ranges. Although Kaplan (1973) considers pseudorabies virus to have one of the widest ranges of cells, Bagust (1971) lists seventeen primary cultures and fourteen cell lines suitable for EHV-1. At the other extreme, equid herpesvirus 3 (EHV-3) can be propagated only in cells of equine origin (Pascoe et al, 1969). Rabbit kidney cells, particularly primary cultures, have proved to be of value for a wide range of herpesviruses, including EHV-1 (Bagust, 1971) EHV-2 (Karpas, 1966), infectious bovine rhinotracheitis virus (IBRV) (Plummer, 1967), malignant catarrhal fever virus (MCFV) (Plowright et al, 1960), goat herpesvirus (Saito et al, 1974) and pseudorabies virus (PRV) (Kaplan and Vatter, 1959). Although multiplication of some of the viruses cited in Table II may occur in a variety of cell cultures, the cytopathic effect is not constant. Cell type and strain of virus play a major role in the extent to which cytopathic effect appears (Plummer, 1967).

Cultivation of herpesviruses in fertile eggs.

Of those viruses in the herpes group which cause disease in the domestic mammals, only two will grow in fertile eggs. White plaques are formed on the chorioallantoic membrane by pseudorabies virus and the virus subsequently invades the central nervous system of the embryo (Bang, 1942). Pseudorabies virus is also reported (Glover, 1939) as causing a haemorrhagic reaction and death of the embryo, usually by the third day. Hamster adapted strains of EHV-1 have been adapted to fertile eggs with growth occurring on the chorioallantoic membrane (Doll and Wallace, 1954) and in the yolk sac and amnion (Randall, 1955).

TABLE I

FIRST ISOLATIONS IN CELL CULTURES OF
HERPESVIRUSES OF DOMESTICATED MAMMALS

Host	Disease/Virus	Reference	
Cat	FVR	Grandell & Maurer	(1958)
	New feline herpes	Fabricant <u>et al</u>	(1971)
Cattle	Allerton	Alexander <u>et al</u>	(1957)
	Mammillitis	Martin <u>et al</u>	(1964)
	DN 599	Mohanty <u>et al</u>	(1971)
	IBR	Madin <u>et al</u>	(1956)
	MCF	Flowright <u>et al</u>	(1960)
Dog	Herpes canis	Carmichael <u>et al</u>	(1964)
Goat	Goat herpes	Saito <u>et al</u>	(1974)
	IBR	Mohanty <u>et al</u>	(1972)
Horse	EHV-1	Randall <u>et al</u>	(1953)
	EHV-2	Plummer & Waterson	(1963)
	EHV-3	Petzoldt	(1967)
Pig	Pseudorabies	Scherer	(1953)
	Inclusion body rhinitis	L'Ecuyer & Corner	(1966)
Sheep	Jaagsiekte	Mackay	(1969)

TABLE II
GROWTH IN CELL CULTURES

Host	Disease/Virus	Cell Type	Reference
Cat	FVR	Feline kidney, lung, testis	Andrewes & Pereira (1972)
	New feline herpes	Feline kidney, embryonic lung. Canine kidney. Embryonic bovine heart, lung, spleen. HeLa.	Fabricant & Gillespie (1974)
Cattle	Allerton /mammillitis	Lamb testis	Andrewes & Pereira (1972)
		Calf kidney	Alexander <i>et al</i> (1957)
		Bovine lymph node, kidney. BHK 21	Martin <i>et al</i> (1964)
		Ovine, porcine, feline kidney. Bovine thyroid. Feline lung.	Kaplan (1973)
	DN 599	Embryonic bovine kidney	Mohanty <i>et al</i> (1971)
	IBR	Embryonic bovine kidney, testis, lung. Porcine kidney Human amnion Hela Rabbit kidney Caprine, ovine, equine kidney. Rabbit testis. Bovine lymph node. Monkey kidney	Madin <i>et al</i> (1956)
			Schwarz <i>et al</i> (1958)
			Cheathan & Grandell (1957)
			Cabasso <i>et al</i> (1957)
			Plummer (1967)
McKercher (1959)			
MCF	Bovine foetal thyroid. Bovine adrenal. Ovine thyroid. Calf testis. Rabbit, wildebeest kidney	Rouhandeh (1963)	
		Flowright <i>et al</i> (1960)	
Dog	Herpes canis	Canine kidney	Spertzel <i>et al</i> (1965)
Goat	Goat herpes	Bovine, canine, caprine, equine, ovine & rabbit kidney. Canine synovial tissue. Bovine bone marrow. Feline embryo.	Saito <i>et al</i> (1974)
		IBR	As for cattle
	Horse	EHV-1	Very wide range; 17 primary cultures, 14 cell lines
EHV-2			
		EHV-3	Equine kidney, testis Equine embryo fibroblasts
Pseudorabies			
	McFerran <i>et al</i> (1972)		
	Inclusion body rhinitis	Pig lung cells	L'Ecuyer & Corner (1966)
Sheep	Jaagsiekte	Lung macrophages	Mackay (1969)

Presence of herpesviruses in respiratory tract.

The concept that infectious diseases are spread by the dissemination of minute, invisible agents was first clearly expressed, over four hundred years ago, by the Italian physician, Fracastorius (Wright, 1958). Since the acceptance of this idea, many routes of excretion of microbes have been examined. Cruickshank, Duguid, Marmion and Swain (1973) recognised four major routes for dissemination of micro-organisms. These were respiratory and faecal excretion plus venereal and arthropod transmission. In a variety of herpesvirus infections the respiratory route is important. Andrewes and Pereira (1972) suggested that nasal excretion of virus might be involved in the transmission of feline rhinotracheitis (FRV), IBR, canine herpes infection, EHV-1, and Aujeszky's disease in pigs. This list is incomplete; over sixty per cent of the herpesviruses recovered from our domesticated animals have been shown to be present in the nasal cavities (Table III).

TABLE III

HERPESVIRUSES RECORDED FROM NASAL CAVITIES

Host	Disease/Virus	Reference
Cat	FVR	Grandell & Maurer (1958)
Cattle	IBR	Blood & Henderson (1974)
	DN 599	Mohanty <u>et al</u> (1971)
Dog	Herpes canis	Karpas <u>et al</u> (1968)
Goat	Goat herpes	Saito <u>et al</u> (1974)
	IBR	Mohanty <u>et al</u> (1972)
Horse	EHV-1	Bagust (1971)
	EHV-2	Plummer & Waterson (1963)
Pig	Pseudorabies	Jubb & Kennedy (1970)
	Inclusion body rhinitis	Cameron - Stephens (1961)

The association of viruses with leucocytes.

The relationship between viruses and leucocytes has been comprehensively reviewed by Gresser and Lang (1966). Of the veterinary viruses, rinderpest was one of the first to be shown to be present in the blood (Nicholle and Adil-Bey, 1899). These authors had some marked success with their transmission experiments and remarked that "Le sang infecte constamment les sujets sensibles à la dose d'une goutte.". Three years later, Nicholle and Adil-Bey demonstrated that in the infectivity of the blood was associated with the centrifuged blood cells (Nicholle and Adil-Bey, 1902). Leucocytes have since been shown to be involved in the transportation of a variety of veterinary viruses including canine distemper, rinderpest, myxomatosis, fowl plague, MCFV, infectious canine hepatitis (Platt, 1967); EHV-2 (Kemeny and Pearson, 1970) and African swine fever (Lucas et al, 1967). In contrast, is the erythrocytic association of the double stranded RNA viruses of bluetongue and African horse sickness.

Besides the two herpesviruses already mentioned, Kaplan (1973) recorded that four others in this genus (IBRV, EHV-1, PRV and canine herpesvirus) have been shown to be present in the leucocyte fraction of the blood. Downie (1963), when discussing the association between blood and viruses, suggested that "at some stages of the viraemic infection, most of the virus seems to be within leucocytes". In a slightly more cautious vein, Wilson and Miles (1975) commented that during the viraemia "viruses may be present in greater amounts and for longer periods in the leucocytes than in the serum". Up until the early nineteen sixties, informed opinion was fairly uniform in its conviction that neutrophils were not implicated as "virus carriers" (Gresser and Lang, 1966). The work/

work of Sommerville and MacFarlane (1964) was therefore of considerable interest when their immunofluorescent techniques demonstrated a high proportion of neutrophils infected with entero- and adeno-viruses.

Separation of leucocytes.

Researchers over the past fifty years have been attracted to the examination of leucocytes and have devised a range of methods for their collection. Unfortunately, from a veterinary point of view, the anthropocentric bias on disease investigation has tended to produce a mass of literature on the separation of human leucocytes and relatively little on the extraction procedures suitable for other animals.

Generally, leucocyte separation methods from blood can be divided into: lysis of erythrocytes, simple and enhanced sedimentation of blood cells, centrifugation and procedures utilising the phagocytic properties of leucocytes or differences in the specific gravity of cells.

Many agents have been used to lyse erythrocytes. In 1956, Phillip used acetic and tartaric acid mixtures on dog, pig, and lamb blood, with some success. Stewart and Ingram (1967) commented on the use of saponin to lyse canine erythrocytes and Kuper, Bignall and Luckock (1961) favoured its use for human cells. In 1941, Dubos and Hotchkiss described the production and extraction of gramicidin but did not initially detect its haemolytic properties. The haemolysis of rabbit and sheep blood by gramicidin was recorded by Heilmann and Herrel (1941). These researchers continued their investigations on gramicidin and reaffirmed its haemolytic activity on sheep erythrocytes, declaring gramicidin to be more actively haemolytic than tyrothricin (Heilmann and Herrel, 1941). Singer, Silberbach and Schwartz (1947) examined the feasibility of using gramicidin and lysolecithin. Other methods of selective red cell destruction/

ion include ammonium chloride solutions (Dioguardi et al, 1963), phosphoric acid-acetate buffer (Prager et al, 1958), streptolysin-O (Ulrich and Moore, 1966) and anti-red blood cell serum (Sanderson, 1967).

Hypotonic lysis of erythrocytes has been the method of choice of several workers. Some (Fallon et al, 1962; Dain and Hall, 1967) favoured hypotonic saline whilst others (Walford et al, 1957; Odajima and Sonoda, 1970) used distilled water.

Sedimentation can be simple or involve the addition of sediment-enhancing agents. The rate of red cell sedimentation is greatly influenced by the size and character of erythrocyte rouleaux formation (Fahraeus, 1921), the packed cell volume (Schalm et al, 1975a) and the ambient temperature (Wartman, 1946). As far as leucocyte collection is concerned, horse blood is the simplest to handle, since equine erythrocytes agglutinate and settle out spontaneously, leaving leucocyte-rich plasma (Bennet and Cohn, 1966). The high erythrocyte sedimentation rates in horses are followed, in descending order, by those in pigs, cats, dogs and sheep. Since cattle and goat red cells show no tendency to rouleaux formation, no gross sedimentation of erythrocytes can be seen during the standard hour of observation. Cutts (1970a) listed the properties which govern the suitability of compounds for use as sedimenting agents. These include reversible rouleaux-forming ability at suitably innocuous concentrations and the possibility of removal of the agent without damaging the cells. Also mentioned is the need to be able to sterilise the material, preferably by autoclaving.

The sediment-enhancing property of fibrinogen is associated with the long asymmetric shape of the molecule. This material has been used by many workers, including Skoog and Beck (1959) who investigated various concentrations/

concentrations and volume ratios. Biggars and McFeely (1963) commented on the ability of fibrinogen to accelerate the sedimentation of bovine erythrocytes. Dextran has proved to be a popular compound for inducing rouleaux formation (Alexander and Sprigg, 1960; Cline, 1966; Skoog and Beck, 1959). Two stage leucocyte extraction procedures frequently use dextran to produce leucocyte-rich plasma, which is subsequently purified by, for example, hypotonic lysis or saline sedimentation (Fallon et al, 1962; Lapin et al, 1958). Phytohaemagglutinin from red beans (Phaseolus vulgaris) gave satisfactory results in the hands of Li and Osgood (1949) and Prager, Goerner and Matthews (1958). Similarly, polyvinylpyrrolidone (PVP) proved useful when tried by the French researchers Grabar, Seligmann and Bernard in 1955. Gelatin, although it requires to be used fresh and forms a gel at room temperature, is not without its protagon- isis (Coulson and Chalmers, 1964; Sanderson, 1967). Greenwalt and Polka (1960) studied the performance of gum acacia as an erythrocyte sediment- ing agent. In more recent times, Lionetti, Hunt, Gore and Kirby (1975) chose hydroxyethyl starch to aggregate human erythrocytes and concentrate the leucocytes in their cryopreservation experiments.

Several other high molecular weight substances have been used and Cutts (1970b) cites the use of globulin, pectins, polybrene and a number of synthetic polymers. Bøyum (1964) examined four synthetic polymers, methylcellulose, ethylhydroxyethylcellulose, methylhydroxypropylcellulose and hydroxyethylcellulose, and demonstrated their ability to cause clump- ing and erythrocytes. He also examined the use of Isopaque/dextran, Isopaque/Ficoll, Ficoll/EDTA and EDTA/dextran mixtures. A particularly satisfactory result was obtained, however, with methylcellulose/Isopaque mixtures. Phytohaemagglutinin can be usefully employed on human blood but is/

is unsatisfactory with bovine blood (Biggers and McFeely, 1963; Pfeiffer, 1963). Sedimentation techniques were critically evaluated in 1958 by Tullis and Baudanza.

Simple centrifugation in conventional centrifuge tubes is unlikely to produce a layer of leucocytes more than 0.5m.m. thick. The separation in sheep blood is even less marked as a result of the small size of the ovine erythrocytes (Dain and Hall, 1967). Many special centrifuge tubes have been designed and most, like the one used by Wright and Douglas (1903), depended on a constricted portion to facilitate the recovery of the buffy coat. Lindahl (1948), however, described the principle of the counter-streaming centrifuge for the separation of particles of different sizes. The distribution of the layers of cells can be derived from Stoke's law and the streaming velocity.

Vallee, Hughes and Gibson in 1947 used salt-free serum albumin (specific gravity 1.079) to isolate leucocytes from whole blood. The contamination with erythrocytes tended to be rather high and lytic methods had to be applied to remove these cells. More complex density gradients, using two concentrations of albumin were described by Agranoff, Vallee and Waugh (1954). More recently, Bennet and Cohn (1966) used a twenty seven per cent albumin solution to separate equine monocytes. The fractionation of bovine leucocytes has also been carried out, without centrifugation, using dense plasma (Burrin et al, 1966).

The non-specific clumping which occurs during albumin floatation techniques is severe and, according to Walford, Peterson and Doyle (1957), may render the leucocytes unusable. In 1952, Minor and Burnett recommended a two per cent solution of Triton WR-1339 as a means of preventing non-specific clumping. Walford et al (1957) also used this compound with some success. Some years earlier, Spear (1948) introduced floatation/

floatation on gum acacia as an alternative to albumin. Gum acacia is not without its disadvantages. Jago (1956) and Biggers and McFeely (1963) noted problems associated with this compound. These difficulties ranged from adjustment of pH and the initial preparation to the problem of separating the cells from the gum acacia.

Ficoll, a sucrose polymer, has received considerable attention and Betts (1967) quotes seventy per cent recovery rates from the blood of cattle and sheep in experiments performed by Biggers and McFeely (1963). On the other hand, the original publication by these two authors does qualify the results to some extent by commenting that most of the cells recovered were mononuclears and that red cell contamination was fairly high. For the separation of lymphocytes, Ficoll is of considerable value and a one step lymphocyte separation procedure has been described by Harris and Ukaejiofo (1970).

Some fairly bizarre methods of leucocyte recovery have been tried. One of the more intriguing procedures, described by Rous and Beard in 1934, utilised the phagocytic properties of the reticulo-endothelial cells. These experimenters collected Von Kupffer cells, which had ingested ferric oxide particles, using an electromagnet. Almost thirty years later, Kuper et al (1961), in their studies on tumour cells in the blood, used the ability of polymorphs to ingest iron particles.

Sodium bicarbonate	4.4 g.
0.4 per cent phenol red	0.5 ml.
Distilled water	100 ml.

The solution was saturated with carbon dioxide until a neutral pH was reached, then autoclaved, in tightly stoppered containers, at 150 pounds per square inch for fifteen minutes.

Phosphate buffered saline

MATERIALS AND METHOD

REAGENTS

Growth Medium.

The following formula was used in the preparation of growth medium:-

Medium 199 (10 x concentrated)	10 ml.
Foetal calf serum	10 ml.
Sodium bicarbonate solution	1.5 - 2.5 ml.
Sodium benzylpenicillin solution (100,000IU/ml)	0.1 ml.
Streptomycin solution (50,000 ug/ml.)	0.1 ml.
Amphotericin B solution (50 ug/ml.)	0.1 ml.
Deionised distilled water	<u>ad</u> 100 ml.

Maintenance medium.

Maintenance medium was prepared using a modification of the basic formula for growth medium, the foetal calf serum content being reduced to a final concentration of five per cent and the sodium bicarbonate solution increased to 2.5 - 3 ml.. Double quantities of the antibiotic solutions were used in the maintenance medium, i.e. 0.2 ml. of each in 100 ml. medium.

Sodium bicarbonate solution.

Sodium bicarbonate	4.4 g.
0.4 per cent phenol red	0.5 ml.
Distilled water	100 ml.

The solution was saturated with carbon dioxide until a neutral pH was reached, then autoclaved, in tightly stoppered containers, at ten pounds per square inch for fifteen minutes.

Phosphate buffered saline./

Phosphate buffered saline.

Dulbecco A phosphate buffered saline tablets were prepared and solutions sterilised according to the manufacturers instructions.¹

Hanks balanced salt solution (HBSS).¹

Immediately before use the pH was adjusted using 1.4 per cent sodium bicarbonate solution (approximately 2.5 ml. per 100 ml. HBSS) and antibiotics added as for growth medium.

Antibiotic solutions.

These were prepared in bulk, dispensed in ten ml. quantities and stored at -20°C .

Trypsin solution.

A 0.25 per cent trypsin solution was prepared by dissolving 0.5 g. of trypsin in two hundred ml. phosphate buffered saline. Sterilisation was by filtration. Before use, antibiotics were added to the trypsin solution at the rates used in the preparation of the Maintenance medium.

Saline trypsin versene (STV).

Five ml. of one per cent trypsin solution in phosphate buffered saline and a similar volume of one per cent versene in distilled water were made up to 500 ml. with phosphate buffered saline.

CELL CULTURES

Primary rabbit kidney (1^YRK).

Neonatal rabbits, one to four days old, were killed with ether and pinned out on a board. The abdominal skin was swabbed with methylated spirits and flamed. After the abdomen was opened, using sterile forceps and scissors, these instruments were discarded.

¹ Oxoid

The kidneys were detached from the peri-renal fat, using fresh instruments, and transferred to a sterile petri-dish. After flaming the instruments, the renal capsules were removed and the kidneys placed in a second sterile petri-dish. Thin strips of the cortex were carefully dissected and added to fifteen ml. of HBSS, plus antibiotics, in a container at 37°C.. This tissue was washed in the Hank's solution, the fluid decanted and after the addition of a further fifteen ml. of HBSS, chopped finely with scissors. The Hank's solution was again discarded and the tissue fragments added to a flask containing a teflon-coated bar magnet and trypsin solution at 37°C.. Depending on the time available when this stage was reached one or other of the following trypsinisation procedures was adopted:-

A. Warm trypsinisation (37°C.).

Minced kidney cortex was added to fifty ml. of 0.25 per cent trypsin solution and agitated for ten minutes at 37°C. on a magnetic stirrer. The trypsin was discarded and a further fifty ml. of trypsin added. After thirty to sixty minutes of stirring the trypsin-cell suspension was carefully decanted. Trypsinisation was repeated until no kidney tissue remained.

B. Cold trypsinisation (4°C.).

The minced kidney cortex was added to 100 ml. of 0.25 per cent trypsin solution at 37°C. and agitated by a magnetic stirrer for fifteen minutes at room temperature. After allowing the tissue fragments to settle and discarding the supernatant fluid, 100 ml. of fresh trypsin at room temperature was added. The flask was then placed on the pre-cooled stage of a magnetic stirrer¹ set to maintain the/

¹ Fryka - Kalttechnik

the contents at four degrees centigrade. Trypsinisation was continued for approximately eighteen hours before the trypsin-cell suspension was collected. Any tissue fragments remaining after this period were subjected to the action of fresh trypsin solution for a further twenty minutes at room temperature.

Decanted cell suspensions, from either of the above procedures, were centrifuged at 150 G. for five minutes and the supernatant fluids discarded. The cells were resuspended in HBSS and the samples pooled. Finally the cells were spun down and resuspended in growth medium in the ratio of one ml. packed cells to 100 ml. growth medium. Cells were counted in an improved Neubauer haemocytometer and cell concentrations adjusted to three million per ml.. Plastic tissue culture tubes¹, Leighton tubes and glass test tubes were seeded with one ml. of cell suspension; 25cm.² plastic flasks¹ received five ml.. All cultures were incubated at 37°C for 48 hours before decanting the growth medium and replacing it with an equal volume of maintenance medium. Confluent monolayers were normally obtained within 48-72 hours. Changes of medium were indicated by alteration of the pH and were carried out as necessary.

Secondary Rabbit Kidney.

After decanting the medium, the primary monolayers were washed twice with PBS at 37°C and the culture vessels returned to the incubator. Inspection of the cultures was carried out every five minutes, with agitation on each occasion, until the majority of the cells appeared to have detached from the vessel walls.

Dispersal/

¹ Nunc UK Ltd.

Dispersal of any clumps or "flakes" of cell was achieved by pipetting the suspension vigorously. Cells were harvested by centrifuging the fluid at 150G for 5-10 minutes and resuspending the sedimented cells in growth medium. The concentration of cells was adjusted, after counting, to one million cells per ml. growth medium and culture vessels seeded as for primary cultures. Confluent monolayers were normally obtained within three days but higher concentrations of cells could be used to give complete sheets in one to two days.

Rabbit Kidney Cell Line (RK 13).

The RK 13 cell line was propagated in 20 oz. flat bottles using culture medium prepared according to the formulae previously described. These stock cultures were split 1:2, using STV solution when dense monolayers were complete. For seeding Leighton tubes, test tubes and 25cm² flasks cell suspensions containing 400,000 cells per ml. were prepared. The frequency of media changes was governed by the alteration of medium colour as a result of falling pH.

Foetal Lamb Kidney (F.L.K.).

Primary foetal lamb kidney cultures were obtained weekly in Roux flasks from the Moredun Institute. Once confluent monolayers were established, the cells were dispersed using STV and cell suspensions containing four to five hundred thousand cells per ml. of growth medium were prepared. These cells were handled in a similar manner to the primary rabbit kidney and RK 13 cell suspensions. Confluent secondary monolayers were normally obtained in 48-72 hours.

TECHNIQUES

A. SamplesDonors in phases I and II

All the sheep examined in this investigation were present on the same farm and were classified into January or April-born lambs. In phase I, twenty January-born Suffolk lambs, varying in age from three to eight weeks, were examined, blood and nasal samples being collected from each. Cheviot lambs constituted the majority of those examined in phase II, with the remainder being Suffolk or Cheviot crosses. Since swabbing and bleeding were not synchronised, a total of 130 animals were examined in phase II. The ages of these lambs varied from two weeks to three months. A total of 150 lambs were sampled by nasal swab or by bleeding or by both procedures, fifty lambs being present in each group (Table IV).

TABLE IV

LAMBS AND SAMPLES

Blood sample only	Nasal swabs only	Both blood and nasal samples
50 April lambs	20 January lambs	20 January lambs
	30 April lambs	30 April lambs

Nasal swabs

Sterile culture swabs¹, with an absorbant cotton bud, of approximately six to seven mm. maximum diameter, were used. The swabs were passed through the anterior nares, care being taken to avoid contact with the skin surrounding the nostrils. Once located in the ventral meatus/

¹ Stayne Laboratories

meatus, the swabs were rotated gently and removed. On withdrawal from the nasal cavity, the bud and approximately one cm. of the applicator stick was snipped off and immersed in three ml. of growth medium, contained in a sterile bottle. Both nasal cavities of 100 lambs were sampled.

Blood samples.

Blood was collected from the jugular veins into seven ml. heparinised (143 USP units per tube) evacuated glass tubes.¹ Immediately after filling, each tube was inverted gently several times to ensure thorough mixing of the blood and anticoagulant. As detailed in Table IV, blood was collected from 100 lambs.

B. Handling and Examination of Samples

Nasal swabs.

The bottles containing the swabs were agitated by hand to encourage any debris adhering to the swabs to free itself in the medium. Rough agitation was avoided, since this caused disintegration of the swab bud. After removal of the cotton buds, the contents of the bottle were centrifuged at 90G for five minutes in order to sediment the heavier debris cells. If any delay was inevitable before inoculation of the cell culture the centrifuged samples were held at 4°C. until required.

Preparation of ovine leucocyte suspensions.

Initial processing of all the blood samples was commenced within two hours of collection. Between collection and processing, the blood samples were maintained at room temperature. The procedure adopted for the separation of the leucocytes was as follows:-

¹ Becton Dickinson & Co.

Five ml. of the heparinised blood was transferred to a flat-bottomed glass bottle into which 7.5 ml. of sterile distilled water had previously been dispensed. The blood-water mixture was shaken gently to ensure thorough mixing. Exactly twenty seconds after the addition of the blood to the water, 1.5 ml. of a 5.4 per cent sterile saline solution was added to restore the isotonicity of the mixture. The haemolysate was then centrifuged at 700G for five minutes to sediment the leucocytes. After removal of the supernatant fluid, the leucocytes were resuspended in growth medium and recentrifuged at 400G for five minutes. The supernatant fluid was again discarded. These "washed" leucocytes were then suspended once again in growth medium. The volume of growth medium added was adjusted, according to the percentage leucocyte recovery rate, to give approximately a two-fold concentration of white cells, per unit volume, compared to the original blood sample. A portion was then subjected to a two-fold dilution procedure, designed to produce a white cell dilution of approximately 10^{-2} of that found in the original sample. In an attempt to determine whether "non-wettable" surfaces offered any significant advantages in the extraction procedure, fifty per cent of the containers were coated internally with a one per cent silicone solution.¹

Leucocyte viability.

The trypan blue exclusion test was used to assess the viability of the recovered leucocytes. A 0.1 per cent solution of trypan blue in normal saline was prepared. Equal volumes of the trypan blue solution and the two times concentrated leucocyte suspensions were mixed and allowed to stand for five minutes to permit the penetration of/

¹ Siliclad, Clay Adams

of the stain into the "dead" cells. The mixture was then treated as normal blood for the purposes of counting in the haemocytometer. The ratio of stained to unstained cells was recorded.

Virus isolation and culture.

Three day cultures in test tubes containing flying coverslips were used for primary isolation attempts. Before inoculation the cultures were screened for uniformity, any tubes showing discrepancies in pH being discarded. A few randomly selected tubes were also examined microscopically. If any abnormal features were observed all tubes were examined under the microscope before inoculation. In the preliminary phase and phase I of the survey, four cultures were inoculated with each prepared sample. A single culture for each prepared sample was used in phase II. The volume of the samples used to seed the cultures varied with the size of the culture vessels, being 0.1 ml. for test tubes and 0.3 ml. for 25 cm.² flasks. The culture medium was removed before the inoculum was added. A period of 45 to 60 minutes incubation at 37°C. was allowed for absorption before maintenance medium was returned to the culture vessels. Incubation, at 37°C., was continued and medium changes were made when necessary to restore altered pH. The cultures were observed daily for evidence of cytopathic effect. The details of the cells used and samples added are summarised in Table V.

Virus passage.

Routine blind passage of cultures was carried out in phase I whereas in the preliminary phase and phase II only those cultures showing cytopathic effect or considered, for some other reason, to contain/

TABLE V
SUMMARY OF CELL TYPES AND SAMPLES

Cell type	Preliminary Phase			Phase I	Phase II
	I ^v RK	RK13	FLK	FLK	FLK
No. of cultures per sample	4	4	4	4	1
EHV - 2 propagated	+	+	+	-	-
Ovine leucocytes	+	+	+	+	+
2 x concentrated	-	-	-	+	+
10 ⁻² x concentrated	-	-	-	-	-
Ovine nasal samples	-	-	-	+	+
Deposit	+	+	+	+	+
Supernatant	-	-	-	-	-
Vol. of inoculum (in ml.)	0.1	0.1	0.1	0.1	0.1
per tube	0.3	0.3	0.3	-	-
per 25 cm ² flask	/*	/*	/*	3	as considered necessary

/* = variable

contain virus were subcultured. The cell sheets were detached from the vessel walls with a flame sterilised wire hoop. Appropriate volumes (0.1 ml. for tubes, 0.3 ml. for 25 cm.² flasks) of the resulting cell suspensions were inoculated into fresh cultures of the same cell type. As was practiced for primary isolation, the maintenance medium was removed before adding the inoculum and 45 to 60 minutes allowed for absorption. Incubation was continued under the same conditions.

Propagation of EHV-2.

Aliquots of EHV-2, previously isolated in rabbit kidney cell cultures and stored in the vapour phase of liquid nitrogen (Roeder 1974), were thawed at room temperature. The virus was inoculated into 1^VRK, RK-13 and FLK. Inoculation dosages and cultures used are summarised in Table V. The procedure for inoculation and subsequent handling of the cultures is described under "Virus isolation and culture".

Light microscopy.

Besides the daily examination of cell cultures under the inverted microscope, coverslips were removed as required and fixed in methanol for 2-3 minutes. The coverslips were then immersed, for three minutes, in Erlich's haematoxylin and rinsed in distilled water. "Blue-ing" was carried out in distilled water to which a little saturated lithium carbonate had been added. After rinsing in distilled water, staining in one per cent eosin for five minutes was followed by a further rinse in distilled water. Differentiation in 95 per cent alcohol had to be performed very rapidly to avoid removal of excessive amounts of stain. The stained cell sheets were then dehydrated in absolute alcohol, cleared in xylol and mounted in D.P.X..

Fluorescent microscopy.

Flying coverslips were stained by the method described by

Hsiung/

Hsiung (1969) and examined under darkground illumination. All reagents were prepared according to the formulae provided by Hsiung (1969) and the recommendation that 0.002M $MgSO_4$ be added to every solution was followed. The stained coverslips were mounted in phosphate buffer (containing 0.067M Na_2HPO_4 and KH_2PO_4) on 0.8-1.0 mm. thick glass slides.

Immunosuppression.

A three and a half month-old crossbred blackface lamb of twenty five kilograms body weight was immunosuppressed with betamethasone.¹ This corticosteroid was administered at the rate of one mg. per kilogram body weight for five days. On the first day of treatment half of the calculated dose was given intramuscularly and the other half intravenously. The full dosage was injected intravenously on each of the following four days. A temperature record of the lamb was maintained along with daily total and differential white cell counts. Seven days after the commencement of treatment nasal swabs were collected.

Electron microscopy.

The nasal swabs collected from the immunosuppressed lamb were agitated in three ml. growth medium to encourage detachment of any adhering material. The cotton buds were removed from the growth medium and discarded. Vigorous pipetting of the medium caused any clumps or masses of nasal secretion to be broken up and to become dispersed. Centrifugation at low speed (40G) for five minutes sedimented the coarse debris, which consisted mainly of cotton fibres from the swab bud. The supernatant fluid was centrifuged at 30,000G for thirty minutes, to pellet any virus present. The pellets were resuspended in a small/

¹ Betsolan Soluble, Glaxo.

a small quantity of growth medium and transferred to a shallow sterile container. Carbon-coated copper grids were floated on the suspensions for thirty seconds and excess fluid was removed by touching the grid edge on a filter paper. Staining was accomplished by floating the grids on phosphotungstic acid solution (PTA) pH 7.7 for thirty, forty-five or sixty second periods. The excess PTA was removed with filter paper.

SURVEY

Preliminary phase.

To assess different techniques for the preparation of cell cultures, leucocyte suspensions, nasal samples, virus isolation and staining methods, an initial investigation was undertaken. Fifteen sheep (ten adults, five lambs) were sampled and EHV-2 passaged. This trial enabled standard procedures to be evolved for use in phases I and II.

Phase I.

Twenty Suffolk lambs varying in age from three to eight weeks were blood sampled and nasal swabbed. Three passages on each sample were carried out routinely.

Phase II.

The final phase of the investigation involved 130 lambs, varying in age from two weeks to three months. The majority were Cheviots but a small number of Suffolk and Cheviot crossbred lambs were also included. Nasal swabs, only, were collected from twenty January born lambs and thirty April born lambs. Fifty April born lambs donated only blood samples. A further thirty April lambs gave nasal and blood samples./

samples. Following preparation of the collected material, one cell culture vessel was inoculated with each sample. Routine blind passage was not undertaken.

The details of the cell cultures and inocula used in the three phases are summarised in Table V.

$$\frac{\text{WBC count per cmm. of processed blood}}{\text{WBC count per cmm. of blood sample}} \times 100$$

The efficacies of two sediment-enhancing agents were assessed in the preliminary phase. Ovine blood was layered on to solutions of six per cent dextran and three per cent gelatin. Five ratios of agent to blood were used:-

2.0:1, 1.5:1, 1.0:1, 0.66:1, 0.5:1.

Neither sediment-enhancing agent induced marked separation. Leucocyte recovery rates were very low and red cell contamination high.

The mean and standard deviation of the leucocyte recovery rates for thirty six samples in non-silicified and a similar number in silicified containers was 46.7 ± 12.8 per cent and 54.8 ± 12.2 per cent, respectively. The difference was not significant ($t(72) = 0.645$ $P > 0.50$). Figures I and II show the distribution of the recovery rates for samples in non-silicified and silicified bottles, respectively. Eleven per cent of the samples, in both cases, had recovery rates of less than fifty per cent. Twenty nine of the thirty six samples in non-silicified bottles and thirty one of those in silicified vessels had recovery rates of between fifty and eighty five per cent. Three blood samples in plain glass bottles and one sample in a silicone treated container gave leucocyte recovery rates greater than eighty five per cent. Stratification of

RESULTS

Leucocyte separation.

The leucocyte recovery rate was defined as the number of leucocyte present in a specified volume of the processed sample, as compared to the number present in a similar volume of the original blood sample, expressed as a percentage; the relevant formula being:-

$$\frac{\text{WBC count per cmm. of processed blood}}{\text{WBC count per cmm. of blood sample}} \times \frac{100}{1} .$$

The efficacies of two sediment-enhancing agents were assessed in the preliminary phase. Ovine blood was layered on to solutions of six per cent dextran and three per cent gelatin. Five ratios of agent to blood were used:-

2.0:1 , 1.5:1 , 1.0:1 , 0.66:1 , 0.5:1 .

Neither sediment-enhancing agent induced marked separation. Leucocyte recovery rates were very low and red cell contamination high.

The mean and standard deviation of the leucocyte recovery rates for thirty six samples in non-siliconised and a similar number in siliconised containers was 66.7 ± 12.8 per cent and 64.8 ± 12.2 per cent, respectively. The difference was not significant ($t_{(74)} = 0.645$ $P > 0.50$). Figures I and II show the distribution of the recovery rates for samples in non-siliconised and siliconised bottles, respectively. Eleven per cent of the samples, in both cases, had recovery rates of less than fifty per cent. Twenty nine of the thirty six samples in non-siliconised bottles and thirty one of those in siliconised vessels had recovery rates of between fifty and eighty five per cent. Three blood samples in plain glass bottles and one sample in a silicone treated container gave leucocyte recovery rates greater than eighty five per cent. Stratification of/

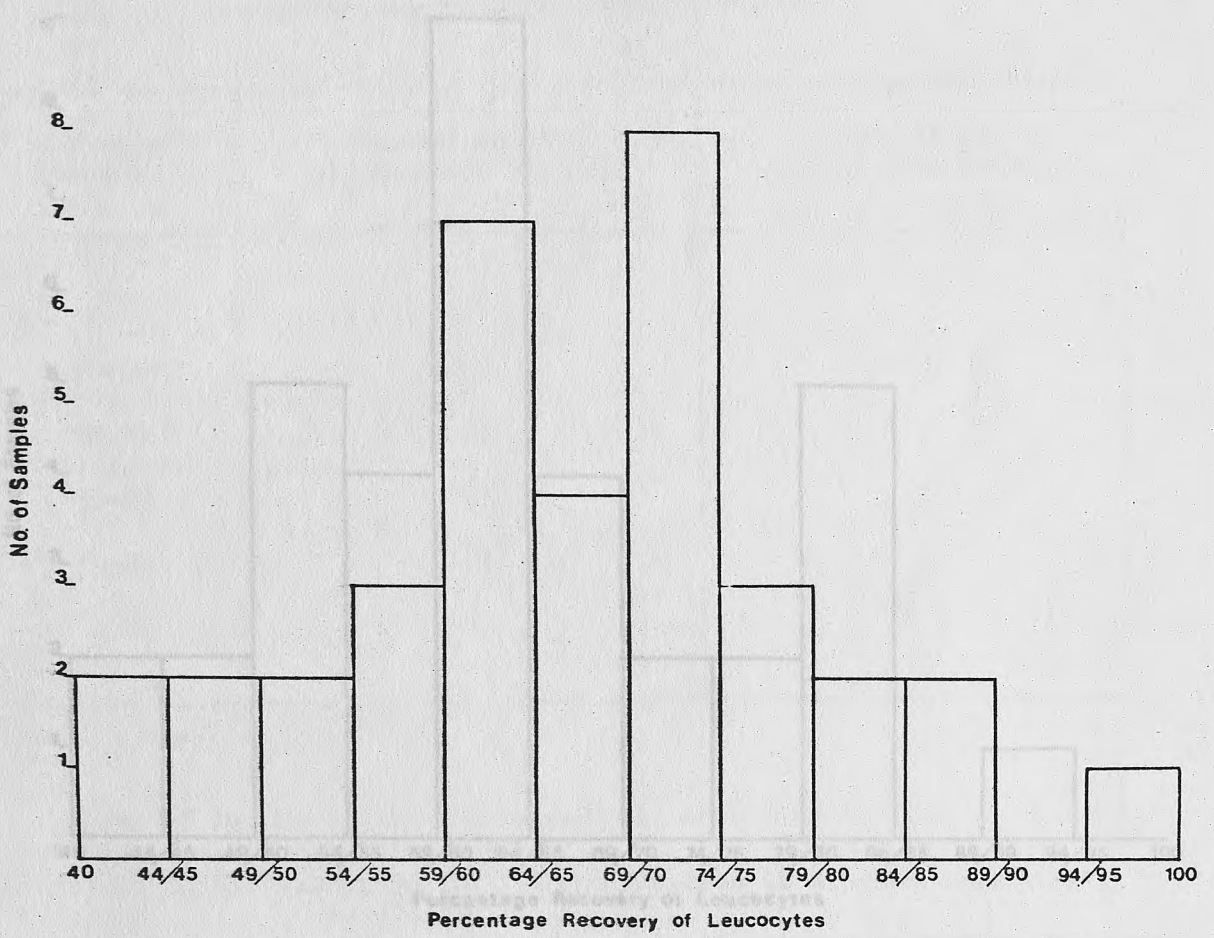


Figure I

Histogram of distribution of leucocyte recovery rates

Histogram of distribution of leucocyte recovery rates
using non-siliconised containers.

of the recovery rates emphasized the similarity of results from non-siliconised and siliconised bottles (Table VI).

TABLE VI

COMPARISON OF LEUCOCYTE RECOVERY RATES

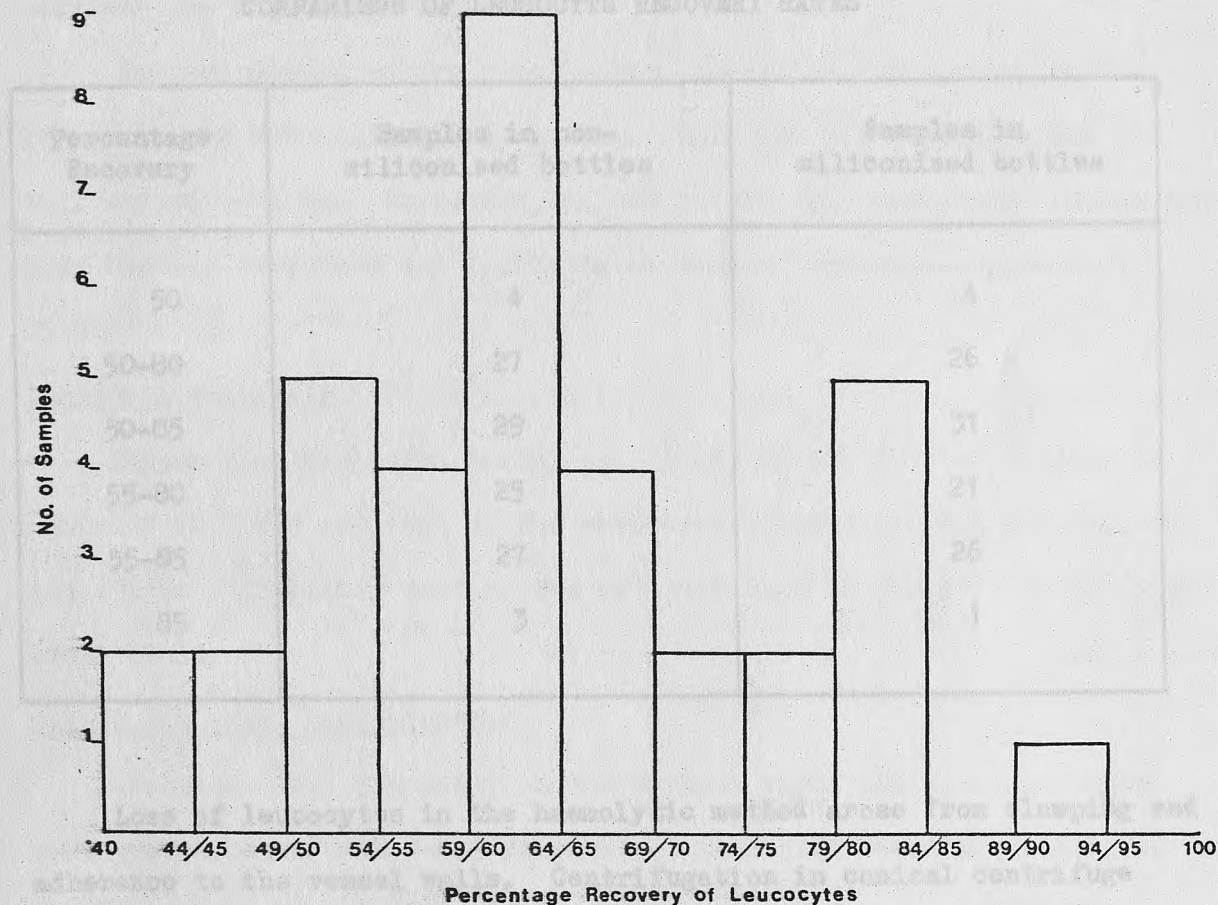


Figure II

Histogram of distribution of leucocyte recovery rates using siliconised containers.

However, the cells were difficult to remove from the surfaces of the bottles. Flat-bottomed glass containers proved to be more satisfactory, with less clumping or adherence to the walls. Leucocytes were easily detached from the surfaces of the siliconised vessels.

Before/

of the recovery rates emphasised the similarity of results from non-siliconised and siliconised bottles (Table VI).

TABLE VI

COMPARISON OF LEUCOCYTE RECOVERY RATES

Percentage Recovery	Samples in non-siliconised bottles	Samples in siliconised bottles
50	4	4
50-80	27	26
50-85	29	31
55-80	25	21
55-85	27	26
85	3	1

Loss of leucocytes in the haemolytic method arose from clumping and adherence to the vessel walls. Centrifugation in conical centrifuge tubes caused packing of the white blood cells and the formation of large clumps. Dispersal of the clumps required prolonged agitation or pipetting. Leucocytes deposited, by centrifugation, in thirty ml. plastic bottles with slightly conical bottoms, formed fewer aggregations. However, the cells were difficult to remove from the surfaces of the bottles. Flat-bottomed glass containers proved to be more satisfactory, with less clumping or adherence to the walls. Leucocytes were easily detached from the surfaces of the siliconised vessels.

Before/

Before total white cell counts were made, in the electronic cell counter, all macroscopic clumps were dispersed by gentle pipetting. This did not disrupt all the microscopic aggregations and these were counted as one cell. Consequently, the cell counter tended to underestimate the numbers present.

During removal of the haemolysate, the white cells stuck to the bottle in the non-siliconised vessels. This did not happen in the siliconised bottles. Therefore, in the latter, the sedimented leucocytes were readily disturbed and liable to be removed with the supernatant fluid.

Leucocyte viability

Trypan blue exclusion tests carried out on the treated leucocytes revealed 84 ± 2.2 per cent of the recovered white cells did not take up the stain. Viability testing was not performed on the leucocytes in the fresh blood.

Fungal and yeast contamination

Fourteen (2.1 per cent) of the primary virus isolation attempts were contaminated with fungi or yeasts. Table VII details the samples which were associated with fungal or yeast contamination during the first passage in culture.

Table VII/

TABLE VII
CONTAMINATION OF CULTURES BY YEASTS AND FUNGI

	Nasal swabs		Leucocytes		Total
	Super-natant	Deposit	2 x conc	10^{-2} Dil	
Fungus	1	5	1	0	7
Yeast	1	1	5	0	7

No monolayers were lost as a result of contamination, the threat being removed by changing the culture medium.

Virus isolation.

In phase I, 320 primary isolation attempts were made (twenty animals, four prepared samples per animal, four cultures per sample). Each culture was subsequently subcultured on two occasions. No virus was isolated.

One hundred and sixty cultures were seeded with material from the nasal swabs from eighty animals. A further 160 cultures received leucocyte suspensions from eighty lambs. In neither of these parts of phase II was any virus detected.

Propagation of EHV-2 in FLK.

EHV-2 was cultivated without difficulty in FLK cells. Times to inception of cytopathic effect varied from five to eight days on first passage. By the third subculture, cytopathic effects were observable three to five days after inoculation.

The/

The non-infected monolayers of FLK were composed of uniform fibroblasts (Fig. III). The early cytopathic effect was recognised, in unstained preparations, by an increasing cytoplasmic granulation and the appearance of rounded refractile cells (Fig. IV). Discrete circular foci developed with three zones; an outer granular band surrounded a zone of refractile cells which formed a rim of varying thickness round a clear central region.

Acridine orange staining of infected monolayers consistently demonstrated altered staining reactions in the cells surrounding a focus of degeneration. The band affected was up to ten cells wide and showed increased orange fluorescence from the cytoplasm (Fig. V). The nuclear fluorescence changed from green to yellow. Multiple indentation of cell boundaries was detected in preparations stained with acridine orange (Fig. VI) but not with haematoxylin and eosin. Cells showing this change were situated at the periphery of foci of degeneration.

Syncytium formation was not uncommon and degenerative changes in syncytia were characteristic. The nuclei appeared amorphous, less clearly separated and gave yellow fluorescence if stained by acridine orange. The cytoplasm fluoresced green and the boundary was hazy. (Fig. VIII).

Figures IX and X show the intranuclear inclusion bodies formed in FLK cells infected with EHV-2. Early inclusion bodies were more easily recognised when stained by haematoxylin and eosin, Cowdry type A inclusions being typical.

Electron microscopy.

Virus particles were not observed by electron microscopy in preparations of nasal swabs collected from the immunosuppressed sheep.

Figure III.

Foetal lamb kidney

Normal secondary cultures. Magnification x 250.

Figure IV.

Foetal lamb kidney.

Early cytopathic effect caused by EHV-2.

Magnification x 250.

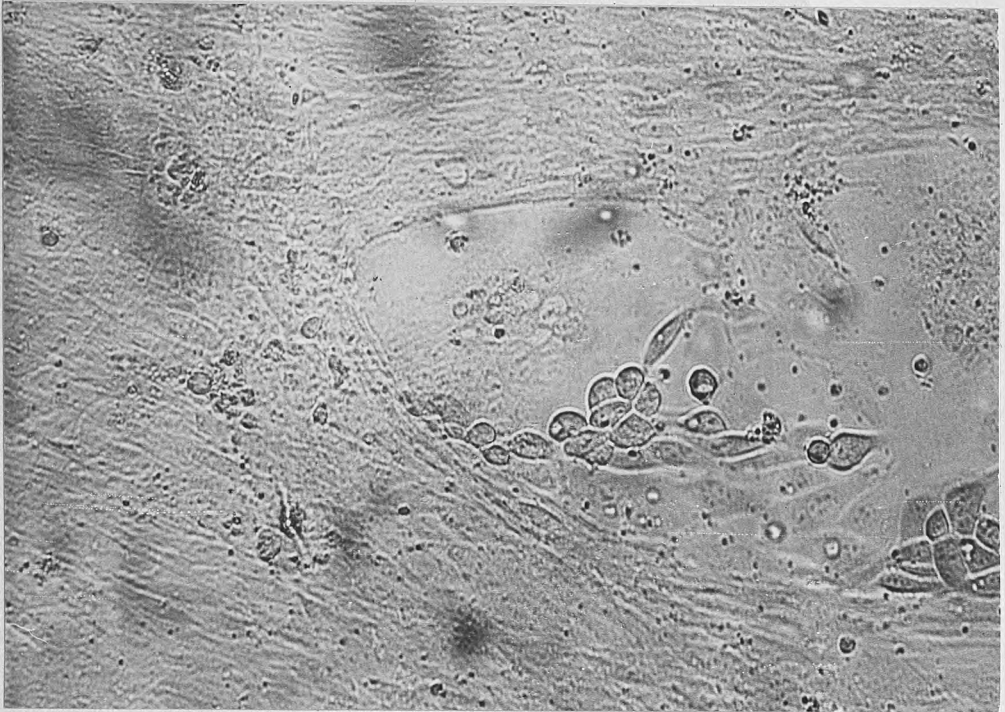
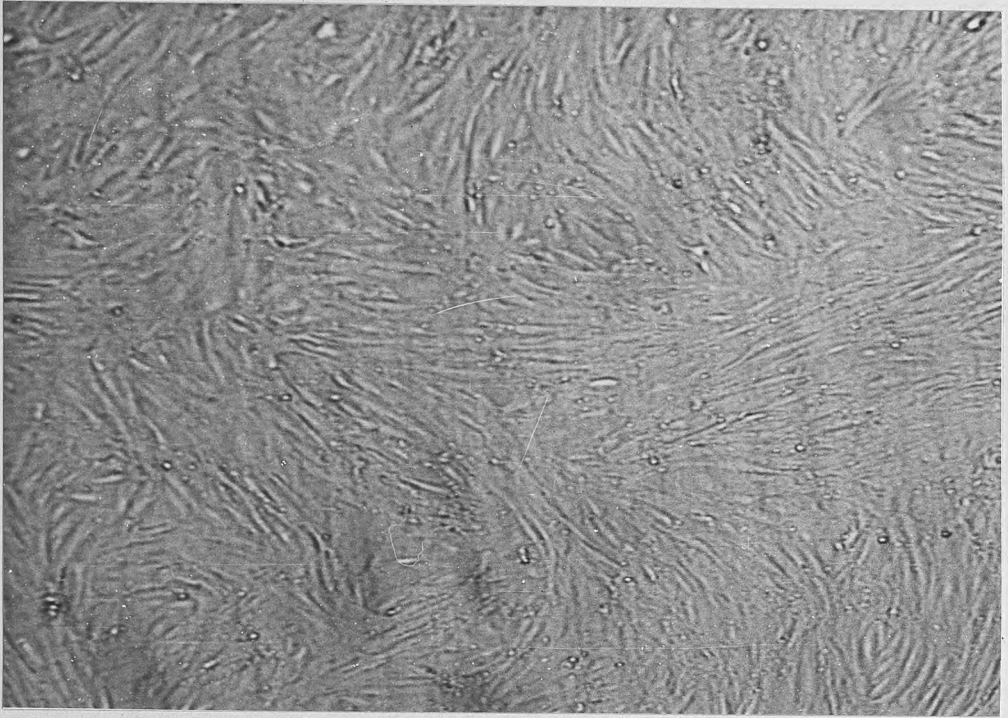


Figure V.

Foetal lamb kidney.

Differentiation of affected and apparently normal cells. Degenerating cells show more intense orange fluorescence of the cytoplasm and decreased green fluorescence of the nuclei. Acridine orange staining. Magnification x 350.

Figure VI.

Foetal lamb kidney.

Multiple indentation of the cell boundaries heralds cell separation and the appearance of typical cytopathic effect. Acridine orange staining. Magnification x 700.

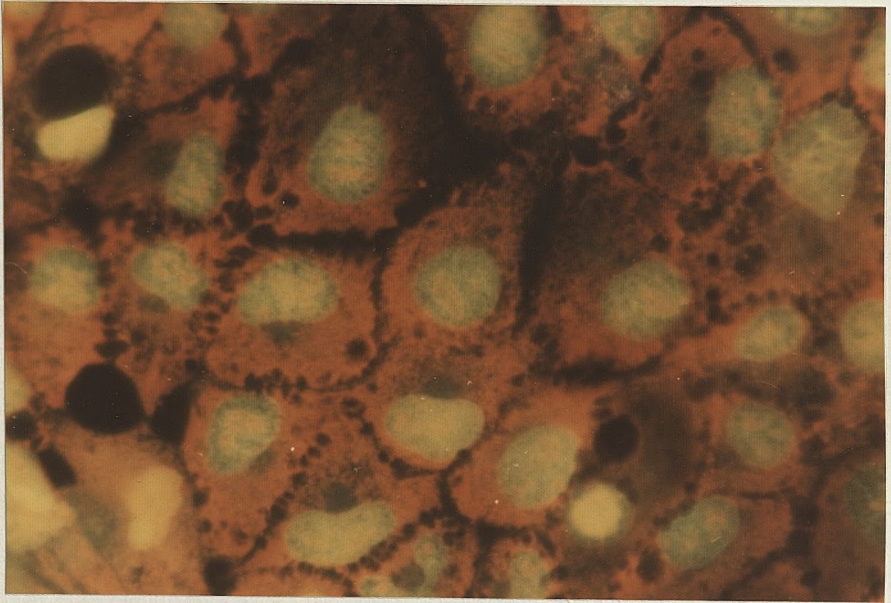
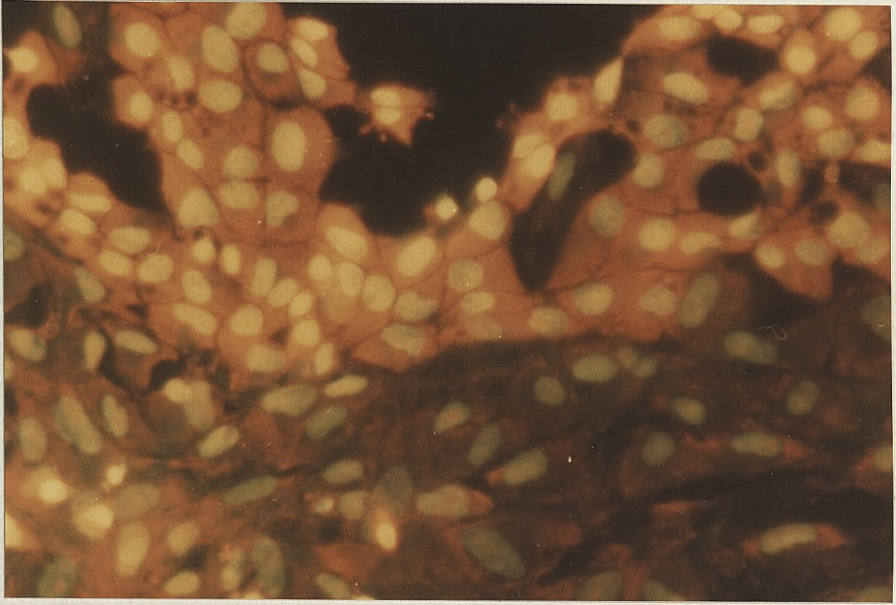


Figure VII.

Foetal lamb kidney.

A syncytium.

Acridine orange staining. Magnification x 350.

Figure VIII.

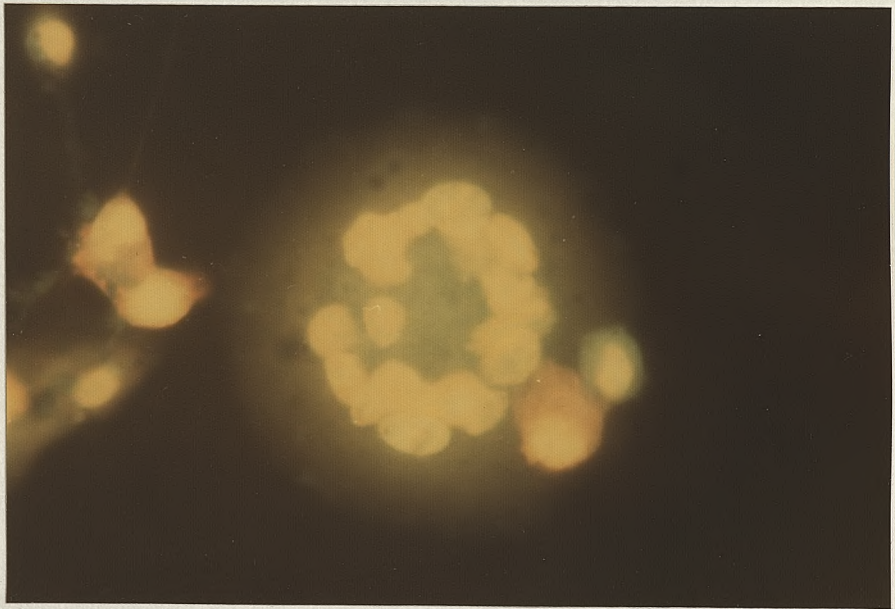
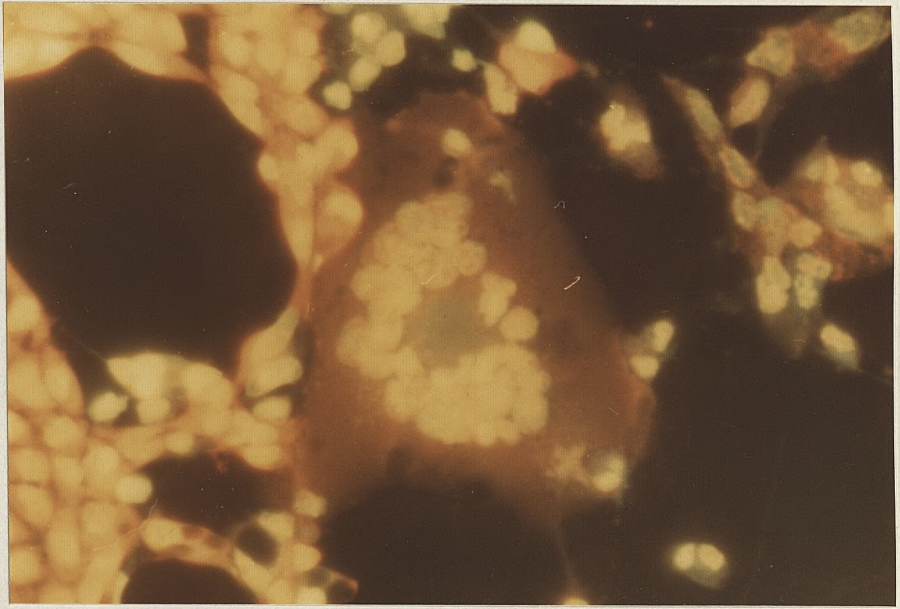
Foetal lamb kidney.

A syncytium showing signs of advanced degeneration.

The nuclei appear amorphous and less clearly separated.

Note, also, the change from orange to green fluorescence of the cytoplasm.

Acridine orange staining. Magnification x 700.



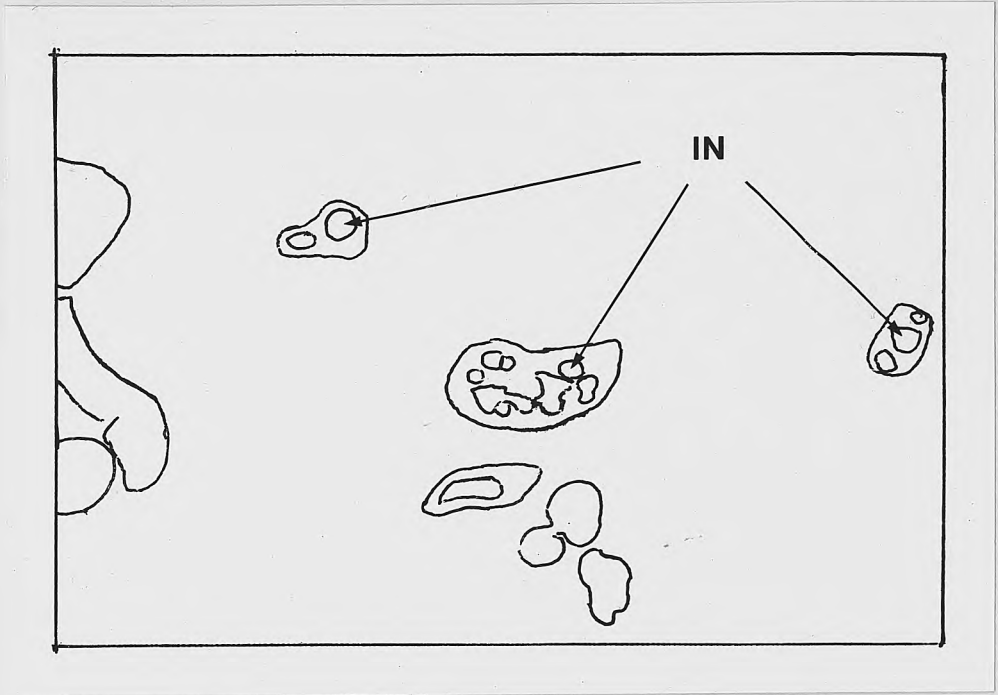


Figure IX.

Foetal lamb kidney.

Intermediate stage intranuclear inclusion bodies (IN).

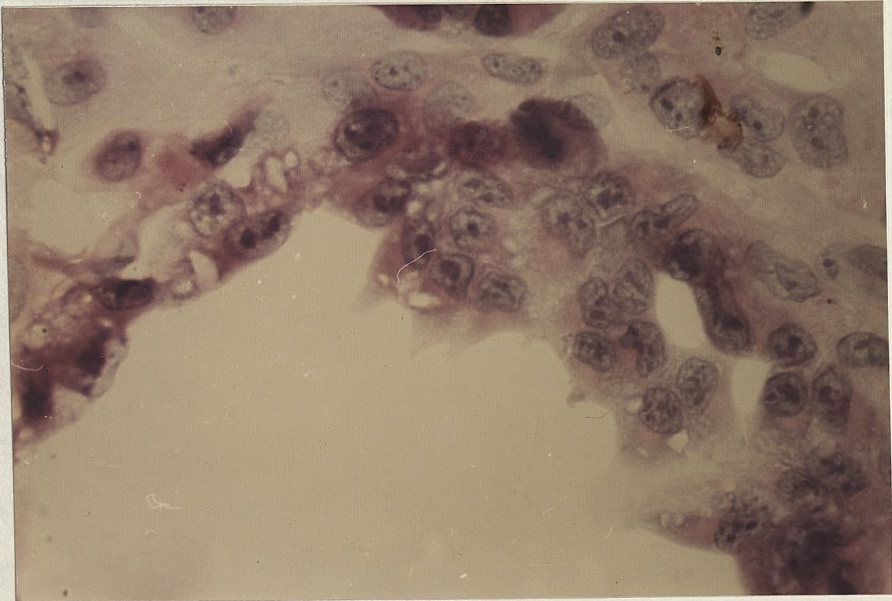
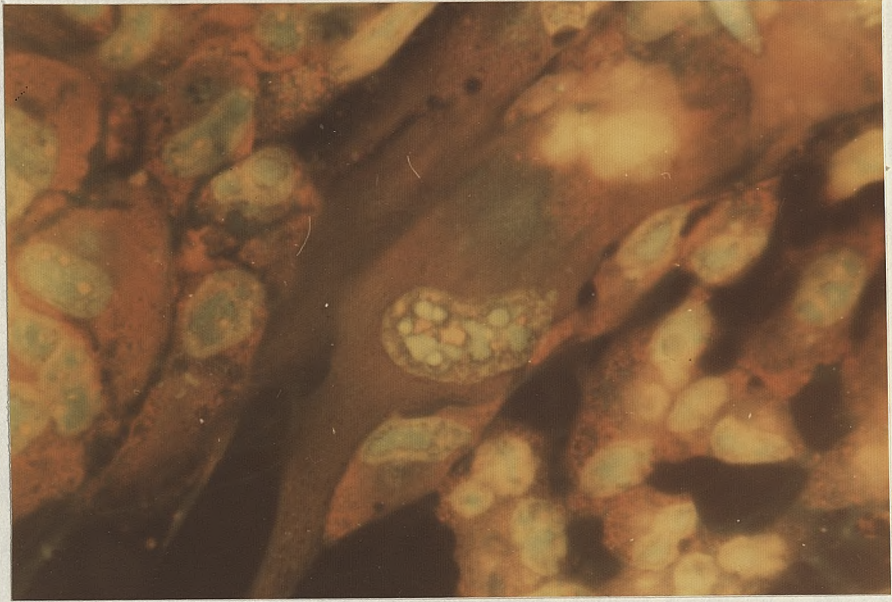
Acridine orange staining. Magnification x 700.

Figure X.

Foetal lamb kidney.

Intermediate and mature intranuclear inclusion bodies.

Haematoxylin and eosin staining. Magnification x 437.



DISCUSSION

Interpretation of the significance of micro-organisms recovered from clinical material depends on a knowledge of which microbes are present in the healthy animal. The lack of such information reduces confidence in the validity of the diagnosis. The results of this survey, therefore, provide basic information against which future herpesvirus isolations from lambs can be assessed.

Virus isolation is, as yet, an imperfect art and the findings of this investigation should be viewed in perspective. Hore (1968) examined nasal swabs from lambs, in eastern Scotland, affected with an upper respiratory tract infection. From eighty-nine lambs sampled, thirteen virus isolations were made. Consequently, it was not unexpected that no virus was recovered from one hundred and fifty healthy lambs in the present study.

Failure to recover virus can be explained in several ways. Firstly, the anterior nasal cavities and bloods of the lambs may have been virologically sterile. Alternatively, the sampling techniques may not have been sufficiently refined to "pick up" very low levels of virus. A third possibility is that viable virus was present but the cell culture system was unsuitable. Finally, since the detection of virus was based on the appearance of cytopathic effect, non-cytopathogenic viruses could have been overlooked.

Although dexamethasone and dexamethasone-prednisolone mixtures are less potent than betamethasone (Anon, 1965), the immunosuppressive activity of these compounds was sufficient to induce recrudescence of herpesvirus infections (Dennett et al, 1973; Gaskell and Povey, 1973; Davies and Duncan, 1974; Darcel and Dorward, 1975, Gibbs et al, 1975). Consequently, /

Consequently, the evidence gathered from the immunosuppressed sheep would suggest that little, if any, virus was present in the nasal cavities of the sheep on this farm. The use of nasal swabs is considered appropriate for upper respiratory tract infections (Fenner and White, 1970a) and is of proven efficacy in the detection of respiratory viruses in sheep (Hore, 1968). Nevertheless, it is not claimed that nasal swabbing is ideal. Nasal washings have been favoured by some investigators for the recovery of respiratory viruses, including herpesviruses (Rose et al, 1974). This procedure was assessed during the preliminary phase of the investigation. The results were disappointing, with the sheep sneezing out or swallowing the bulk of the fluid.

The cell-associated nature of some members of the herpesvirus group influenced the handling of the nasal swabs. Centrifugation deposited any cellular material collected from the nasal cavities. It was then possible to culture the deposit for cell-associated virus and the supernatant fluid for "free" virus.

A feature which influenced all stages of the investigation was the lack of detailed knowledge of the properties likely to be possessed by an ovine herpesvirus. Documentation of the properties of the herpesviruses is uneven, with a considerable volume of literature being available on certain viruses. Most others in the group have not received similar attention. This unevenness is only too apparent in the section by Kaplan, (1973) on the resistance of herpesviruses to inactivation. Herpes simplex virus (HSV) is discussed at some length, under various headings, with only occasional reference being made to other herpesviruses. It was imperative, therefore, that a leucocyte collection procedure was evolved which required the addition of minimal quantities of/

of potentially noxious chemicals.

There is a dearth of information available on satisfactory methods for the collection of ovine leucocytes. Slightly more experimentation has been carried out on bovine blood. The relative similarity in the problems faced during the extraction of leucocytes in these two species, permits tentative extrapolation of results from bovine to ovine blood. Although sheep erythrocytes have a greater tendency to rouleaux formation than bovine red cells, the erythrocyte sedimentation rate of ovine blood is only 3.0 to 8.25 mm. over twenty four hours (Bunce, 1954). Simple sedimentation is therefore valueless as an aid to leucocyte concentration. Enhanced sedimentation is a useful procedure with human blood and has been used in other species. Bovine erythrocytes can be sedimented by fibrinogen. However, the high concentrations of fibrinogen required lead to problems of filtration and, consequently, of sterility (Biggers and McFeely, 1963). The other commonly used sediment-enhancing agents have been shown to be inefficient in inducing rouleaux formation of bovine erythrocytes. Experiments on ovine blood, during the preliminary phase, confirmed the unsuitability of dextran and gelatin sedimentation techniques.

Red cell contamination of the leucocyte fraction was high in albumin flotation methods (Vallee *et al*, 1948). A similar situation was encountered by Burrin *et al*, (1966) during the separation of bovine leucocytes, using plasma at specific gravity 1.06 to 1.07. Ficoll flotation has proved its value under a number of circumstances but, in the light of the findings of Sommerville and MacFarlane, (1964), was considered unsuitable for this study. These authors stressed the possible importance of neutrophils in the transportation of virus. Ficoll, /

Ficoll, on the other hand, favours the concentration of monocytes with a low recovery of granulocytes. Biggers and McFeely (1963) also report moderately high red cell contamination of bovine and ovine leucocyte fractions following Ficoll flotation.

Many methods for the collection of leucocytes involve selective destruction of red blood cells. The unknown fragility of any virus present necessitated caution in selection of a method requiring the addition of unusual chemicals. Although successful in recovering leucocytes from lambs' blood, the technique of Phillipu (1956) required the use of acetic and tartaric acids and the restoration of pH by the addition of potassium hydroxide. Sheep blood has also been lysed using gramicidin but Singer et al (1947) stressed the difficulty in removing all traces of this compound from the leucocytes after collection. None of the haemolytic methods is ideal and Chen and Palmer (1958) considered that all haemolytic methods altered morphologic and physiologic characteristics of leucocytes. Methods describing the use of streptolysin-0 were criticised by Kuper et al (1961) and those using saponin by Walford et al (1957). According to Dioguardi et al (1963), the ammonium chloride technique favoured the recovery of granulocytes. Lapin et al (1958) and Thorsby (1967) forwarded criticisms of hypotonic lysis but satisfactory results were obtained in sheep by Dain and Hall (1967) and in cattle by Odajima and Sonoda (1970).

Hypotonic lysis depends on the limited capacity of erythrocytes for expansion. The erythrocyte cell membrane is flexible but essentially non-elastic. Consequently, the cell ruptures if water is taken into the cell beyond its critical volume (Schalm et al, 1975b). The susceptibility to hypotonic lysis is, in part, related to red cell size, /

size, with increasing fragility correlating with decreasing red cell volume (Stone et al, 1953; Schalm et al, 1975b). This agrees with the findings of Perk et al (1964b), who maintained that, of the common domestic and laboratory animals, sheep erythrocytes had the second highest osmotic fragility rating. It seemed reasonable, therefore, to make use of this property when selecting a method for collection of ovine leucocytes. The choice between the use of distilled water or a hypotonic saline solution was influenced by the simplicity of the water method described by Odajima and Sonoda (1970).

During the preliminary phase, experiments conducted on blood collected from Suffolk ewes and lambs yielded more consistent results with the lambs' blood. It was during these trials that the twenty second period of exposure to water was determined to be the most efficient. The blood of neonatal and young animals contains two erythrocyte populations which have differing osmotic fragilities (Perk et al, 1964b). The haemoglobin type and content normally influences the osmotic fragility but a striking correlation could not be demonstrated, in sheep, between the more osmotically resistant populations and the percentage of foetal haemoglobin (Perk et al, 1964a). Even so, the erythrocytes of young animals are generally more osmotically resistant than those of adults. However, in this investigation such a tendency was not particularly noticeable.

The demonstration of viability in eighty four per cent of the recovered white cells, accompanied by the knowledge that no noxious chemicals were added, during the technique, gave a degree of confidence in predicting only minor losses of infectivity of leucocyte-associated virus.

Two dilutions of leucocytes were prepared to avoid the problems associated with minimal and excessive virus concentration in the samples. The appearance of a recognisable cytopathic effect in cultures infected with low virus concentration might have been sufficiently delayed to be obscured by non-specific degeneration of the monolayers. This eventuality was anticipated and FLK cultures were exposed to the concentrated leucocyte suspensions. An equal number of cell cultures were inoculated with the diluted suspensions since it has been the experience of some workers (Roeder, 1974) that, on occasion, virus was recovered only from the well-diluted leucocytes. The reasons given for this phenomenon have varied from autointerference (Fenner, 1968) to the presence of neutralising antibody (Roeder, 1974).

All chemicals added should be considered as possible causes for the lack of virus replication. Washing and diluting the leucocyte suspensions had the effect of reducing the concentration of any added chemicals or gammaglobulin present. This was important since heparin had been used during the collection of the leucocytes. Heparin, although considered by Martin and Green (1958) to be the best anti-coagulant, was shown to block the absorption of HSV (Vaehri and Cantell, 1963) and to remove absorbed HSV from host cells (Hochberg and Becker, 1968). Vaehri and Cantell (1963) also demonstrated some restoration of virus infectivity after diluting the virus and heparin. Although the effect of heparin on veterinary herpesviruses is not known, the use of diluted leucocyte suspensions was considered prudent.

Three main considerations guided the selection of a suitable cell culture system. Primary and secondary cultures were considered by Hoskins (1967) to be more sensitive to viruses than serially passaged homologous/

homologous cell systems. Hoskins (1967) also stated that cells derived from the host species harbouring the virus were more likely to support virus growth than cells derived from phylogenetically distant animals. The third factor which affected the decision was the general acceptance of the value of foetal or neonatal kidney cells in virus isolation. Ovine kidney cells have been used in the propagation of several herpesviruses, EHV-1, EHV-2, Allerton or mamillitis virus, IBR, PRV and goat herpesvirus (Table II).

Virus isolation from faeces is frequently attempted in human medicine. Veterinary virologists, however, tend to be more discerning in their requests for material for virus isolation. Faecal examination is usually carried out if there is reasonable evidence to suggest viral involvement in alimentary disease. Herpesviruses can, in some diseases, be present in the faeces. The recently described (Saito et al, 1974) herpesvirus in goats, responsible for enteritis in kids, was recovered from the faeces. However, faecal examination for herpesvirus infection is likely to be less rewarding than using other clinical material. Obviously, there are gaps in our knowledge, partially filled by McFerran et al, (1969) who examined the faeces of sheep for the presence of virus. Primary ovine kidney cultures were inoculated with faecal samples collected at post-mortem and specimens submitted for parasitological examination. On the basis of morphology, five picorna viruses, eight adenoviruses and two reoviruses were isolated. No herpes-like particles were seen. These findings reinforced the view that the detection of faecal excretion of herpesviruses in healthy sheep was unlikely. Unfortunately, McFerran and his co-workers did not cite the total number of samples examined.

Herpesviruses/

Herpesviruses commonly show two types of cytopathic effect: focal areas of rounding and degeneration and the formation of syncytia. However, both types of cytopathic effect do not always appear. The kind of effect seen is dependent on the genetic constitution of the infecting virus. Variants of PRV, for example, were isolated by Tokumaru (1957) which produced one or other effect. Foetal lamb kidney infected with EHV-2 developed both types of cytopathic effect. Daily observation of the infected monolayers provided useful experience in detecting the presence of herpesvirus.

The replication of EHV-2 in FLK cultures also provided the opportunity to assess staining techniques. Haematoxylin and eosin, and acridine orange were used. Several methods for acridine orange staining were tried, with the procedure of Hsiung (1973) giving the most satisfactory results. Hsiung (1973) claimed that the addition of 0.002M $MgSO_4$ to all solutions was, in part, responsible for the excellent colour differentiation obtained.

Intranuclear inclusions are frequently encountered in herpesvirus infections. These inclusion bodies were considered by Fenner and White (1970b) to be late degenerative changes which conferred altered staining characteristics on the cell. When stained by haematoxylin and eosin, early inclusions appeared bluish. It was at this stage in the development of the intranuclear bodies that a feulgen positive reaction was demonstrated by Crouse, Coriell, Blank and Scott (1950). Later, after the virus particles had crossed the nuclear membrane into the cytoplasm, the inclusions became feulgen negative and eosinophilic. The green fluorescence of the early inclusions in FIK cultures, stained by acridine orange, confirmed the presence of a high DNA content.

A feature not readily detectable by haematoxylin and eosin staining was the multiple indentation of certain cell margins. It is suggested that the pitting of the cell boundaries in infected cultures is a manifestation of very early cytopathic effect. This alteration in cell morphology may be an indication of impending cell separation. The detection of slowly replicating herpesviruses can be hindered by the appearance of non-specific degeneration of the monolayers, before conventional techniques have demonstrated virus activity. A procedure which could detect very early cytopathic changes would assist in assessing the usefulness of further subcultures.

In those cells showing well-defined degeneration, the intensity of orange fluorescence from the cytoplasm was increased (Fig.V). A halo, up to ten cells wide, surrounding the focus of degeneration also showed this change in colour. Haematoxylin and eosin staining did not reveal any abnormalities in the latter cells. The sharp differentiation between "affected" and healthy cells is shown in Figure V. The nuclei of the brightly fluorescing cells were less green and emitted a yellow light. Two factors may have been involved in the change of nuclear fluorescence. Firstly, alterations in the chemical composition of the nuclear contents, as a result of degenerative changes could have been responsible. Secondly, the optical effect of superimposition of cytoplasmic emission of increased intensity on the green fluorescence of the nucleus must be considered. The colour appreciated by the human eye depends on the wavelength of light reaching it. Orange has a relatively long wavelength whereas green is somewhat shorter. Between these, is the wavelength which gives the naked eye the impression of yellow. Consequently, the superimposition of increased/

increased orange fluorescence on the green of the nucleus would present to the eye a combination of wavelengths more likely to be interpreted as yellow than green.

The hazard of bacterial and fungal contamination is constantly present when inoculating cultures with clinical material. Such material is frequently contaminated with large numbers of micro-organisms. The problem was to a large extent lessened, in this investigation, by the initial preparation of the material. The nasal swabs were transported and processed in growth medium containing twice the normal concentration of antibiotics and antifungal agent. During the separation of the blood cells the fluid suspending the leucocytes was changed three or five times depending on the final concentration of white cells. Table VII highlighted the advantage gained by diluting the leucocytes, no fungi or yeasts being recorded in the diluted fractions.

Bacterial contamination was not observed during the study but fungal and yeast growth was encountered on fourteen occasions. The antifungal agent amphotericin B has been shown to be effective against a variety of yeasts and fungi. Included in its spectrum of activity were Candida albicans, C.guilliermondi, C.krusei, C.parakrusei, C.stellatoidea, C.tropicalis, Rhodotorula glutinis, R.mucilagenosa, Saccharomyces cerevisiae, Aspergillus fumigatus and Aspergillus spp. (Gold et al, 1956; Hemphill et al, 1958; Littman et al, 1958).

Hemphill et al (1958) also examined the suitability of using this agent in six different cell culture systems and gave a favourable report. The ever-present spectre of antibiotic resistance inevitably springs to mind when tube contamination is encountered. Littman and his colleagues (1958) studied this problem with six species of Candida. They/

They demonstrated that resistance to amphotericin B could only be induced after many transfers. Candida albicans did not show significant resistance.

The incorporation of amphotericin B into the maintenance medium at marginally effective concentrations would also have a bearing on the sporadic appearance of contamination. However, the quantities of amphotericin B added were considerably above the minimum recommended dosage. In 1961 Perlman et al found that concentrations of two to five ug. per ml. provided an amount of antifungal agent in excess of that necessary for inhibition of growth of yeasts and fungi. Although it would generally be considered a contamination hazard, Perlman et al (1961) recommended changing fungal infected medium. It was their experience that removal of contaminated medium and replacement with fresh medium containing amphotericin B at the same concentration was sufficient to inhibit further fungal growth. Contamination of subsequent subcultures did not occur. The experience of these authors was borne out by the fourteen contaminated tubes in this study. After replacement of the medium, no further growth of contaminants was observed nor were any monolayers lost or destroyed.

yeast growth.

CONCLUSIONS

Herpesviruses were not isolated from samples collected from one hundred and fifty healthy lambs.

EHV-2 multiplied in FLK cultures and its growth was accompanied by the appearance of a typical herpesvirus cytopathic effect. Acridine orange staining offered advantages, over conventional staining methods, in the determination of the extent of degenerative changes and the recognition of early cytopathic effect. The early changes were manifested by cytoplasmic fluorescence of increased intensity and multiple indentation of the cell margins.

Hypotonic lysis, using distilled water, was demonstrated to be a suitable method for recovering ovine leucocytes. The degree of red cell contamination, mean recovery rates and percentage viability of leucocytes compared favourably with other methods.

Finally, it was confirmed that, when using amphotericin B, a change of the contaminated medium was sufficient to inhibit further fungal or yeast growth.

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REFERENCES

- AGRANOFF, B.W., VALLEE, B.L. and WAUGH, D.F. (1954). Centrifugal subfractionation of polymorphonuclear leucocytes and erythrocytes. *Blood*, 9, 804-809.
- ALEXANDER, R.A., PLOWRIGHT, W. and HAIG, D.A. (1957). Cytopathogenic agents associated with lumpy skin disease of cattle. *Bulletin of Epizootic Diseases of Africa*, 5, 489-492.
- ALEXANDER, R.F. and SPRIGGS, A.I. (1960). The differential diagnosis of tumour cells in circulating blood. *Journal of Clinical Pathology*, 13, 414-424.
- ANDREWES, C. and PEREIRA, H.G. (1972). *Viruses of Vertebrates*. 3rd edition. Bailliere, Tindall, London.
- ANON (1965). *Glaxo veterinary guide to corticoid therapy*. 3rd edition, p.3. Glaxo laboratories, Middlesex.
- AUJESZKY, A. (1902). *Zentralbl. Bakteriolog., Parasitenk., Infektionskr. Hyg., Abt.1:Orig.*, 32, 353. Cited by Kaplan (1973).
- BAGUST, T.J. (1971). The equine herpesviruses. *Veterinary Bulletin*, 41, 79-92.
- BANG, F.B. (1942). Experimental infection of the chick embryo with the virus of pseudorabies. *Journal of Experimental Medicine*, 76, 263-270.
- BENNET, W.E. and COHN, Z.A. (1966). The isolation and selected properties of blood monocytes. *Journal of Experimental Medicine*, 123, 145-159.
- BETTS, A.O. (1967). *Viral and Rickettsial Infections of Animals*. Vol., 1. Ed. A.O. Betts and C.J. York. p. 87. Academic Press, London.
- BIGGERS, J.D. and McFEELY, R.A. (1963). Simple method for the display of chromosomes from cultures of white blood cells with special reference to the ox. *Nature*, 199, 718-719.
- BLOOD, D.C. and HENDERSON, J.A. (1974). *Veterinary Medicine*. 4th edition. p. 522. Bailliere, Tindall, London.
- BOYUM, A. (1964). Separation of white blood cells. *Nature*, 204, 793-794.
- BUNCE, S.A. (1954). Observations on the blood sedimentation rate and the packed cell volume of some domestic farm animals. *British Veterinary Journal*, 110, 322-328.

BURRIN, /

- BURRIN, D.H., KEEPIE, J. and SMITH, H. (1966). The isolation of phagocytes and lymphocytes from bovine blood and the effects of their extracts on the growth of Brucella abortus. *British Journal of Experimental Pathology*, 47, 70-75.
- CABASSO, V.J., BROWN, R.G. and COX, H.R. (1957). Infectious bovine rhinotracheitis (IBR) 1. Propagation of virus in cancer cells of human origin (HeLa). *Proceedings of the Society for Experimental Biology and Medicine*, 95, 471-476.
- CAMERON-STEPHENS, I.D. (1961). Inclusion body rhinitis of swine: Clinical aspects. *Australian Veterinary Journal*, 37, 87-91.
- CARMICHAEL, L.E., FABRICANT, J. and SQUIRE, R.A. (1964). A fatal septicaemic disease caused by cytopathogenic organisms with characteristics of Mycoplasma. *Proceedings of the Society for Experimental Biology and Medicine*, 117, 826-833.
- CHEATHAM, W.J. and CRANDELL, R.A. (1957). Occurrence of intranuclear inclusions in tissue cultures infected with virus of infectious bovine rhinotracheitis. *Proceedings of the Society for Experimental Biology and Medicine*, 96, 536-538.
- CHEN, H.P. and PALMER, G.K. (1958). A method for isolating leucocytes. *American Journal of Clinical Pathology*, 30, 567-569.
- CLINE, M.J. (1966). Isolation and characterisation of RNA from human leucocytes. *Journal of Laboratory and Clinical Medicine*, 68, 33-46.
- COULSON, A.S. and CHALMERS, D.G. (1964). Separation of viable lymphocytes from human blood. *Lancet* i, 468-469.
- CRANDELL, R.A. and MAURER F.D. (1958). Isolation of a feline virus associated with intranuclear inclusion bodies. *Proceedings of the Society for Experimental Biology and Medicine*, 97, 487-490.
- CROUSE, H.V., CORIELL, L.L., BLANK, H. and SCOTT, T.F.McN. (1950). Cytochemical studies on the intranuclear inclusions of Herpes simplex. *Journal of Immunology*, 65, 119-128.
- CRUICKSHANK, R., DUGUID, J.P., MARMION, B.P. and SWAIN, R.H.A. (1973). *Medical Microbiology*, 12th edition. Vol.1, 119-122. Churchill Livingstone.
- CUTTS, H.J. (1970a). Cell Separation. *Methods in Haematology*. p. 49. Academic Press. New York and London.
- CUTTS, H.J. (1970b). *Ibid.* p.65.
- CUTTS, H.J. (1970c) *Ibid.* p.72.
- DAIN/

- DAIN, A.R. and HALL, J.G. (1967). A method for the isolation of white cells from the blood of sheep by differential lysis with hypotonic saline solution. *Vox Sanguinis*, 13, 281-284.
- DARCEL, C.Le Q. and DORWARD, W.J. (1975). Recovery of infectious bovine rhinotracheitis virus following corticosteroid treatment of vaccinated animals. *Canadian Veterinary Journal*, 16, 87-88.
- DAVIES, D.H. and DUNCAN, J.R. (1974). The pathogenesis of recurrent infections with infectious bovine rhinotracheitis virus induced in calves by treatment with corticosteroids. *Cornell Veterinarian*, 74, 340-366.
- DENNETT, D.P., ALLAN, P.J. and JOHNSON, R.H. (1973). The use of corticosteroids to aid detection of bulls carrying infectious bovine rhinotracheitis virus. *Australian Veterinary Journal*, 49, 594.
- DIOGUARDI, N., AGOSTONI, A., FIORELLI, G. and LOMANTO, B. (1963). Characterisation of lactic dehydrogenase of normal human granulocytes. *Journal of Laboratory and Clinical Medicine*, 61, 713-723.
- DOLL, E.R. and WALLIS, M.E. (1954). Cultivation of equine abortion and equine influenza viruses on the chorioallantoic membrane of chicken embryos. *Cornell Veterinarian*, 44, 453-461.
- DOWNIE, A.W. (1963). *Mechanisms of Virus Infection*. Ed. Wilson Smith, p.131. Academic Press, London and New York.
- DUBOS, R.J. and HOTCHKISS, R.D. (1941). The production of bacteriocidal substances by aerobic sporulating Bacilli. *Journal of Experimental Medicine*, 73, 629-640.
- ERASMUS, B.J. (1970). Equine cytomegaloviruses. Proceedings of the second International Conference on Equine Infectious Diseases, Paris, 1969. p.46-55. S. Karger, Basel.
- FABRICANT, C.G. and GILLESPIE, H.J. (1974). Identification and characterization of a second feline herpesvirus. *Infection and Immunity*, 9, 460-466.
- FABRICANT, C.G., GILLESPIE, J.H. and KROOK, L. (1971). Intracellular and extracellular mineral crystal formation induced by viral infection of cell cultures. *Infection and Immunity*, 3, 416-419.
- FAHRAEUS, R. (1921). The suspension-stability of the blood. *Acta Medica Scandinavica*, 55, 3-228.
- FALLON, H.J., FREI, E., DAVIDSON, J.D. TRIER, J.S. and BURK, D. (1962). Leucocyte preparations from human blood; Evaluations of their morphologic and metabolic state. *Journal of Laboratory and Clinical Medicine*, 59, 779-791.

- FENNER, F. (1968). The Biology of Animal Viruses. Vol. 1. Molecular and Cellular Biology, p. 358-359. Academic Press, London.
- FENNER, F. and WHITE D.O. (1970a). Medical Virology, p. 195-196. Academic Press, New York, London.
- FENNER, F. and WHITE D.O. (1970b). Ibid. 76-78.
- GASKELL, R.M. and POVEY, R.C. (1973). Re-excretion of feline viral rhinotracheitis virus following corticosteroid treatment. Veterinary Record, 93, 204-205.
- GIBBS, E.P.J., PITZOLIS, G. and LAWMAN, M.J.P. (1975). Use of corticosteroids to isolate IBR virus from cattle in Cyprus after respiratory disease and ataxia. Veterinary Record, 96, 464-466.
- GIRARD, A., GREIG, A.S. and MITCHELL, D. (1968). A virus associated with vulvitis and balanitis in the horse. A preliminary report. Canadian Journal of Comparative Medicine, 32, 603-604.
- GLOVER, R.E. (1939). Cultivation of the virus of Aujeszky's disease on the chorioallantoic membrane of the developing egg. British Journal of Experimental Pathology, 20, 150-158.
- GOLD, W., STOUT, H.A., PAGANO, J.F. and DONOVICK, R. (1956). Amphotericin A and B, antifungal antibiotics produced by a streptomycete. I. In vitro studies. Antibiotics Annual. 1955-1956. p. 579-586.
- GRABAR, P., SELIGMANN, M. and BERNARD J. (1955). Methodes de preparation d'extraits leucocytaires et de serums anti-leucocytaires susceptibles d'etre utilises pour des etudes immunochemiques. Annales de l'Institut Pasteur, 88, 548-562.
- GREENWALT, T.J. and POLKA, R. (1960). Gum acacia for preparing suspensions of leucocytes. American Journal of Clinical Pathology, 33, 358-361.
- GRESSER, I. and LANG, D.J. (1966). Relationships between viruses and leucocytes. Progress in Medical Virology, 8, 62-130. Karger, Basel, New York.
- HARRIS, R. and UKAEJIOFO, E.O. (1970). Tissue typing using a routine one-step lymphocyte separation technique. British Journal of Haematology, 18, 229-235.
- HELLMAN, D. and HERRELL, W.E. (1941). Haemolytic effect of gramicidin. Proceedings of the Society for Experimental Biology and Medicine, 46, 182-184.
- HEMPHILL, J.J., HERMAN, Y.F. and YOUNG, V.M. (1958). Comparative antifungal activity of nystatin and amphotericin B in tissue culture for virus propagation. Antibiotics Annual, 1957-58. p. 961-966.
- HERRELL/

- HERRELL, W.E. and HEILMAN, D. (1941). Experimental and clinical studies on gramicidin. *Journal of Clinical Investigation*, 20, 583-591.
- HOCHBERG, E. and BECKER, Y. (1968). Adsorption, penetration and uncoating of herpes simplex virus. *Journal of General Virology*, 2, 231-241.
- HORE, D.E. (1968). Some aspects of the pathogenesis of pneumonia in sheep. Ph.D. Thesis, Edinburgh.
- HOSKINS, J.M. (1967). *Virological Procedures*. p. 121-122. Butterworth and Co., London.
- HSIUNG, G.D. (1973). *Diagnostic Virology. An illustrated handbook*. p. 46-47. Yale University Press. New Haven and London.
- JAGO, M. (1956). A simple method for the separation of living lymphocytes from normal human blood. *British Journal of Haematology* 2, 439-444.
- JUBB, K.V.F. and KENNEDY, P.C. (1970). *Pathology of Domestic Animals*. 2nd edition. Vol. 2. p.416.
- KAPLAN, A.S. (1973). *The Herpesviruses*. Academic Press, New York and London.
- KAPLAN, A.S. and VATTER, A.E. (1959). A comparison of herpes simplex and pseudorabies viruses. *Virology*, 7, 394-407.
- KARPAS, A. (1966). Characterization of a new herpes-like virus isolated from foal kidney. *Annales de l'Institut Pasteur*, 110, 688-696.
- KARPAS, A., KING, N.W., GARCIA, F.G. CALVO, F. and CROSS, R.E. (1968). Canine tracheobronchitis; Isolation and characterization of the agent with experimental reproduction of the disease. *Proceedings of the Society for Experimental Biology and Medicine*, 127, 45-52
- KEMENEY, L.J. and PEARSON, J.E. (1970). Isolation of herpesvirus from equine leucocytes: Comparison with equine rhinopneumonitis virus. *Canadian Journal of Comparative Medicine*, 34, 59-65.
- KUPER, S.W.A., BIGNALL, J.R. and LUCKOCK, E.D. (1961). A quantitative method for studying tumour cells in blood. *Lancet* i, 852-853.
- LAPIN, J., HORONICK, A. and LAPIN, R.H. (1958). An improved method for isolating viable human leucocytes from peripheral blood. *Blood*, 13, 1001-1005.
- L'ECUYER, C. and CORNER, A.H. (1966). Propagation of porcine cytomegalic inclusion disease virus in cell cultures: Preliminary Report. *Canadian Journal of Comparative Medicine*, 30, 321-326.

- LI, J.G. and OSGOOD, E.E. (1949). A method for the rapid separation of leucocytes and nucleated erythrocytes from blood or marrow with a phytohaemagglutinin from red beans (Phaseolus vulgaris). *Blood*, 4, 670-675.
- LINDAHL, P.E. (1948). Principle of a counter-streaming centrifuge for the separation of particles of different sizes. *Nature*, 161, 648-649.
- LIONETTI, F.J., HUNT, S.M., GORE, J.M. and CURBY, W.A. (1975). Cryopreservation of human granulocytes. *Cryobiology*, 12, 181-191.
- LITTMAN, M.L., PISANO, M.A. and LACINCASTER, R.M. (1958). Induced resistance of Candida species to nystatin and amphotericin B. *Antibiotics Annual*, 1957-58. p. 981-987.
- LUCAS, A., HAAG, J. and LARENAUDIE, B. (1967). La Peste Porcaine Africaine. p.78. *L'Expansion Scientifique Francaise*. Paris.
- MacKAY, J.M.K. (1969). Tissue culture studies on sheep pulmonary adenomatosis (Jaagsiekte). *Journal of Comparative Pathology*, 79, 147-154.
- MADIN, S.H., YORK, C.J. and McKERCHER, D.G. (1956). Isolation of the infectious bovine rhinotracheitis virus. *Science*, 124, 721-722.
- MARTIN, S.P. and GREEN, R. (1958). Methods for the study of surviving leucocytes. *Methods in Medical Research*, 7, 136-141.
- MARTIN, W.B., MARTIN, B. and LAUDER, I.M. (1964). Ulceration on cows' teats caused by a virus. *Veterinary Record*, 76, 15-16.
- McFERRAN, J.B., CLARKE, J.K., KNOX, E.R. and CONNOR, T.J. (1972). A study of cell lines required to detect a variety of veterinary viruses in routine diagnostic conditions. *British Veterinary Journal*, 128, 627-735.
- McFERRAN, J.B., NELSON, R., McCRACKEN, J.M. and ROSS, J.G. (1969). Viruses isolated from sheep. *Nature*, 221, 194-195.
- McKERCHER, D.G. (1959). Infectious bovine rhinotracheitis. *Advances in Veterinary Science*, 5, 299-328.
- MINOR, A.H. and BURNETT, L. (1952). A method for separating and concentrating platelets from normal human blood. *Blood*, 7, 693-699.
- MOHANTY, S.B., HAMMOND, R.C. and LILLIE, M.G. (1971). A new bovine herpesvirus and its effects on experimentally infected calves. *Archiv fur die Gesamte Virusforschung*, 33, 394-395.
- MOHANTY/

- MOHANTY, S.B., LILLIE, M.G., CORSELIUS, N.P. and BECK, J.D. (1972). Natural infection with infectious bovine rhinotracheitis virus in goats. *Journal of the American Veterinary Medical Association*, 160, 879-880.
- NICHOLLE, M. and ADIL-BEY (1899). Etudes sur la peste bovine. *Annales de l'Institut Pasteur*, 13, 319-336.
- NICHOLLE, M. and ADIL-BEY (1902). Etudes sur la peste bovine. *Annales de l'Institut Pasteur*, 16, 56-64.
- ODAJIMA, M. and SONODA, M. (1970). Haematological observations of leucocytes collected by water method from bovine peripheral blood. *Japanese Journal of Veterinary Research*, 18, 173-179.
- PASCOE, R.R., SPRADBROW, P.B. and BAGUST, T.J. (1969). An equine genital infection resembling coital exanthema associated with a virus. *Australian Veterinary Journal*, 45, 166-170.
- PERK, K., FREI, Y.F. and HERZ, A. (1964a). The osmotic resistance and haemoglobin pattern of erythrocytes obtained from growing lambs. *American Journal of Veterinary Research*, 25, 68-73.
- PERK, K., FREI, Y.F. and HERZ, A. (1964b). Osmotic fragility of red blood cells of young and mature domestic and laboratory animals. *American Journal of Veterinary Research*, 25, 1241-1248.
- PERLMAN, D., JIUFFRE, N.A. and BRINDLE, S. (1961). Use of Fungizone in control of fungi and yeasts in tissue culture. *Proceedings of the Society for Experimental Biology and Medicine*, 106, 880-883.
- PETZOLDT, K. (1967). Isolierung verschiedener herpesviren von pferden und ihre epidemiologische bedeutung. *Centrallblatt fur Bakteriologie, Referate*, 206, 540.
- PFEIFFER, R.A. (1963). Preparation of bovine chromosomes. *Nature*, 199, 719.
- PHILLIPU, A.J. (1956). A method for the separation of the different morphologic forms of the blood leucocytes. *Blood*, 11, 1041-1044.
- PLATT, H. (1967). *In* *Viral and Rickettsial Infections of Animals*. Vol.1. Ed. A.O. Betts and C.J. York. p. 187. Academic Press, London.
- PLOWRIGHT, W., FERRIS, R.D. and SCOTT, G.R. (1960). Blue wildbeest and the aetiological agent of bovine malignant catarrhal fever. *Nature*, 188, 1167-1169.
- PLUMMER, G. (1967). Comparative virology of the herpes group. *Progress in Medical Virology*, 9, 310-314.
- PLUMMER/

- PLUMMER, G. and WATERSON, AP. (1963). Equine herpesviruses. *Virology*, 19, 412-416.
- PRAGNER, M.D., GOERNER, M. and MATTHEWS, N. (1958). Purification of human leucocytes. *Proceedings of the Society for Experimental Biology and Medicine*, 97, 726-728.
- RANDALL, C.C. (1955). Propagation of equine abortion virus in the chick embryo. *Proceedings of the Society for Experimental Biology and Medicine*, 90, 176-178.
- RANDALL, C.C., RYDEN, F.W., DOLL, E.R. and SCHELL, F.S. (1953). Cultivation of equine abortion virus in fetal horse tissue *in vitro*. *American Journal of Pathology*, 29, 139-153.
- ROEDER, P.L. (1974). A survey of the prevalence of equid herpesvirus 2 infections. M.Sc. Thesis, Edinburgh.
- ROIZMAN, B. (1969). The herpesviruses - A biochemical definition of the group. *Current Topics in Microbiology and Immunology*, 49 1-79.
- ROSE, M., HOPES, R., ROSSDALE, P.D. and BEVERIDGE, W.I.B. (1974). Virus infections of horses at Newmarket, 1972 & 1973. *Veterinary Record*, 95, 484-488.
- ROUHANDEH, H. (1963). Propagation of infectious bovine rhinotracheitis virus in monkey kidney tissue culture cells. *Nature*, 200, 386-387.
- ROUS, P. and BEARD, J.W. (1934). Selection with the magnet and cultivation of reticulo-endothelial cells (Kupffer cells). *Journal of Experimental Medicine*, 59, 577-591.
- SAITO, J.K., CRIBBLE, D.H., BERRIOS, P.E., KNIGHT, H.D. and McKERCHER, D.G. (1974). A new herpesvirus isolate from goats: Preliminary report. *American Journal of Veterinary Research*, 35, 847-848.
- SANDERSON, A.R. (1967). Tissue typing in man. *Nature*, 215, 23-25.
- SCHALM, O.W., JAIN, N.C. and CARROLL, E.J. (1975a). *Veterinary Hematology*, 3rd edition. p.41.
- SCHALM, O.W. JAIN, N.C. and CARROLL, E.J. (1975b). *Ibid.* p.76-78.
- SCHERER, W.F. (1953). The utilisation of a pure strain of mammalian cells (Earle) for the cultivation of viruses *in vitro*. *American Journal of Pathology*, 29, 113-134.
- SCHWARZ, A.J.F., ZIRBEL, L.W., ESTELA, L.A. and YORK, C.J. (1958). Propagation and modification of infectious bovine rhinotracheitis virus in porcine kidney tissue culture. *Proceedings of the Society for Experimental Biology and Medicine*, 97, 680-683.

SINGER/

- SINGER, T.P., SILBERBACH, I. and SCHWARTZ S. (1947). The preparation of morphologically intact leucocytes from peripheral blood by means of gramicidin and lysolecithin. *Blood*, Special Issue I, 88-97.
- SKOOG, W.A. and BECK, W.S. (1959). Studies on the fibrinogen, dextran and phytohemagglutinin methods of isolating leukocytes. *Blood*, 11, 436-454.
- SOMMERVILLE, R.G. and MacFARLANE, P.S. (1964). The rapid diagnosis of virus infections by immunofluorescent techniques applied to blood leucocytes. *Lancet* i, 911-912.
- SPEAR, F. (1948). Separation of leucocytes from whole blood by flotation on gum acacia. *Blood*, 3, 1055-1056.
- SPERTZEL, R.O., HUXSOLL, D.L., McCONNELL, S.J., BINN, L.N. and YAGER, R.H. (1965). Recovery and characterisation of a herpes-like virus from dog kidney cell cultures. *Proceedings of the Society for Experimental Biology and Medicine*, 120, 680-683.
- STEWART, C.C. and INGRAM, M. (1967). A method for counting phytohaemagglutinin stimulated lymphocytes. *Blood*, 29, 628-639.
- STONE, E.C., ADAMS, M.F. and DICKSON, W. (1953). Electrophotometric determination of osmotic resistance of ovine erythrocytes. *Cornell Veterinarian*, 43, 3-9.
- THORSBY, E. (1967). Cell specific and common antigens on human granulocytes and lymphocytes demonstrated with cytotoxic hetero-antibodies. *Vox Sanguinis*, 13, 194-206.
- TOKUMARU, T. (1957). Pseudorabies virus in tissue culture: Differentiation of two distinct strains of virus by cytopathogenic pattern induced. *Proceedings of the Society for Experimental Biology and Medicine*, 96, 55-60.
- TULLIS, J.L. and BAUDANZA, P. (1958). Sedimentation technics. *Methods in Medical Research*, 7, 130-135.
- ULRICH, K. and MOORE, G.E. (1966). Separation of viable leucocytes from normal human blood. *Acta Haematologica*, 35, 338-343.
- VAHERI, A. and CANTELL, K. (1963). The effect of heparin on herpes simplex virus. *Virology*, 21, 661-662.
- VALLEE, B.L., HUGHES, W.L. and GIBSON, J.G. (1947). A method for the separation of leukocytes from whole blood by flotation on serum albumin. *Blood*, Special Issue I, 82-87.
- WALFORD, R.L., PETERSON, E.T. and DOYLE, P. (1957). Leucocyte antibodies in human sera and in immune rabbit sera. *Blood*, 12, 953-971.

WARTMAN/

- WARTMAN, W.B. (1946). Effect of room temperature on sedimentation rate of red blood cells of man. *American Journal of Medical Sciences*, 212, 207-210.
- WILSON, G.S. and MILES, A. (1975). *Topley and Wilson's Principles of Bacteriology, Virology and Immunity*. 6th edition. Vol. 1. p. 1224. Edward Arnold.
- WRIGHT, A.E. and DOUGLAS, S.R. (1903). An experimental investigation of the role of the blood fluids in connection with phagocytosis. *Proceedings of the Royal Society (London)*, 72, 357-370.
- WRIGHT, G.P. (1958). *An Introduction to Pathology*. 3rd edition. p.46. Longmans, London.

