CONTAGIOUS ECTHYMA VIRUS INFECTION OF SHEEP: VIROLOGIC AND IMMUNOLOGIC INVESTIGATIONS

by

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INTRODUCTION

Contagious ecthyma (CE) infection of sheep has been reported to cause considerable economic loss (1) and, in 1976 was rated as a top health priority problem by the U.S. Sheep Industry Development Program (2). Because of the contagious nature and economic importance of the disease as well as the public health aspect, it is essential that effective procedures be developed for its control. Although, CE virus vaccines have been available since 1923 (3) immunity following vaccination is not absolute (4).

An understanding of the development of immunity in sheep following inoculation of CE virus could provide useful information for improving vaccination methods and production of new vaccines. However, for effective control of CE a thorough knowledge of the epidemiology of the disease is required as is information pertaining to the possibility of latent infection. The importance of colostral immunity in protecting lambs against CE viral infection should also be investigated. A further aspect of control relates to prevention of CE outbreaks in vaccinated sheep. Several authors (4) (5) consider that a multiplicity of strains of CE virus could explain such outbreaks. Investigations are needed to determine if there are antigenic differences among CE isolates and to establish the role of such differences in the etiology of CE outbreaks in vaccinated sheep.

The objectives for the research were as follows:

1) Study the immune response of sheep to CE virus.

2) Determine the importance of colostral immunity for protection of lambs against CE.

3) Investigate the occurrence of latent CE infections in sheep and determine their importance in the initiation of outbreaks of CE.

4) Study the possible causes of CE vaccination failures in sheep.

LITERATURE REVIEW

Definition.

Contagious ecthyma (CE), an acute viral disease of sheep and goats, is characterized by the sequential development of papules, vesicles, pustules and scabs on the skin of the lips, nostrils, eyelids, teats and udder. The disease, produced by a poxvirus, occurs in all countries where sheep are raised, and is commonly called "sore mouth" or "scabby mouth" in this species. The effect of the disease is to cause a marked loss of body condition, due to affected animals experiencing difficulties in suckling or grazing. CE is readily transmissible to man and constitutes a public health problem for people working directly with infected sheep. Medical physicians frequently refer to the condition in man as "orf".

History.

Although several reports of a contagious stomatitis of lambs had been made in England and France between 1888 and 1923, French workers first demonstrated that the disease was produced by a virus (3). The typical lesions could be reproduced in scarified skin of susceptible sheep by inoculation of a bacteria-free filtrate. Furthermore, these workers prepared a vaccine from infected scabs which was used to successfully immunize 10,000 sheep against field exposure to the disease.

Moussu (6) considered that the disease should be described as contagious ecthyma of the lips, rather than using the term stomatitis, as the lesions are often confined to skin without involving the mucosa of the mouth. In an intensive study, Glover (7) reported that the virus existed in great abundance in the lesions and was highly resistant to

desiccation. Vaccination induced a definite, solid immunity that endured for at least eight months.

In the United States, Howarth (8), in California, described "sore mouth" in lambs, which he considered identical with the condition reported by Aynaud. Broughton and Hardy (9) reported the success of a large scale field vaccination program in Texas.

Etiology.

Contagious ecthyma virus has been classified by the International Committee on Taxonomy of Viruses (10) under the genus Parapoxvirus in the family Poxviridae. Other viruses in the genus Parapoxvirus include bovine papular stomatitis, chamois contagious ecthyma and milker's node virus. Electron microscopic investigations have revealed that the parapoxviruses differ morphologically from the other viruses in the family Poxviridae (11). Parapox virions are cylindrical structures with convex ends and are symmetrical about both major and minor axes. They have a conspicuous crisscross or woven pattern on their surface made by a series of parallel dense stripes of material running diagonally from each side. Several studies have indicated the viruses are 158-200 nm by 250-270 nm (11, 12, 13).

Growth Characteristics.

Greig (14) demonstrated that the CE virus could be grown outside the sheep host, and passaged the virus through 10 serial transfers in monolayer cultures of embryonic sheep skin. Contagious ecthyma virus has now been successfully propagated in a variety of cell cultures, including cell cultures of bovine and caprine embryonic kidneys and of calf and lamb testes (15). Specific host adaptation of sheep CE strains was noted by Nagington (16) with CE virus from sheep lesions initially able

to grow only in sheep cells, whereas CE virus recovered from human lesions had lost this specificity. After several cell passages most sheep strains lost their specificity for sheep cells and grew as readily in the heterologous cells.

Contagious ecthyma virus produced plaques of rounded cells in the majority of the cell monolayers in which the virus grew (14, 15, 16). Infected cells become rounded and highly refractile and readily detach from flasks to leave a clear central area, surrounded by cells in the process of rounding. Time of onset of cytopathic change was dependent on the number of virus particles in the inoculum and the cytopathogenicity of the isolate and ranged from 1 to 18 days. In stained preparations of infected cells the appearance of large, granular intracytoplasmic inclusions have been described, with a differentiation into an eosinophilic, paranuclear part and an outer arc of more basophilic staining reaction (16, 17). Immunofluorescent staining has revealed that parapoxvirus-specific antigens are only found in the cytoplasm of infected cells (18).

Contagious ecthyma virus does not readily grow in chick embryos and several authors have reported that those strains which grew initially, failed to do so after 3 or 4 consecutive passages (19, 10). However, Sawhney (21) was able to passage the virus through 15 serial transfers on chorioallantoic membranes of chick embryos, observing specific lesions in most passages.

Immunological Reactions and Antigenic Properties.

A variety of immunological techniques have been developed to help confirm the diagnosis of CE in disease outbreaks and to differentiate the CE virus from other poxviruses. The complement-fixation test was

utilized by Glover (22) to detect the presence of viruses in suspected lesions. It was necessary to use hyperimmune serum because sera of recovered animals only fixed complement to a slight degree. In contrast, the precipitin or flocculation test using scab material as the antigen was considered to be of little practical value for detection of CE virus (22, 23, 24). However, Romero-Mercado et al. (25) utilized an immunodiffusion test for the detection of precipitins present in infectious scab material and found the method sensitive and efficient in confirming a diagnosis of CE when scabs were collected within the first 19 days after experimental infection. Thereafter, the complement-fixation test and electron microscopic detection of the virus in scabs were the most efficient techniques.

Although hemagglutinins are produced by other poxviruses such as vaccinia (26) variola (27), cowpox (28) and ectromelia (29), most attempts to demonstrate hemagglutination by CE virus have been unsuccessful or unreliable (24, 30). Sawhney (21) was able to demonstrate hemagglutination by CE virus with human group "O", fowl, guinea pig and mouse red blood cells, but only after the CE virus had been passaged 6 times on the chorioallantoic membrane of chick embryos.

In the initial virus-neutralization tests, the virus-antisera mixtures were inoculated into test animals and several investigators (22, 23, 24) demonstrated that virus-neutralizing (VN) antibodies were present in the sera of sheep recovered from CE infection. Demonstration of the growth and cytopathogenicity of CE virus in cell culture encouraged more detailed investigations into VN antibody response. Plowright et al. (15) determined the neutralization index of sera from sheep recovered from CE virus infection, titrating the virus-antisera mixtures in tubes of embryonic

sheep kidney cells. The sera caused a variable degree of neutralization with the majority of sera containing very low levels of VN antibody. A plaque reduction test was utilized by Nagington and Whittle (31) and neutralizing antibody titers of 1:40 to 1:60 were detected in sheep recovered from CE infection. Poulain et al. (32) concluded that sensitivity of the plaque reduction test could be increased by the addition of 10 percent guinea pig serum as a source of complement. A constant virus-variable serum dilution neutralization test has also been attempted and VN antibody titers of approximately 1:200 were observed in convalescent goat sera using an inoculum of 10 cell culture infecting dose 50 percent ($CCID_{50}$) of virus/microtiter well (1).

Cross-immunity experiments have been used to determine whether there are multiple strains of CE virus. Seddon and McGarth (33) tested English and Australian strains and found them to be equally protective. In an extensive series of cross-immunity tests with ovine strains from England, France and California, Glover (22) found that they conformed to a single type; however, there were marked variations in the potency of viruses obtained from different sources. Lambs immunized against a mild virus were not always protected against strains which had been recently isolated. Glover postulated that there may be a positive correlation between virulence and immunizing potency. Horgan and Haseeb (34) confirmed Glover's findings and, in a series of cross protection tests in sheep, indicated that cross-immunity varied from strong to complete. They concluded that the clinical disease was caused by a series of strains of virus, some of which were immunologically identical while others were closely related.

Relationships among CE isolates have also been studied in the laboratory by serum neutralization tests (21, 35, 36). On the basis of these tests it has been suggested that there is more than one antigenic form of the CE virus. However, in many cases the virus-neutralization titers for the homologous virus and antiserum were very low and the results must be interpreted with caution. Wittek et al. (36) compared isolates of CE virus by DNA restriction analysis and observed an extensive heterogeneity of restriction patterns for the different isolates which suggests antigenic differences.

Serological tests have clearly indicated that CE virus is not closely related to vaccinia as determined by complement-fixation (37) and immunodiffusion tests (38). However, the relationships between CE, goatpox and sheeppox viruses are more complex. In cross-immunization trials, Bennett et al. (39) reported that goatpox virus protected goats on subsequent challenge with CE virus, but the reverse was not true. Sharma and Dhanda (40) were unable to demonstrate any cross protection between goatpox and sheeppox viruses with CE virus and vice versa. However, by cross complement-fixation tests, cross reactions were revealed between CE virus and both goatpox and sheeppox viruses. Dubey and Sawhney (41) observed that hyperimmune serum prepared against CE and goatpox viruses yielded 8 precipitable factors with their homologous antigens and 6 factors were common to both viruses as revealed on cross-precipitation reactions. In the serum neutralization test, the goatpox antiserum neutralized both the homologous and heterologous antigens, while CE antiserum failed to neutralize the goatpox virus.

Transmission.

Transmission of CE is by direct contact with infected sheep or by contact with objects contaminated by the virus (42). In feedlots the virus contaminates equipment, fences, manure, bedding and feed, with crowding facilitating direct transmission. Infected scab material contains very large quantities of CE virus and serves as a source of infectious virus. Glover (7) reported "takes" with 1:50,000 dilution of infectious scabs, while Broughton and Hardy (9) transmitted the infection to a susceptible kid with scab material diluted 1: 100,000 in sterile normal saline. Hart et al. (43) demonstrated the resistance of the virus to desiccation by detecting the presence of infectious virus in scab material after storage of the scabs at room temperature for 15½ years.

Broughton and Hardy (9) observed that CE often reappeared on a particular ranch after an absence of 2 or 3 years indicating that the virus was extremely resistant to climatic conditions. They found that scabs, exposed to the weather on the ground during the fall and winter months, retained their virulence when tested in the spring. This allowed the virus to carry over from the early fall until spring, when a new crop of lambs was born. They detected that during the hot Texas summer virus remained viable in scabs on pasture for only 30 to 60 days.

In experimental infection, Glover (7) established that CE lesions could be produced by inoculation of virus intradermally or applying virus onto scarified skin, whereas intravenous or subcutaneous inoculation failed to produce lesions. While slight irritation or scarification of the skin may favor entrance of the virus and subsequent establishment of the disease, Broughton and Hardy (9) observed CE lesions following application of the virus to the unbroken skin. However, severe CE outbreaks in

the field may follow extensive scarification to mucous membranes. Gardiner et al. (44) reported that in a severe CE outbreak in an Australian flock, the rapid spread, severe disease and high morbidity were attributed to the concentration of sheep around a palatable shrub, Templetonia retusa, which caused extensive trauma to mucous membranes during grazing.

Beck and Taylor (4) reported that infection in man usually occurred as a result of attempts to shear, vaccinate, feed or treat infected sheep.

Pathogenesis.

In natural outbreaks, Broughton and Hardy (9) observed that the lesions of CE were confined to the lips, muzzle, teats and udder, suggesting a localized infection resulting from direct infection of the affected area. Teat and udder lesions in ewes resulted from direct infection by the swollen, inflammed lips of lambs during suckling. Glover (7) reported that there was no generalized eruption over the hairless parts of the body as in sheeppox and there was no evidence of a viremia.

In studies of disease pathogenesis in sheep, Wheeler and Cawley (45) biopsied experimentally induced lesions at intervals from 4 to 42 days after inoculation. By days 5 to 6 post-inoculation the prickle cells of the epidermis began to balloon and multiply, with leukocytes and endothelial cells accumulating in the derma. The ballooning degeneration of the prickle cell layer resulted in the development of multiloculated, superficially placed vesicles and pustules. Between the 11th and 17th days the cells of the pustule disintegrated and the epidermis showed a marked pseudo-epitheliomatous hyperplasia. The rete pegs grew downwards and the dermal papillae grew upwards to produce a finger-like papillomatous appearance. Scabs formed by days 17 to 22 and separated from healed skin on days 22 to 42. On gross appearance, red macules developed successively

into red papules, gray pustules and brown crusts which overlay warty, papillomatous bases.

Bacteria may penetrate the vesicles or pustules and cause secondary infection, extensive necrosis, and ulceration of lips, mouth and forestomachs (42). These infections may lead to otitis media, liver abscesses, pneumonia, inanition and occasionally death.

The highest concentration of virus in lesions has been reported to occur 2 weeks after inoculation, but virus has been detected in lesions up to 9 weeks after inoculation (25).

Limited studies have been conducted on the development of antibodies in sheep recovering from CE infection. Renshaw and Dodd (1) detected virus-neutralizing antibody in sera of goats 10 days after inoculation which reached a peak at 30 days post-inoculation. On reexposure to CE virus, the secondary antibody response occurred more rapidly and was of greater magnitude than the primary response.

Clinical Signs.

The first signs noted in natural outbreaks of CE are moist, warty scabs on the lips, mouth, nostrils or eyelids (9). These scabs are usually brownish gray and moist in the early stages, but later dry out, becoming hard and very often cracked. When removed manually or torn off by brush or weeds, they leave an elevated, raw, bleeding surface which in certain areas attracts screw-worm flies. If not infested with screw-worm larvae, the scabs gradually dry up and, finally drop away, leaving the skin of the lips smooth and without scars.

Glover (7) reported that the development of lesions was best studied in inoculated animals. He observed that following an incubation period

of 2 to 4 days a zone of inflammation could be seen surrounding the scarified area. Twenty-four hours after the appearance of the inflammation, small circular papules appeared, and in a further 24 hours small vesicles filled with a clear fluid protruded from the papules. The vesicle remained for only a few hours, and rapidly developed into a pustule which was opaque-yellow in color and easily ruptured. By day 11 a scab began to form and a firm brown mass persisted for 1 to 2 weeks.

Occasionally, marked edema of the lips and intermandibular spaces has been observed and lesions may be found on buccal, gingival and lingual mucosae, in addition to the lips (44).

Although the virus infects sheep of all ages, the disease more commonly develops among lambs 3-5 months old. The morbidity rate may reach 90 percent or more of feedlot lambs, but mortality is low and due to secondary bacterial infection. In fatal cases, necrotic tissue and ulcers may be present on the tongue, gums, palate, pharynx, rumen or omasum and show evidence of a spreading bacterial infection. Liver abscesses or pneumonia may develop in terminal stages. Uncommonly, lesions may exist on the teats or udder and be accompanied by mastitis (42).

Infectivity for Other Species.

Although guinea pigs and mice are not susceptible to CE virus, divergent views have been expressed on the susceptibility of rabbits (20). Many investigators (3, 7, 8, 9) failed to infect the skin of rabbits with the virus, whereas Bennett et al. (39) and Abdussalam (20) succeeded in producing mild skin lesions in rabbits by applying a massive dose of virus to scarified skin.

The natural disease of CE is usually seen only in sheep, goats and occasionally man, but Wilkinson et al. (46) reported that natural CE lesions

developed in a pack of hound dogs and the virus was transmitted to susceptible sheep. Experimentally induced CE lesions have been produced in virus-inoculated monkeys (39), calves (3, 39) and cats (47).

Immunization.

The studies of Aynaud (3) established that solid immunity could be produced by artificial transmission of CE virus. This method was successful when applied to a large number of animals in the field by Broughton and Hardy (9), using a vaccine consisting of finely powdered dried scab material suspended in 50 per cent glycerin. The vaccination procedure used, has remained unchanged to the present time and consists of brushing a drop of vaccine over a lightly scarified area of skin in non-wooled areas such as the medial surface of the thigh (4).

The duration of immunity after vaccination has been estimated by several investigators to be greater than 8 months (7, 9, 43). Although immunity is not always absolute, these investigators observed that lesions which developed when vaccinated lambs were exposed, were usually slight and of little significance. Lambs are normally vaccinated during the first month or two of life. However, Kerry and Powell (48) observed that lambs could be successfully vaccinated at 24 to 48 hours of age. Some protection is provided by vaccination of clinically unaffected lambs even after signs of CE have appeared in the flock (49).

Although LeJan et al. (50) observed that colostral antibodies can be transferred to lambs from ewes previously vaccinated with CE virus, the transfer of colostral immunity may not be of great importance for protection of the lambs. Broughton and Hardy (9) and Rossi et al. (51) were unable to demonstrate that lambs born to mothers vaccinated during pregnancy possessed protective immunity.

A major problem with the use of the traditional vaccine is that the virus contained in the vaccination scab, drops on the pasture and contaminates the premises. Hence, the use of the vaccine is limited to animals on farms where the disease is already prevalent. Several investigators have developed vaccines which may be administered intramuscularly or subcutaneously, hoping to prevent contamination of farms with live virus (51, 52). Rossi et al. (51) attenuated the CE virus by 10 passages in duck fibroblasts and inoculated the virus intramuscularly into 5,000 sheep. The vaccine proved to be effective in healthy animals and had a favorable influence on affected animals. Mayr (52) reported the development of an attenuated CE virus vaccine (140 alternate passages in secondary cultures from fetal ovine and bovine lungs) which was safe for lambs and could be administered subcutaneously without loss of protective ability.

MATERIALS AND METHODS

Animals

The lambs used in these investigations came from the Sheep Unit, College of Agriculture and Life Sciences, Virginia Tech, and from the Shenandoah Valley Research Station, Steeles Tavern, Virginia. The lambs had had no previous contact with CE virus, but the dams of one group of lambs from the Sheep Unit had been vaccinated against CE six months prior to the birth of these lambs. Lambs used in the CE studies ranged from 1 day to 10 months of age and were maintained in isolation rooms at the Veterinary Medicine Research Center, Virginia Tech.

One year old rams from the Shenandoah Valley Research Station were used for the vaccination studies reported in chapter one.

Cell Culture

Ovine testis monolayers were prepared by a modification of a method described by Ferris and and Plowright (53). Lambs were castrated at 2 to 5 weeks of age and the testes were processed within three hours. Each testis enclosed in the tunica albuginea was rinsed in several changes of phosphate-buffered saline (PBS) (54) containing 100 units of penicillin/ml and 100 micrograms (mcg) dihydrostreptomycin /ml. Seminiferous tissue was scraped from the tunica albuginea and minced with scissors. After several rinses in PBS, prewarmed (37° C) trypsin-versene solution (0.25 percent trypsin and 0.02 percent versene) prepared in PBS, was added to the testicular tissue at a concentration of approximately 100 ml per 100 grams of tissue. The mixture was stirred slowly for 5 minutes and then the supernatant fluid was discarded. A similar volume of fresh trypsin-versene solution was added and the mixture stirred vigorously. Dispersed cells

were collected every 20 minutes, followed by the addition of fresh trypsin-versene solution. These dispersed cells were filtered through several layers of surgical gauze and the action of trypsin was inhibited by the addition of fetal bovine serum (FBS). The cell suspension was centrifuged for 5 minutes at 300 xg and resuspended in growth medium. Centrifugation was repeated after pooling all the dispersed cells and the volume of the packed cells measured. The cells were then resuspended in a small volume of PBS before dispensing at a concentration of 1 ml of packed cells per 200 ml of growth medium. The growth medium used was Eagle's minimal essential medium with Earle's salts, plus 2mM L-glutamine, 8 percent FBS (heat inactivated at 56° C for 30 minutes), with 100 units penicillin, 100 mcg dihydrostreptomycin and 2 mcg amphotericin B per ml.

Primary though 8th subpassage cells were used for studies with CE virus. Cell subcultures were prepared by using trypsin-versene solution (0.05 percent trypsin and 0.02 percent versene) with the cells passed at a ratio of 1:2. Growth medium was replaced by maintenance medium when the confluent monolayers had developed. Maintenance medium was the same as the growth medium except that the FBS was reduced to 4 percent. Components for these media were purchased from Flow Laboratories Inc.[a] Cells were grown in 96-well and 24-well tissue culture plates, and in 25 cm^2 flasks for virus titration and isolation. Cells intended for antigen preparation were grown in 75 cm^2 flasks. The plates and flasks were purchased from various commercial sources. For all cell and virus studies, the plates and flasks were incubated at 36°C in a humidified 3 percent carbon dioxide and air atmosphere.

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[a]Flow Laboratries Inc., Rockville, Maryland.

Viruses

Eleven isolates of CE virus denominated (Har/1, Har/2, TZ, ST, PF, Pu, Shoe, Mu, Am, Cu, CSL) were obtained from various locations in the United States. The first five CE viruses were isolated from sheep showing clinical signs of CE in 5 separate disease outbreaks in Virginia. Virus designated Pu was isolated from ovine skin biopsy provided through the courtesy of Dr. J. Baker.[b] The Shoe isolate was received from Dr. L. D. Pearson,[c] and was originally isolated from a skin lesion on the finger of a veterinary student who had been in recent contact with an infected lamb. This virus was at the twelfth passage level when received.

The Mu virus was isolated from a skin biopsy supplied by Dr. R. L. Zarnke,[d] and this biopsy had been excised from the muzzle of an infected muskox. A cell culture adapted isolate (Am) of unknown passage history was received from the Diagnostic Laboratory, National Animal Disease Center.[e] Two viruses were obtained from commercial CE vaccines. The Cu isolate was isolated from a CE vaccine[f] prepared from lyophilized infectious scab material, while the CSL isolate was obtained from a cell culture CE vaccine.[g]

Virus isolations were made in ovine testis cell cultures and, after

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[c]College of Veterinary Medicine, Colorado State University, Fort Collins, Colorado.

[d]Department of Fish and Game, Fairbanks, Alaska.

[e]National Animal Disease Center, Ames, Iowa.

[f]Cutter Animal Health Laboratories, Shawnee, Kansas.

[g]Colorado Serum Laboratories, Denver, Colorado.

3 to 6 consecutive cell culture passages, all isolates were plaque-purified or purified by three limiting dilutions. Infected cell cultures with 80 percent cytopathic effect (CPE) were frozen and thawed 3 times and cellular debris pelleted by centrifugation at 600 xg for 10 minutes. Inoculum for a 25 cm^2 flask of consisted of 0.5 ml of a 10^{-1} dilution of infected cell culture fluid. After 1 hour incubation at 36° C the inoculum was poured off, the culture was rinsed with PBS and 5 ml of maintenance medium was added per flask.

Virus Titration

A microtiter method was developed to assay the infectivity of viral isolates. Ten-fold serial dilutions of virus were prepared and 0.05 ml of the different dilutions were added to each of 8 wells in a 96-well microtiter plate. Each well was seeded with approximately 1 x 10^4 cells in 0.15 ml of growth medium and then the plates were incubated for 5-7 days. Cytopathic changes were scored and the 50 percent end-point ($CCID_{50}$) was calculated by the method of Kärber (55).

Virus Isolation

Skin biopsies and scabs from sheep were tested for the presence of CE virus by grinding 1 or 2 small pieces of tissue (3-5 mm diameter) in a Ten-Broeck grinder in 5 ml of maintenance medium with 100 mcg/ml Gentamicin added. Tissue suspensions were centrifuged at 1,000 xg for 10 minutes (4°C) and 0.5ml aliquots of the undiluted supernatant fluids were absorbed for 1 hour on sheep testis monolayers in 25 cm^2 flasks before adding maintenance medium. Inoculated cultures were examined for CPE over a 14 day period and 2 further passages were made before considering the samples negative for CE virus. To confirm the isolation of CE virus, the isolates were examined by electron microscopy.

Skin biopsies were tested for the presence of persistent virus by maintaining intact skin fragments *in vitro* and tested periodically for virus release. Two methods were used to test for persistent virus utilizing modifications of methods described by Beran *et al.* (56) In the first method, the tissue-fragment culture technique, skin biopsies were minced with scissors and six to nine 1 mm^3 portions of tissue were suspended in 0.5 ml of growth medium in a 25 cm^2 flask for 1 hour at 36° C to aid their adherence to the flask. A further 2.5 ml of growth medium with Gentamicin (100 mcg/ml) was then added and the flask was incubated. Aliquots of medium were removed after 7 days and 0.5 ml of undiluted medium from each aliquot was tested for virus by inoculation onto ovine testis monolayers. Culture medium was replaced at weekly intervals and a tissue sample was considered negative for presence of virus if no virus was detected in spent medium collected over a 3 week period.

The second method used to test for virus release was the coculture technique. Six to nine 1 mm^3 tissue fragments were placed on an ovine testis monolayer in a 25 cm^2 flask with 1 ml of growth medium for a 1 hour absorption period. An additional 2 ml of growth medium containing Gentamicin (100 mcg/ml) was then added and the cell culture was examined daily for CPE. Tissue fragments were transferred to a fresh testis monolayer when the one in use started to deteriorate, usually after 12 days of culture. Cell cultures which had been cocultured with tissue fragments were frozen and thawed three times and after centrifugation at 600 xg for 10 minutes, the supernatant fluids were inoculated on fresh testis monolayers. Tissue fragments were considered negative for CE virus after three serial coculture passages without observing CPE.

Animal Inoculation

Sheep were inoculated with CE virus in the axilla, medial aspect of the groin or on the muzzle. Only the muzzle required to be clipped free of wool. Inoculation sites were scarified with a commercial CE vaccine applicator[h]. The metal applicator consisted of a 2 pronged fork which was used for scarifing the skin and a small brush on the opposing end for applying the virus inoculum onto the scarified area. The scarification abraded the epidermis, and very little bleeding occurred. Virus inocula were prepared from CE infected cell cultures or from scabs collected from CE infected sheep. Contagious ecthyma infected cell cultures, showing 80 percent CPE, were frozen and thawed 3 times for use as the virus inoculum. The sheep-passaged virus inoculum was prepared by grinding infected material in a Ten-Broeck grinder with maintenance medium containing Gentamicin (100 mcg/ml) and pelleting the debris by centrifugation at 1,000 xg for 10 minutes. Dilutions of the virus inocula were prepared in maintenance medium. Sterile maintenance medium was applied to scarified areas of control sheep.

Preparation of Antisera

Antisera were prepared in sheep by inoculating cell culture-passaged or sheep-passaged CE virus onto scarified skin in the axillary region and the medial aspect of groin and repeating this inoculation 3 to 6 weeks later. After a further 2 weeks, sheep were inoculated intravenously at weekly intervals for 4 weeks with cell culture-passaged virus, and the animals were bled 10 days after the last inoculation. Serum was separated from the whole blood and stored in 2 ml aliquots at -20°C.

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[h]Cutter Animal Health Laboratories, Shawnee, Kansas.

Virus Neutralization Tests

Serum samples were taken from sheep prior to exposure to CE virus and at various intervals thereafter. Virus neutralization tests were performed using microtiter equipment. Serial two-fold serum dilutions were prepared and an equal volume (0.05 ml) of virus containing 10-30 $CCID_{50}$ was added. Serum dilutions were prepared in triplicate in maintenance medium and the serum was not heat-inactivated. Serum-virus mixtures were incubated for 2 hours at 36° C, ovine testicular cells were added and the cultures observed daily with an inverted microscope for 5 days. The antibody titer was determined as the reciprocal of that serum dilution which neutralized 50 percent of the test virus as calculated by the method of Kärber (55). The cross virus neutralization studies were also carried out in this manner. Appropriate cell culture and virus controls and known positive and negative sera were included in each test.

Lymphocyte Transformation Studies

The culture medium used in these studies as well as for the buffy cell cultures was RPMI 1640[i] with 10 percent FBS, penicillin (100 units/ml) and dihydrostreptomycin (100 mcg/ml) and will be referred to as RPMI 1640 medium unless otherwise stated.

Lymphocyte transformation experiments were carried out by using whole blood which was collected from sheep before and after exposure to CE virus. The blood was collected from the jugular vein by venipuncture in heparinized Vacutainer tubes.[j] Triplicate 0.05 ml samples of blood were mixed

[i]Grand Island Biological Company, Grand Island, New York.

[j]Becton-Dickinson, Rutherford, New Jersey.

in microtiter plates with 0.25 ml of RPMI 1640 medium alone (control medium) or containing of phytohemagglutinin(PHA), viral antigen or control antigen and incubated.

PHA (PHA-M) was prepared at a concentration of 0.03 ml per ml of RPMI 1640 medium. Viral antigen was prepared from CE infected ovine testis monolayers as described for the preparation of virus for passage in cell culture. The viral antigen was diluted 1/40, 1/400 and 1/4,000 in RPM1 1640. Uninfected cell cultures were treated in a similar manner to the CE infected cell cultures and a 1/40 dilution served as the control antigen.

The whole blood cultures were incubated for 4 days, the optimal incubation period as determined in preliminary studies. For the final 18 hours of incubation [H^3] thymidine[k] μ Ci per microtiter well (specific activity, 5 Ci/mmol) was added. Incubation was terminated by freezing the cultures at -20° C. Cultures were harvested on filters of glass microfiber paper[l] by means of an automated sample harvester. The dried filter disks were placed in scintillation bags and 3 ml of scintillation fluid was added. Scintillation fluid consisted of toluene plus 6 gm/liter 2-5 diphenyloxazole and 0.3 gm/liter dimethyl-pop-op. Mean counts per minute (cpm) of three replicates were determined in a Beckman LS 8100 liquid scintillation spectrometer. Results were expressed as a stimulation index (SI), which is the ratio of cpm in PHA or virus-exposed cultures to cpm of cultures containing RPMI 1640 alone.

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[k] Radiochemical Centre, Amersham, England.

[l] Whatman Inc., Clifton, New Jersey.

Delayed Hypersensitivity Skin Test

Sheep were tested for specific delayed-type hypersensitivity (DTH) by intradermal injection of 0.1 ml inactivated CE antigen into the medial surface of a hind leg. The skin test CE antigen was prepared as described for the preparation of virus for passage in cell culture but was inactivated by heating for 1 hour at 65° C. Samples of the heated virus preparations were tested in cell cultures for presence of viable virus. No evidence of viral replication was detected. Control antigen was prepared in a similar manner from uninfected testis monolayers. Sheep were observed prior to and 6, 24 and 48 hours post-inoculation. Calipers were used to measure the skin thickness at the inoculation site.

Hematology

Total and differential leukocyte counts were determined on heparinized blood samples collected from sheep before and at intervals after dexamethasone treatment. Total leukocyte counts were obtained using a Coulter counter[m] (Model ZBI) and differential counts were estimated by viewing 100 to 200 cells stained with a modified Wright-giemsa stain.

Buffy Coat Cell Cultures

Twenty ml of heparinized blood was collected by venipuncture from CE infected and non-infected sheep and the buffy coat was collected after centrifugation at 1,000 xg for 10 minutes. Sterile distilled water was added at a ratio of 2:1 to the suspension of the buffy coat cells to lyse erythroeytes and after mixing well for 30 seconds 1.7 percent sodium chloride was added at an equivalent volume to the distilled water to restore osmolarity. The buffy coat cells were washed twice with PBS and then

[m]Coulter Electronics, Hialeah, Florida.

resuspended to 1.5 x 10^6 cells/ml in RPMI 1640 medium. The cells were cultured in 24-well tissue culture plates with each well containing 1 ml of the cell suspension. Viability of the cells was determined by the trypan blue exclusion method (57).

To study the replication of CE virus in buffy coat cells from CE infected and non-infected sheep, medium was aspirated from the cell cultures 24 hours after seeding and 0.1 ml of RPMI 1640 medium containing 10^3-10^4 $CCID_{50}$ of CE virus was added. After 1 hour absorption at 36° C, a further 0.9 ml of RPMI 1640 medium was added to each well. At various intervals after inoculation the cells were scraped from the tissue culture wells with wooden applicator sticks and cells were aspirated from the wells. Control wells containing buffy coat cells without CE virus and CE virus without buffy coat cells were also harvested at the same times. All samples were stored at -70°C and subsequently frozen and thawed three times before virus titrations were performed.

Purification of Virus for Protein Analysis

A modification of the method described by Joklik(58) was used for the virus purification. Infected cultures with 80 percent CPE were scraped from flasks with a rubber policeman and disrupted by sonication (sonic dismembrator)[n] for four minutes. Sonications were always carried out at the maximum setting in an ice bath. Coarse debris was pelleted by centrifugation at 600 xg for ten minutes and the pellet resuspended in 1 ml of 0.01 M Tris buffer (pH 8.0) and 0.001M ethylenediamine tetracetate (EDTA). After sonication for 30 seconds the suspension was recentrifuged at 600

[n]Quigley-Rochester Inc., Rochester, New York.

xg for ten minutes. Supernatant fluids from the first and second centrifugations were pooled and concentrated by centrifugation at 35,000 xg in a Ti70 rotor for 30 minutes using a Beckman L5-75 ultracentrifuge. Pooled pellets were resuspended in a small volume of buffer and sonicated for 30 seconds to disperse the virus.

The partially purified virus suspension was centrifuged through 36 percent sucrose in 0.01M Tris buffer (pH 8.0) at 45,000 xg for 30 minutes (SW-60 rotor, 4°C). After resuspension in 1ml of the Tris buffer the virus was layered on a sucrose step gradient of 40, 50 and 60 percent sucrose in Tris buffer and centrifuged at 45,000 x g for 30 minutes (SW-60 rotor, 4°C). The virus accumulated in the 50 percent band and this entire band was collected. The pellet, if any, was resuspended, sonicated for 20 seconds and again put on through the step gradient. The pooled virus harvest was dialyzed against 0.001M Tris (pH 8.0) overnight before concentration by centrifugation at 45,000 xg for 30 minutes (SW-27, 4°C).

Controlled Degradation of CE Virus

A modification of the method described by Easterbrook (59) was used. A purified virus suspension (10^9 $CCID_{50}$/ml) in 0.001 M Tris buffer (pH 8.0) was treated with 1 percent Nonidet P-40[o] and 0.05M 2-mercaptoethanol (2-ME) for 1 hour at 37°C with constant agitation. The detergent treated virus suspension was sonicated at the maximum setting for 10 seconds to dissociate the surface material of the virions before centrifugation at 30,000 x g for 30 minutes (4°C, SW-60 rotor). The supernatant fluid was carefully removed and the pellet was resuspended in 4 ml 0.001 M Tris

[o]Sigma Chemical Company, St. Louis, Missouri

buffer (pH 8.0). The suspension was recentrifuged at 30,000 x g for 30 minutes (4°C) and the pellet was finally resuspended in 0.2 ml of 0.01 M Tris buffer (pH 8.0).

Supernatant fluid was dialyzed against distilled water, lyophilized and redissolved in 0.1 ml of 0.01 M Tris buffer (pH 8.0). Samples of the supernatant were further purified by passage through a DEAE A-50 column. The detergent was eluted with 0.1 M Tris buffer (pH 7.4) and the viral polypeptides were eluted by increasing the molarity of the Tris buffer to 1 M. The fraction eluted with 1 M Tris buffer was dialyzed, lyophilized and redissolved in 0.1 ml of 0.01 M Tris buffer (pH 8.0).

The surface tubules released from CE virions were partially purified as described by Stern and Dales (60). Material released from purified CE virions treated with Nonidet P-40, 2-ME and sonication as described above was layered on 2ml of 40 percent sucrose prepared in 0.001 M phosphate buffer (pH 7.2). The samples were centrifuged (100,000 x g, 20 minutes, 4°C SW-60 rotor); the material at the interface and above the cushion was pooled, dialyzed, lyophilized and resuspended in 0.1 ml of 0.01 M Tris buffer (pH 8.0). To prepare samples of the surface tubule for electron microscopy, the material above the sucrose cushion (including the interface) was diluted 10 times with 0.001 M Tris buffer (pH 8.0), centrifuged at 100,000 x g for 18 hours (4°C, SW-60 rotor) and the pellet was resuspended in 0.1 ml of 0.001 M Tris buffer.

For analysis by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis 50 µl samples were mixed with 50 µl of 0.063 M Tris buffer (pH 6.7) containing 2 percent SDS, 0.2 percent 2-ME and 20 percent (v/v) glycerol.

Polyacrylamide Gel Electrophoresis (PAGE)

Two OD_{260} units of purified virus were suspended in 100 µl of 0.063M Tris buffer (pH 6.7) containing 1 percent SDS , 0.1 percent 2-ME and 10 percent (v/v) glycerol. Bromocresol blue (0.002 percent) was used as the tracking dye and the virus suspension was solubilized by immersion in boiling water for 2 minutes. Electrophoresis was performed using a discontinuous buffer system described by Laemmli (61). Gels containing 4 percent (stacking gel) and 8-12 percent acrylamide were prepared by polymerizing a solution containing acrylamide and N,N'-methylene bis acrylamide at a ratio of 30:0.8 by the addition of 0.05 percent (w/v) N,N,N',N'-tetramethylenediamine and 0.05 percent (w/v) ammonium persulfate. The separation slab gel (144 cm^2) contained 0.1 percent SDS in 0.5M Tris buffer (pH 8.8) while the stacking gel contained 0.1 percent SDS in 0.14M Tris buffer (pH 6.8).

The 100 µl of sample was layered directly into each well and the electrophoresis was run at 15 mA for the first hour, 22 mA for the second hour and then at 30 mA until the bromophenol blue marker reached the bottom of the gel (about 3 hours). Gels were stained overnight with 0.3 percent solution of Coomassie brillant blue R-250, prepared in 10 percent acetic acid, 45 percent distilled water, and 45 percent ethanol and destained by repeated washings in an aqueous solution containing 10 percent acetic acid and 25 percent ethanol. The molecular weight (mol. wt.) of viral polypeptides was estimated by the method of Weber and Osborn[62]. Protein markers included phosphorylase a (mol. wt. 94,000), transferrin (76,000), egg albumin (45,000), deoxyribonuclease (31,000), trypsinogen (24,000), β-lactoglobulin (18,400) and ribonuclease (13,700).

Electron Microscopy

Electron microscopy was used to detect CE virus in skin lesions, confirm the identification of CE virus in cell culture, assess the purity of virus preparations for SDS-PAGE and monitor the controlled degradation of the CE virus. To release CE virus from skin biopsies, the tissue was ground in a Ten Broeck grinder, with 5ml PBS followed by sonication at the maximum setting on ice for 1 minute. Virus was released from cell cultures by freezing and thawing 3 times or disrupting the cell monolayer with a rubber policeman and sonicating the suspension for 1 minute.

Cellular debris from skin or cell culture suspensions was pelleted by centrifugation at 600 xg for 10 minutes and the supernatant fluids were concentrated by centrifugation in a Ti70 rotor at 35,000 xg for 30 minutes. Pellets were resuspended in one or two drops of distilled water, and a drop of the suspension was deposited on a carbon-coated nitrocellulose film on a electron microscope (EM) grid. The EM grid had been pretreated with 0.01 percent bovine serum albumin (BSA) to ensure that the film was hydrophilic. After the virus suspension had remained on the grid for 5 minutes the excess fluid was drawn off with filter paper and the grid allowed to air dry. Grids were stained with a drop of 0.5 percent uranyl acetate for 30 seconds, blotted and air-dried.

When skin lesions consisted of papules or vesicles a needle was inserted into the lesion and the point of the needle then touched to a drop of distilled water on a waxed slide. The drop of specimen-contaminated water was placed on a EM grid pretreated with BSA and as described for the skin biopsy and cell culture grid specimens.

To assess the purity of virus preparations and monitor the controlled degradation, samples were placed directly on BSA pretreated grids and

processed as described above. The grid specimens were examined in a JEOL 100C electron microscope and specimens were photographed at magnifications of 20,000 - 100,000X.

CHAPTER 1: CONTAGIOUS ECTHYMA INFECTION OF SHEEP: PATHOGENESIS AND EPIDEMIOLOGY

RATIONALE

The host response to a viral infection involves many non-specific components, including resistance of individual host cells, macrophages and leukocytes to infection, local factors such as temperature, formation of interferon, and specific components involving humoral and cell-mediated immune responses (63). In spite of many investigations aimed at understanding the measurable immune reactions to CE virus, the mechanisms responsible for recovery from, and resistance to CE viral infections have not been clarified. An understanding of these immune mechanisms would be helpful for developing optimal vaccination methods against the disease.

To reach this goal studies were conducted to compare the cell mediated and humoral immune responses induced by the inoculation and reinoculation of CE virus and to determine how these immune parameters correlate with the clinical manifestations of CE. Whole blood lymphocyte blastogenesis in response to CE antigen was used as an indication of a cell-mediated immune response and virus neutralizing (VN) antibody titers were used as a measure of the humoral immune response.

Since mononuclear phagocytes have been shown to play a significant role in the pathogenesis of infections by a number of viruses including poxviruses (64). It appeared important for this study to determine if CE virus can replicate in macrophages. This knowledge would be helpful in establishing the feasibility of parenterally administering live CE vaccines,

since macrophages have been described to limit virus spread. Several authors (64, 65, 66) have observed that strains of poxviruses which replicated poorly in host macrophages tended to produce self-limiting diseases, whereas those that replicated readily often produced fatal disease. The studies included the comparison of virus replication in macrophages from CE-inoculated and uninoculated sheep. Buffy coat cells were used as a practical source of macrophages since these preparations contain a high proportion of monocytes (67) and the cells are easily obtainable.

Conflicting evidence exists regarding the protection of lambs against CE infection by colostral immunity (9, 50). Hence, the value of colostral immunity for protection of lambs against CE was investigated. Ewes which had been vaccinated against CE six months prior to parturition were used for these experiments.

Spontaneous recrudescence of latent CE infections could be a mechanism for the initiation of new outbreaks of CE, particularly with the transfer of CE-vaccinated or naturally infected sheep to new flocks. A study was undertaken to investigate the possibility of latent CE infections in sheep by administering synthetic corticosteroids which can induce the recrudescence of many latent viral infections (68).

Many studies and field observations indicate that CE vaccination does not assure protection. Outbreaks of CE in vaccinated sheep have been attributed to many different causes. Peddie (69) suggested that the majority of failures were due to improper administration of CE vaccine. The efficacy of the current CE vaccination technique was evaluated by vaccination and revaccination of 2 large groups of sheep and the immune parameters and size of the vaccination lesion were closely monitored to

determine if a single parameter could be used to predict the effectiveness of a CE vaccine.

EXPERIMENTAL DESIGN

Immune Response of Sheep to CE Virus

Three 8 months old lambs were inoculated with the ST viral isolate in scarified skin in the left axilla. Five additional lambs were inoculated in a similar manner with the Cu viral isolate. Both groups of lambs were reinoculated with a CE virus (ST isolate) in scarified skin in the right axilla four weeks after the first inoculation. Blood samples were collected for serologic and lymphocyte transformation studies on 0, 2, 4, 7, 10, 14, 21, and 28 days post-inoculation (PI) and at the same intervals after the reinoculation. Development of the clinical lesions was followed by examination of the lambs at two day intervals.

Virus-neutralizing antibody titers to ST virus were calculated for sera from the group of 3 lambs, while VN antibody titers to Cu virus were calculated for sera from the other group of 5 lambs. In the lymphocyte transformation studies, the appearance of sensitized lymphocytes to ST and Cu viral antigens were measured after the inoculation and reinoculation.

Replication of CE Virus in Buffy Coat Cells

The ability of buffy coat cells to support CE virus replication was measured in two experiments in cells collected from lambs inoculated with CE virus and from uninoculated lambs. In the first experiment, buffy coat cells were obtained from the blood of a lamb 35 days after inoculation with the Cu virus and from an uninoculated lamb. Twenty-four hours

after seeding the buffy coat cells in 24-well tissue culture plates, Cu virus was added to the cells and duplicate samples of the cells were harvested on days 0, 1, 2, 3, 4, 6, 8, and 10 after the addition of virus. Controls which included buffy coat cells without virus and virus without cells were harvested at similar intervals. The concentration of virus was determined in virus titrations with end-points read after 7 days incubation.

In the second experiment, blood samples were collected from four uninoculated lambs and again two weeks later after two of these lambs had been inoculated with the CSL virus. The CSL virus was added to the 24-hour buffy coat cell cultures and the cells were harvested on days 0, 2, 3, 4, 6, 8, and 10 after the addition of virus. Virus titrations were read after 6 days and rechecked after 10 days incubation.

Colostral Immunity

In the first of two experiments, sera were collected from seven 1-2 day old lambs from CE-vaccinated ewes and from four lambs of a similar age from non-vaccinated ewes. Colostrum and serum samples were taken from the ewes at the same time. The vaccinated ewes had been inoculated with Cutter Animal Health Laboratories CE vaccine six months prior to the birth of the lambs and all lambs were separated from the ewes when 1 to 2 days old and fed milk-substitute. A natural CE disease outbreak occurred when the lambs were 5 to 8 weeks of age and delayed-type hypersensitivity tests to CE virus were performed on the lambs 3 to 4 weeks after the onset of this outbreak.

In a second experiment all the lambs were born to ewes vaccinated as described above, but the lambs suckled the ewes throughout the 12 weeks

of the experiment. At 6 to 8 weeks of age these lambs were inoculated with a CE virus isolate (ST or CSL) prepared from infectious scab material. Neutralizing antibody titers to the Cu virus were determined for serum and colostrum samples for the two experiments.

The Spearman Rank Correlation Test (70) was used to determine if there were correlations between the level of antibody in the sera of the lambs and their dams and between the serum antibody titers of the lambs and their ability to resist CE infection.

Latent CE Infections

The experiment design used for the investigation of latent CE infections in sheep is presented in Table 8. The lambs were exposed to CE virus at 7-8 months of age in experiments A, B and C and at 5-8 weeks of age in experiments D and E. Corticosteroid treatment consisted of the inoculation of 0.15 mg/Kg dexamethasone[a] intramuscularly on 5 consecutive days, except in experiment B where the inoculations were intravenous. In addition to the parenteral dexamethasone, in experiments A, C and E 0.5 percent hydrocortisone cream[b] was applied to the original CE inoculation sites on each of the 5 treatment days.

In experiment A, blood samples were collected from the lambs for hematologic, serologic and lymphocyte transformation studies prior to, during and after the corticosteroid treatment. Lambs in all the experiments were examined for the development of lesions at 2 day intervals for a period of 3 weeks after the corticosteroid treatment. Isolation

[a]Cutter Animal Health Laboratories, Shawnee, Kansas.
[b]Dow Hickman Inc., Houston, Texas.

and identification of CE virus from lesions was attempted by direct culture on testis cell monolayers, coculture of tissue fragments, tissue fragment culture, inoculation of sheep and examination of biopsy samples by electron microscopy.

Vaccination Studies

Two studies were conducted with data obtained from the CE vaccination and revaccination of 280 one year old rams at the Shenandoah Valley Research Station. One study was conducted in 1979 and one in 1980. The rams originated from a number of farms and many had received a CE vaccination 4-8 months prior to arrival at the station. One day after arrival the rams were vaccinated with a CE vaccine (Cutter Animal Health Laboratories) by applying 2 drops of reconstituted vaccine to a scarified area of skin in the right axilla. The vials of vaccine were reconstituted to the volume recommended by the manufacturer and one vial was sufficient to vaccinate 80 to 100 rams. The rams were revaccinated, in the left axilla 5 weeks after the first vaccination. Fresh vials of vaccine were used for revaccination.

The vaccination lesions were inspected 9 days after the initial vaccination and 14 days after revaccination. In the 1979 study, diameters of vaccination lesions were grouped into 4 size ranges, whereas in the 1980 study the diameters of lesions were measured in millimeters (mm). In the 1980 study blood samples were randomly collected from a group of rams which had not been vaccinated with CE prior to the vaccination trial. These samples were collected on days 0, 9, 13, 16 and 23 after the initial vaccination and were used for serologic and lymphocyte transformation studies.

The Chi squared test was used to determine if CE vaccinated sheep developed smaller lesions following CE inoculation compared to non-vaccinated sheep and if the diameter of the primary lesion was related to the number of sheep with a lesion after CE reinoculation. The Spearman Rank Correlation test was utilized to decide if there were significant correlations between the parameters illustrated in Table 14.

RESULTS

Immune Response of Sheep to CE Virus

Development of the clinical lesions, the appearance of VN antibody response and increase in virus-specific lymphocyte stimulation indices (SI) are compared in Figure 1. Experimental infection of lambs with the ST and Cu viruses resulted in similar lesions. Three to four days following CE virus inoculation a zone of inflammation was seen in and surrounding the scarified area and in some cases small red inflamed areas appeared along the lines of scarification. Within 24-48 hours small circular papules developed in the zone of inflammation (Figure 2). Forty-eight to seventy-two hours later, small vesicles filled with clear fluid were noted protruding from the papules. The amount of vesicular fluid rapidly increased and became pustular in character (Figure 3). The pustules ruptured between 10-13 days post inoculation (PI), resulting in a raw and intensely inflamed area. A thick scab rapidly covered the ulcerous area (Figure 4). Scabs dried up and dropped off 13-23 days after scab formation had started and left the areas smooth and without scars other than the marks of the scarification. The disease process lasted 23-33 days.

When the two groups of lambs were inoculated with the ST virus 4 weeks after exposure to the ST or Cu virus no clinical signs of disease were detected in any of the 8 lambs.

Results of the virus neutralization tests are presented in Table 1 and the mean values are illustrated in Figure 5. Neutralizing antibody was not detected prior to the first CE inoculation of the lambs. Antibody was first detected between 10-14 days PI and the highest titers developed between 14-28 days (median 21 days) PI. The VN antibody response was similar for the lambs inoculated with the Cu or ST virus.

A secondary antibody response was observed in both groups of lambs after reinoculation with CE virus. The highest antibody titers occurred between 4-21 days (median 9 days) PI, however, only 2 lambs (2054 and 2237) developed higher titers during the secondary antibody response compared to the primary response. Twenty-eight days following the second CE inoculation all the antibody titers had decreased to low levels.

The inoculation of the Cu virus in 5 lambs resulted in a rapid increase in the mean whole blood lymphocyte SI to the Cu viral antigen (Figure 6). Individual stimulation indices indicated that the increase occurred between 10-14 days (median 10 days) PI and the highest indices were recorded between 10-21 days (median 10 days) PI (Table 2). For the 3 lambs receiving the ST primary inoculation the increase in the stimulation indices to the ST virus antigen and highest indices were observed 14 days (median 14 days) PI (Table 3). The lymphocyte transformation responses to Cu and ST viral antigens for the 2 uninoculated sheep revealed that the stimulation indices ranged from 0.6 - 1.9 during the 28 day sampling period (Table 4).

When sheep previously inoculated with Cu virus were inoculated 28 days later with ST virus there was a rapid increase in the mean lymphocyte SI to Cu viral antigen 4 days PI (Figure 7). The highest stimulation indices for the individual sheep occurred between 2-21 days (median 4 days) PI (Table 5). The only response to the ST viral antigen was 14 days PI, however, low mean cpm were noted for the control antigen in all 5 sheep on this day. The reinoculation of the ST virus in three sheep, 28 days after the primary ST inoculation did not induce an increase in the lymphocyte stimulation indices to the ST or Cu viral antigen.

Replication of CE Virus in Buffy Coat Cells

Buffy coat cells from a CE-inoculated and uninoculated sheep supported CE virus replication in culture with 7.5-fold increases in virus titer (Figure 8). The most rapid increase in virus titer occurred between 1-3 days after addition of virus in the cells from the uninoculated sheep and between 6-8 days in cells from the CE-inoculated sheep. In the absence of cells the virus titer decreased by 3 fold after 10 days incubation at 37°C. No virus was isolated from the cell controls.

In the second experiment, cells from the control sheep supported CE virus replication with increases in virus titer ranging from 3-17 (11.7 mean)-fold (Figure 9). The most rapid virus replication occurred between 3-6 days after the addition of virus. Cells collected 14 days after inoculation of the 2 sheep with CE virus still supported virus replication, but the increases in virus titer were only 1.7 and 3-fold. The 1.7-fold increase in virus titer was not a sustained increase and the 3-fold increase was a delayed response occurring 4-8 days after the addition of virus.

In the absence of cells the virus titer decreased 6-fold after 10 days incubation at 37°C and no virus was isolated from the cell controls. Following 6 days incubation the cells in the control wells became enlarged and spindle or stellate shaped. The virus-infected cells did not change in shape throughout the 10 days of incubation.

Colostral Immunity

As seen in Table 6 there was significant positive correlation ($P < 0.05$) between the levels of antibody in the sera of the lambs and their dams. Only 3 ewes had colostral VN antibody titers of greater than 1:30 and these ewes had the highest serum antibody titers. Serum antibody titers of the lambs had decreased to very low levels by 5-6 weeks. When they were 5-8 weeks old a natural CE outbreak was detected in the lambs. The lambs were housed in adjacent rooms (5-6 lambs/room) and the infection spread to both rooms within 3 days. Nine of the eleven lambs developed lesions on the lips and/or muzzle within one week of the start of the outbreak, but after a further 3 weeks the majority of the lesions had healed completely. No lesions were observed in 2 of the lambs throughout the study.

The severity of the lesions varied from small lesions (less than 10 mm in diameter) on the lips or muzzle (Figure 10) to multiple larger lesions (10-20 mm diameter) covering the muzzle and lips (Figure 11). The severe lesions were only seen in 2 lambs and these lambs were born to non-vaccinated ewes. One lamb had received no colostrum while the dam of the other lamb had a low level of serum VN antibody. There was no correlation between the antibody titers of the lambs at 1-2 days of age and the ability of lambs to resist infection at 5-8 weeks of age.

Biopsy samples taken from lesions of 3 lambs were examined by direct electron microscopy and virus isolation was attempted in lamb testis monolayers. Parapox virions were observed by direct electron microscopy in a sample from one lamb (Figure 12) and after 3 passages in lamb testis monolayers CE virus was isolated from lesions of all 3 lambs.

Delayed-type hypersensitivity skin tests were carried out on 8 of the lambs three weeks after the start of the CE outbreak and 7 of the lambs gave a positive reaction to the CE antigen. A positive reaction was represented by erythema at the site of the intradermal inoculation and a 2-4 fold increase in skin thickness after 24-48 hours. There was no reaction to the control antigen. Lamb 2079, which had no detectable skin lesions, gave a positive skin test reaction, suggesting exposure to CE virus and one lamb (902) with skin lesions failed to react to the CE antigen.

In the second experiment there was a significant positive correlation ($P < 0.01$) between levels of antibody in the sera of the lambs and their dams (Table 7). When 6-8 weeks old, the lambs were inoculated with ST or CSL virus. All lambs developed lesions 5-10 mm in diameter at the site of inoculation. Direct electron microscopy of biopsy samples from these lesions revealed large numbers of parapox virions.

Latent CE Infections

Two sheep previously inoculated with ST virus in experiment A developed lesions at the inoculation sites 7 and 10 days after the start of the corticosteroid treatment (Table 8). The lesions commenced as small circular zones of inflammation (1-2 mm diameter) and developed into small

red papules (2 mm diameter). Distribution of the papules was identical to the location of the original ST viral lesions (Figures 12 and 13). Seventeen days following the start of corticosteroid administration the lesions developed into scabs and biopsy samples were taken.

Examination by direct electron microscopy and 3 passages in lamb testis monolayers failed to reveal the presence of CE virus. Triturated samples of the skin biopsies were inoculated in scarified skin of a 3 months old lamb. No lesions developed after this inoculation and 18 days later the lamb was inoculated with ST virus. The lamb was shown to be fully susceptible to challenge with ST virus as compared with control lambs inoculated with ST virus.

No differences were observed in the lymphocyte transformation response to CE viral antigen and in the VN antibody response in CE-inoculated lambs treated or not treated with corticosteroids (Figures 15 and 16). Changes in the leukocyte count were monitored in sheep which received corticosteroids and those without this treatment. The mean absolute neutrophil count for the corticosteroid-treated animals increased two-fold 2 days after starting treatment, but returned to the pretreatment levels 5 days thereafter (Figure 17). Individual leukocyte counts are presented in Table 9. The mean absolute neutrophil count for the control group and the mean absolute lymphocyte counts for corticosteroid treated and control groups did not change significantly throughout the experiment.

Recrudescence of lesions following corticosteroid treatment was observed in experiment E where 3 of 7 treated lambs developed lesions at the site of the original ST virus inoculation. Small papules (1 mm diameter) were detected at the inoculation sites 13 days following the start

of corticosteroid treatment. After a further 2 days skin biopsies were taken from these sites. The biopsies were tested for virus unassociated with cells by direct culture in lamb testis monolayers and tested for the presence of persistent virus by tissue fragment culture and coculture of fragments with lamb testis monolayers. No virus was isolated by these techniques and no virions were detected by direct electron microscopy. Tissue from the biopsies was inoculated in scarified skin of a lamb. No lesions developed and this lamb was later found to be susceptible to infection with ST virus.

Vaccination Studies

In the 1979-80 studies, 271 of 280 rams developed lesions equal or greater than 2 mm in diameter at the site of CE vaccination. The lesions were significantly smaller for rams vaccinated 4 to 8 months previously ($P<0.001$, $P<0.05$) (Tables 10 and 11). The mean size of vaccination lesions could only be calculated for the 1980 study. Rams vaccinated on their farm of origin had a mean lesion diameter (plus or minus standard error) of 7.4 ± 0.5 mm while the remaining rams had a mean lesions diameter of 10.5 ± 0.8 mm.

Revaccination of the rams 5 weeks after the first vaccination resulted in lesions ($\geq$ 2 mm diameter) in 21 of 150 rams in the 1979 study and in 28 of 130 rams in 1980. In the 1980 study the mean lesion diameter following revaccination was 3.8 ± 0.1 mm. There was no relationship between size of lesion from the first vaccination and development of a lesion after revaccination (Tables 12 and 13).

The clinical response to CE vaccination and revaccination and influence of the primary vaccination on virus-specific lymphocyte transfor-

mation and VN antibody responses are presented in Table 14. The maximum lymphocyte SI at 9 and 13 days PI for the individual sheep did not relate to the diameter of the primary or second vaccination lesion. However, it is interesting to point out that the 3 sheep (57, 107, 114) which had indices greater than 2 on day 9 or 13 PI and had large primary vaccination lesions (>5 mm diameter) developed no significant lesions (<2 mm diameter) after the second vaccination. There was no relation between diameters of lesions produced after the primary or second CE vaccination. Further, the maximum VN antibody titer at 16 and 23 days PI for individual sheep did not relate to the maximum lymphocyte SI at 9 and 13 days PI, the diameter of the primary lesions or diameter of lesions from the second vaccination.

DISCUSSION AND CONCLUSIONS

Initial detection of VN antibodies and an increase in the virus-specific lymphocyte SI occurred in the same period, 10-14 days PI. However, the median value for the peak SI occurred 7 days before the peak antibody titer for the ST and Cu viral infections. The rapid onset of the peak for the virus-specific lymphocyte transformation response was consistent with observations for vaccinia (71), infectious bovine rhinotracheitis (63) and influenza viruses (72).

Lymphocyte transformation in response to an antigen in vitro does not exclusively reflect T-cell reactivity and in certain systems stimulation of B-cell lymphocytes may also occur (73, 74). However, the test is usually considered primarily to reflect cell-mediated immunity (CMI)

and has been applied and recommended by several workers to study CMI in viral infections (68, 73, 75).

Healing of CE lesions was considered to commence with the development of scabs 10-13 days PI. Hence, the rapid onset of the peak for the lymphocyte transformation response suggests that the start of the healing process more closely corresponds to an increase in CMI than to humoral immunity. However, both CMI and humoral immunity may be important in recovery from CE infection, since the first increase in lymphocyte transformation and antibody responses was detected 10-14 days PI.

The peak VN antibody titers for the primary CE infections were very low and antibody titers were only detected when a small quantity of virus was used in the test (10-30 $CCID_{50}$/0.05 ml). After reinoculation with CE virus the secondary antibody response was very variable, but all animals had a rise in antibody titer.

There is little published information on the immune response to CE virus and considering the similar vaccination procedure used with CE and vaccinia viruses, it is advantageous to compare the findings of this study to the immune response to vaccinia virus. Protective immunity to smallpox has been correlated with VN antibody to vaccinia virus (76), although it has also been noted that previously vaccinated individuals without VN antibody have apparently been protected when exposed to natural disease (77). Revaccination with vaccinia has been shown to produce slightly higher antibody levels than in the primary response (78).

The increase in the virus-specific lymphocyte stimulation indices after Cu virus inoculation occurred predominantly 10 days PI, while the increase after ST virus inoculation occurred 14 days PI. Testing larger

numbers of animals and more frequent sampling would be required to determine if the onset of the increase in the virus-specific lymphocyte SI was dependent on the viral isolate used for the inoculation.

The difference in the lymphocyte stimulation indices for ST and Cu antigens following reinoculation with a heterologous virus (ST) was not expected and may be due to a difference in the presentation of the antigens on the virions. The absence of a secondary response in the group of sheep receiving 2 inoculations of the ST virus was also not anticipated. VN antibody was detected in the plasma of these immunized animals and the use of whole blood cultures in the lymphocyte transformation assay may have resulted in the formation of antigen-antibody complexes. Scriba (79) observed that the stimulation indices to herpes simplex virus (HSV) could be reduced by the addition of anti-HSV antibodies.

Buffy coat cells collected from sheep supported the replication of CE virus in vitro. Virus replication was not as rapid as reported for many poxviruses such as vaccinia and myxoma viruses where 100-fold increases in virus titer have been recorded within 48 hours of incubation with rabbit peritoneal macrophages (66). The normal physiology of the buffy coat cells appeared to be affected with the CE virus-infected cells losing the ability to enlarge and form a spindle or stellate shape after 6 days of incubation. The results suggest that CE virus replicates in macrophages and parenterally administered live vaccines might induce an enhanced immune response.

Incubation of CE virus with buffy coat cells from CE-inoculated sheep resulted in a less rapid increase in virus titer. Schultz et al. (80) observed that although vaccinia replicated in macrophages obtained from

the peritoneal cavity of normal rabbits, cells obtained from rabbits infected with vaccinia virus did not support replication of the virus *in vitro*. This inhibition of virus replication was lost if the macrophages were incubated *in vitro* 7 days before being exposed to virus. The increase in CE virus titer 4 to 8 days after the addition of virus to CE-inoculated sheep cells could also represent a loss of the restriction of virus replication in cells with prolonged incubation at 37°C. The restriction in vaccinia virus replication in activated macrophages has been found to be a block in a late step of virus replication (81). In activated macrophages viral antigens were produced, but very few virus particles were formed.

The comparative small 3-6 fold decreases in virus titer after 10 days incubation at 37°C in the absence of cells emphasizes the resistance of CE virus to the extracellular environment.

Antibodies absorbed from colostrum failed to protect lambs against natural or experimental exposure to CE virus at 5-8 weeks of age. Although there was a correlation between the level of antibodies in the sera of lambs and of their dams, the serum antibody levels in the lambs decreased to low levels by 5-6 weeks of age and this may be the cause for the low protection at this age. Vaccination of ewes at the end of the previous milking period or 1-2 months prior to parturition may result in higher antibody levels in colostrum and a longer period of protection for the lambs (82).

The two lambs which developed severe lesions during the CE natural outbreak received no colostrum or colostrum from a ewe with very low levels of serum VN antibody. This suggests that transfer of colostral VN anti-

bodies may be important in decreasing the severity of CE infection. An outbreak of CE was reported at the Sheep Unit, Virginia Tech in lambs born to ewes vaccinated with CE virus 6 months prior to parturition. Forty percent of the lambs developed clinical lesions which healed rapidly and very few severe lesions were observed compared to previous outbreaks when the ewes had not been vaccinated. These observations further support the view that high levels of colostral antibodies may decrease the severity of clinical lesions in lambs, although the less severe lesions may be reflecting an outbreak due to a less virulent strain of CE virus.

Recrudescence of lesions occurred at the sites of previous CE virus inoculation following dexamethasone treatment, but no virus could be isolated and the existence of latent CE infections could not be confirmed. The development of a zone of inflammation, papules and scabs suggested a possible CE viral infection. However, recrudescence of a bacterial infection originating from the secondary infection of the initial CE lesion could not be excluded as a possible cause of these new lesions. No recrudescence of lesions was observed when more than 75 days elapsed between CE inoculation and dexamethasone treatment, after a natural CE infection, in CE-inoculated sheep not treated with dexamethasone or in uninoculated sheep.

Neutralizing antibody levels did not increase after recrudescence of lesions but this does not exclude CE virus as the etiological agent. Bader et al (83) reported that following the spontaneous recrudescence of herpes simplex viral lesions in man, only 4 of 41 patients developed a four-fold or greater rise in antibody titer. The absence of an increase in the virus-specific stimulation indices following the recrudescence of

lesions may also not be significant since increases in these indices were not always seen after reinoculation of a CE virus.

The neutrophilia observed 2 days after the start of the cortiocosteroid treatment was a normal response to the injection of dexamethasone. Duncan and Prasse (84) have reported that corticosteroids cause a mobilization of marginated neutrophils, the impediment of random loss of neutrophils from blood and an increased rate of release of neutrophils from the bone marrow.

In the study to investigate the efficacy of the CE vaccination procedure, vaccination of sheep did not produce absolute immunity against reinfection by inoculation of CE virus. The majority of rams vaccinated 4-8 months previously developed lesions 2 mm diameter or larger following CE virus inoculation. Virus isolations from vaccination lesions had earlier demonstrated that large amounts of CE virus could only be isolated from lesions 2 mm or larger diameter. Lesions less than 2 mm diameter may not have resulted from the CE infection and may have been caused by an immune reaction or bacterial infection. Vaccination 4-8 months previously induced an immune response which resulted in smaller lesions after CE inoculation compared to lesions seen in non-vaccinated sheep.

The CE vaccination procedure used at the Research Station was very effective in inducing a CE lesion ($\geq$ 2 mm diameter) at the site of inoculation. Only 1 of 164 nonvaccinated sheep failed to produce a CE lesion following the CE vaccination. However, 5 weeks after the CE vaccination 17 percent of the 280 rams were susceptible to CE challenge. It is unknown if the susceptible population represents the vaccinated animals which cannot be protected or that five weeks post-vaccination represents the time at which protection is rapidly decreasing.

The size of the primary vaccination lesion, the maximum VN antibody titer at 16 and 23 days PI and the maximum virus-specific lymphocyte SI at 9 and 13 days PI could not be used to predict the clinical reactions following revaccination. CE vaccination induced an increase in the VN antibody titer in all sheep tested, but only 3 of 10 sheep produced virus-specific lymphocyte stimulation indices of greater than 2 following CE vaccination. The difficulty of predicting clinical reactions following revaccination with vaccinia virus using a variety of tests measuring humoral and cell-mediated immunity has been reported by Moller-Larsen and Haahr (85).

Study of the immune response to CE virus infection indicated that both CMI and humoral immunity may be important in recovery from infection. However, measurement of the immune parameters suggested that there was a minimal systemic immune response following CE virus inoculation. Reinoculation of CE virus induced a secondary immune response in most animals, but the response was very variable. Buffy coat cells from sheep supported the replication of CE virus, although there was a restriction in CE virus replication in cells collected from sheep previously inoculated with CE virus.

Vaccination of ewes with CE virus, 6 months prior to parturition, did not result in the induction of sufficient colostral immunity to protect the lambs from subsequent exposure to CE virus. However, the severity of CE lesions in the lambs may have been reduced. Dexamethasone treatment induced the recrudescence of lesions at sites of previous CE virus inoculation, but no virus could be isolated from these lesions. Existence of latent infections could not be confirmed and it is unlikely that latent infections are important for the initiation of CE disease outbreaks.

The CE vaccination procedure was shown to be effective in inducing a CE lesion, however, the protection induced by vaccination was not absolute. Size of the primary vaccination lesions, VN antibody titers and virus-specific lymphocyte stimulation indices could not be used to predict clinical outcome following revaccination.

CHAPTER 2: CLASSIFICATION OF CONTAGIOUS ECTHYMA VIRAL ISOLATES

RATIONALE

Immunity resulting from vaccination with CE virus is well recognized (7, 9), but the appearance of an eruptive disease similar to CE in vaccinated animals can raise questions about possible variation of antigenic types of CE virus. In a comparison of 5 European and Asian isolates of CE virus, Sawhney (21) concluded that there were antigenic differences. No detailed studies have been carried out to determine if there are antigenic differences among CE viral isolates in the U.S.A. and this study was undertaken to investigate the question. Cross virus-neutralization tests have been used extensively to study antigenic relationships between viral strains (86) and this test was used in the present study to compare 5 CE viruses isolated from the U.S.A.

Reactions of delayed-type hypersensitivity (DTH) provide a sensitive *in vivo* technique for measuring cell-mediated immunity (CMI) to microbial infections (87). Tizard (88) reported that a comparative DTH test employing avian and bovine tuberculins inoculated at separate sites could distinguish animals infected with *Mycobacterium avium* or *M. paratuberculosis* from those infected with *M. tuberculosis* or *M. bovis*. An investigation was undertaken to decide if the DTH test could measure immunity to CE virus and if a comparative contagious ecthyma DTH test could be used to classify CE isolates into groups.

The identification of the CE isolate, using an in vivo test would greatly facilitate the determination of the source of CE disease outbreaks.

Recently, Arita and Tagaya (89) analyzed the structural polypeptides of a number of poxviruses using SDS-polyacrylamide gel electrophoresis (PAGE). Variation within cowpox and vaccinia strains was detected by comparing the profiles of the structural polypeptides. A comparison of the structural polypeptides of eleven CE viral isolates was carried out to determine if this method could be used to detect differences among CE viral isolates.

EXPERIMENTAL DESIGN

Virus-Neutralization Tests

The effects of heat-inactiviation of antiserum (56°C, 30 min) and the addition of complement (5 percent guinea pig serum final volume) were investigated to determine optimal conditions for the virus-neutralization test. In the cross-neutralization test, 5 viral isolates (ST, CSL, Har/2, Am, Cu) which had been passed 8 to 12 times in lamb testis cells and virus inocula of 8-25 $CCID_{50}$/0.05 ml were used for the tests. For analysis of cross-neutralization test results a method similar to that described by Archetti and Horsfall (90) was used. This analysis permits the calculation of the unilateral and bilateral relationships between 2 viruses by initially determining the titer ratios. The ratio (r_1) was found by dividing the heterologous titer obtained with virus 2 by the homologous titer obtained with virus 1, and the ratio (r_2)

was found by dividing the heterologous titer obtained with virus 1 by the homologous titer obtained with virus 2. Ratios r_1 and r_2 represent the unilateral relationships between viruses 1 and 2, while the bilateral relationship is given by $R = (r_1 \times r_2)^{\frac{1}{2}}$. Because the ratio R is the geometric mean of the two ratios obtained with the heterologous viruses and the homologous sera, it yields a value which gives equal weight to differences found in either direction.

Delayed-Type Hypersensitivity (DTH) Tests

The appearance and development of clinical reactions were recorded after intradermal inoculation of control and CE viral antigens in sheep previously inoculated with CE virus. The effect of varying the concentration of CE viral antigen was also studied. Sheep were tested 1 to 13 months following inoculation of CE virus to measure the duration of detectable sensitization. Lambs exposed to a natural CE outbreak were also tested.

In the comparative DTH tests 1/10 dilutions of 5 CE viral isolates (ST, CSL, Cu, Har/1, Am) were prepared in PBS and tested in CE-inoculated and uninoculated sheep. The diluted viral preparations contained 10^5 to 10^6 $CCID_{50}$/ml prior to heat inactiviation. The control antigen was prepared from lamb testis cell cultures. Three viral antigens and a control antigen were tested on the medial surface of both hind legs of each sheep using the inoculation pattern illustrated in Figure 18. To insure that there were no differences in skin sensitivity in the 2 areas tested, 1 viral antigen and the control antigen were inoculated in both areas.

Analysis of Contagious Ecthyma Virus Structural Polypeptides

Two viral isolates (Har/2 and Cu) were repeatedly passed in lamb testis cells to determine if there was phenotypic stability with passage in cell culture. Analysis of the structural polypeptides of the eleven CE viral isolates was carried out using virus which had been passed 5 to 12 times in lamb testis cells. To identify some of the polypeptides observed in the polyacrylamide gels, purified suspensions of 2 isolates (Cu and Pu) were subjected to controlled degradation using Nonidet P-40 and 2-ME and the polypeptides released were compared to untreated virions by SDS-PAGE. Surface tubules released from virions by this degradation method were partially purified to allow identification of their position in the SDS-polyacrylamide gels.

RESULTS

Virus-Neutralization Tests

Heat-inactivation of antiserum decreased the VN antibody titers to very low levels (Table 15). However, the addition of 5 percent guinea pig serum as a source of complement restored the VN titers to levels only slightly below those detected prior to heat-inactivation. Titration of guinea pig serum did not reveal evidence of direct virus neutralization, although the concentration of viable virus calculated in the virus titration was lower in the presence of complement than without complement. The investigations of heat-inactivation of antisera and addition of complement were repeated for 3 other viral isolates (ST, Cu, Har/2) and the results were very similar to those illustrated

in Table 15. In some tests the addition of complement decreased the concentration of viable virus by more than $10^{0.5}$ $CCID_{50}$/ml. Since the amount of virus used in the virus-neutralization test was very low (8-30 $CCID_{50}$/0.05 ml), the addition of complement made the calculation of the end-points for the VN titer very difficult.

Results of the cross-neutralization tests are presented in Table 16. For these tests the antisera were not heated and no complement was added. The homologous titer for the Cu viral isolate was only 1:20 and this level was considered too low for inclusion of this isolate in the cross-neutralization tests. There were large differences in the homologous titers among the remaining isolates but all were neutralized to some extent by all of the antisera. Titer ratios (heterologous titer divided by homologous titer) provide an indication of the unilateral relationships between 2 viruses. There were four titer ratios of greater than 0.25 (Table 16) and these included Har/2 antiserum with ST, CSL and Am viruses and Am antiserum with CSL virus. The only bilateral relationship greater than 0.25 was between the Har/2 and ST isolates, however this relationship was influenced by the high antibody titer between the ST isolate and Har/2 antiserum.

Delayed-Type Hypersensitivity Tests

In most cases inoculation of the control antigen produced a transient skin thickening which disappeared within 8 hours PI. By contrast detectable reactions to CE viral antigens in the CE-inoculated sheep first appeared 12 to 18 hours PI. Erythema was seen between 12 to 48 hours PI with a maximum intensity at 30 hours PI (Figure 18). Maximum

skin thickness was detected between 48 to 96 hours PI and the development of the increase in skin thickness can be observed in Figure 19. The swelling at the site of inoculation of the viral antigen was indurated.

The intensity of the skin reactions was dependent on the concentration of the viral preparation (Table 17). Two sheep which had been inoculated with CE virus 13 months prior to the skin test gave positive reactions (skin thickness at 48 hours PI/skin thickness prior to inoculation, was greater than 2). No difference was detected in skin reactions to the viral antigen in sheep inoculated with CE virus between 1 to 13 months prior to the skin test. In 3 non-infected sheep there were no skin reactions to the viral antigen. However, 3 sheep (1 CE-inoculated and 2 previously uninoculated) developed immediate-type hypersensitivity skin reactions to the control and viral antigen preparations. An area of circumscribed edema was detected at the inoculation sites one hour PI. This response was probably induced by a reaction to the lamb testis cell antigens which were present in the control and viral antigen preparations. Inoculation of cell culture growth medium containing 8 percent fetal calf serum did not induce a response in these animals.

Delayed-type hypersensitivity skin tests were performed on 8 lambs three weeks after exposure to CE virus during a natural disease outbreak. Seven lambs had developed CE lesions and six of these lambs gave a positive DTH skin reaction to CE viral antigen. The one lamb without lesions also gave a positive reaction to the CE antigen, suggesting a possible sub-clinical CE infection. None of the lambs reacted to the control antigen.

The results of the comparative DTH skin tests are summarized in Table 18. The CE-inoculated sheep gave positive skin reactions to all of the CE viral isolates tested, while there were no positive reactions in the previously uninoculated sheep. There was no correlation between the intensity of the skin reactions to the different viral isolates and the virus initially used to immunize the sheep.

Analysis of CE Virus Structural Polypeptides

SDS-polyacrylamide gel electrophoresis of purified CE virus gave a complex pattern of at least 31 polypeptides with molecular weights ranging from 200,000 to 18,000 daltons. Because different electrophoresis methods were used, direct comparison with the results of others was not possible, but the patterns were generally similar to those recently reported for parapoxviruses (89, 91).

The phenotype stability of 2 isolates was tested after the viruses had been passed more than 30 times in lamb testis cells. Passage numbers 8, 14 and 33 were compared for the Har/2 isolate (Figure 20), while passage numbers 8 and 32 were tested for the Cu isolate (Figure 21). No differences were observed between the different passages of each isolate.

An electrophoretogram of the structural polypeptides of eleven CE viral isolates in 12 percent polyacrylamide gel is shown in Figure 22. Differences were observed in the 37,000 to 44,000 molecular weight (mol. wt.) region and on the basis of these differences the isolates were divided into 4 groups. Three isolates (Pu, ST and TZ) showed a distinct band of about 37,000 daltons and 6 isolates (Am, Shoe, Har/1,

Cu PF and CSL) had a distinct band of about 44,000 daltons. Two isolates (Har/2 and Mu) formed 2 intermediate groups with the Mu isolate exhibiting a distinct band of 39,000 daltons and the Har/2 isolate showed distinct bands of 37,000 and 44,000 daltons. Three isolates (Pu, TZ and Har/1) may more correctly be grouped with the Har/2 isolate, since they possess both the 37,000 and 44,000 dalton polypeptides. However, one of these polypeptide bands was not distinct; the less distinct band for the Pu and TZ isolates was the 44,000 dalton isolate, and the 37,000 dalton band for the Har/1 isolate. These less distinct bands were observed in SDS-polyacrylamide gels when a range of concentrations of virus was used.

Profiles of the structural polypeptides of the different isolates were also compared using 8 and 10 percent SDS-polyacrylamide gels, but no additional differences were found. Five of the eleven isolates were independently purified on two occasions and subjected to electrophoresis at least two times and in each instance the electrophoretic profiles were identical.

The Cu isolate was treated with Nonidet P-40 and 2-ME and then the incubation mixtures were sonicated and fractionated by centrifugation into a pellet and supernatant fraction. The different fractions were examined by electron microscopy and are illustrated in Figure 23. The effect of the treatment was to remove the surface components of the virus particles from the subviral particles or cores (Figure 23B). The surface components did not appear to be completely solubilized and frequently remained partially associated with the viral cores. When samples of the supernatant fraction were examined by EM, threadlike

structures were observed associated with or in some cases separate from material organized into flat sheets (Figures 23C). PAGE analysis of the material in the supernatant fraction (Figure 24, column 1) showed that 11 polypeptides were released with nonionic detergent and 2-ME. The pellet fraction contained surface components partially associated with viral cores (Figure 23B) and the profile of the polypeptide bands was similar to the whole virus, except that the concentration of the 44,000 dalton polypeptide was reduced.

The material in the supernatant fraction was further fractionated as described by Stern and Dales (60) and examined by EM. Organized structures resembling the surface tubules isolated from vaccinia virus (60) and milker's node virus (91) could be observed (Figure 23D). PAGE analysis of the material present in this preparation showed enrichment of the 44,000 dalton polypeptide and only traces of the other polypeptides present before fractionation. The fractionated sample was contaminated with the nonionic detergent, resulting in smearing of the 44,000 dalton polypeptide band and the PAGE analysis is represented schematically in Figure 25.

The Pu isolate was also treated with Nonidet P-40 and 2-ME, sonicated and centrifuged. The pellet and supernatant fractions were analyzed by PAGE (Figure 26). The 37,000 dalton polypeptide, which was present in the whole virus preparation was greatly reduced in the pellet fraction. However, the supernatant fraction showed an enrichment of the 37,000 dalton polypeptide as well as showing 14 other polypeptides also released from the virions. Confirmation that the enriched polypeptide was the 37,000 dalton polypeptide was achieved by passing the

supernatant fraction through a DEAE A-50 column prior to PAGE analysis (Figure 26, column 4). Smearing of the bands was avoided and the molecular weight of the major polypeptide band was estimated to be 37,000 daltons. Comparison with the Cu whole virus preparation in Figure 26 confirmed that the 37,000 dalton polypeptide was not present in the Cu virus.

DISCUSSION AND CONCLUSIONS

Results of cross-neutralization tests did not permit the 4 CE viral isolates to be grouped distinctly. The study revealed serological cross-reactivity between the isolates, thus confirming the conclusions drawn by others (21, 35, 36). Interpretation of the antigenic relationships between the viruses was difficult because of the large variations among the homologous titers in the cross-neutralization tests. The large variations made it necessary to use the titer ratios instead of neutralizing titers to indicate cross-reactions. The homologous titers are incorporated in the titer ratios.

Although four of the titer ratios suggested cross-reactions between viral isolates, no extensive bilateral cross-reactions were apparent. The Har/2 antiserum cross-reacted with all of the heterologous viruses tested and there was a cross-reaction between the Am antiserum and CSL virus. The observance of only one way cross-reactions did not allow the classification of the isolates.

The DTH test was shown to be a sensitive test for detecting previous CE infection in sheep. However, in the comparative DTH test there was no correlation between the isolate used to inoculate the sheep and the intensity of the reactions to the different CE viral isolates. Thus, a comparative test could not be used to classify the CE isolates. The DTH reaction is probably a response to several antigens which are common to all CE viral isolates. Small variations in the antigen concentration may affect the results of a comparative DTH test. This test could be improved by using purified virus and estimating the concentration by optical density. In addition, purification of the control and virus preparations would reduce the possibility of immediate-type hypersensitivity reactions to lamb testis cell antigens.

The DTH test could be useful for diagnosing a CE disease outbreak or confirming exposure to virus in non-vaccinated lambs. The major disadvantage of the test may be that repeated skin testing in the same animal may modify the immune response, inducing or boosting the immune reaction of the animal and lead to false positives.

Analysis of the structural polypeptides of eleven CE isolates permitted the isolates to be classified into 4 groups. The profiles of the polypeptides were similar except in the 37,000 to 44,000 mol. wt. region. These results were in agreement with observations reported by Esposito et al (92) and Arita and Tagaya (89) for the orthopox group of viruses. The structural polypeptides were quite similar throughout the orthopox group, but those in the 30,000 to 40,000 mol. wt. region were characteristic of variola, monkeypox, cowpox or vaccinia viruses.

Wittek et al (36) observed that there was a small difference in the DNA restriction pattern for high and low passage CE viral isolates. However, results from the present study did not reveal differences in the structural polypeptide profiles of 2 isolates which had been passed 30 times in lamb testis cells.

The controlled degradation of CE virions with Nonidet P-40 in the presence of 2-ME did not result in solubilization of the surface components as described for vaccinia virus (59). The surface components of the CE virus remained in a highly polymerized state and, although sonication disrupted many of these complexes, some surface components remained attached to the viral cores. Eleven polypeptides were released from the Cu virions treated with Nonidet P-40 and 2-ME, whereas 15 polypeptides were released from the Pu virions. Turner and Baxby (93) observed that polypeptides from closely related orthopox viruses differed in the ease with which they could be extracted by Nonidet P-40. These authors suggested that the polypeptides may have slightly different locations on the virion or that some polypeptides may be less firmly bound in certain viruses.

The finding that the 44,000 dalton polypeptides from the Cu virion and the 37,000 dalton polypeptides from the Pu virion were located in the surface component suggested that the differences in polypeptide profiles may be immunologically important. Further fractionation of the surface components as described by Stern and Dales (60) significantly enriched the 44,000 dalton polypeptide of the Cu virion. In addition, EM analysis of the material in this fraction revealed fragments of organized structures which resembled purified surface tubules.

The milker's node virus, a parapoxvirus, has been found to possess a 42,000 to 45,000 dalton polypeptide as a major component of the surface tubule structure (91) which was similar in mol. wt. to the polypeptide fractionated from the Cu virus. This evidence suggested that differences in the polypeptide profiles of the CE isolates may represent differences in the polypeptide which is a component of the surface tubules. Isolated surface tubules of vaccinia have been shown to elicit neutralizing antibody which inhibited vaccinia infectivity (60). Hence, variations in the surface tubule polypeptides may affect the degree of cross-protection to the different CE viral isolates in sheep.

It is important to determine if the unilateral cross-reactions from the cross-neutralization tests correlated with variations in profiles of the structural polypeptides. The Har/2 isolate possessed the 37,000 and 44,000 dalton polypeptides and Har/2 antiserum cross-reacted with the ST isolate (37,000 dalton polypeptide) and CSL and Am isolates (44,000 dalton polypeptide). The absence of cross reactivity in the reverse direction may be because the 37,000 and 44,000 dalton polypeptides of Har/2 virus are associated, defining an antigenic structure which is distinct from the one given by each individual polypeptide and neutralizing antibody must be directed against both associated components to be effective. The cross-reaction between Am antiserum and CSL virus supported the structural polypeptide classification of CE isolates, however, it is not known why this cross-reactivity was not bilateral. In conclusion, cross-neutralization and DTH tests could not be used to classify CE viral isolates. However, analysis of the structural polypeptides permitted the isolates to be classified into

four groups. The polypeptides which differed among groups were shown to be located in the surface component of the virion and may be important in inducing an immune response in sheep. A correlation between the cross-reactions in the cross-neutralization tests and the structural polypeptide profiles provided further evidence that a classification of CE isolates, based on similarity of structural polypeptides is a valid grouping.

CHAPTER 3: CROSS-IMMUNITY EXPERIMENTS IN SHEEP WITH CONTAGIOUS ECTHYMA VIRUSES

RATIONALE

Cross-immunity experiments have suggested that CE viral isolates from many countries are immunologically very similar, however, these conclusions are based on information published between 1931 to 1947 (22, 33, 34). In the U.S.A. there is little current information on antigenic differences between CE viral isolates and Trueblood and Chow (5) have stated that a multiplicity of CE viral strains may play a major role in the causation of various dermal infections of sheep. In Texas CE outbreaks in vaccinated sheep have been considered to be due to the presence of strains of CE virus which are immunologically different from strains incorporated in the CE vaccine currently prepared at the Texas Agricultural Experiment Station, Sonora, Texas. This vaccine contains at least 8 strains of virus (Livingston, pers. comm.). When CE outbreaks do occur in vaccinated sheep, scab material is collected from infected animals and is incorporated into the virus stock used for vaccine production.

In three of four consecutive years (1975-78) outbreaks of CE were observed in vaccinated sheep at a ram-testing station operated by the Virginia Sheep Breeders Association. The development of lesions following vaccination of sheep suggested that the vaccine did contain viable virus and that the CE outbreak may have resulted from exposure of vaccinated sheep to a field isolate of marked virulence or of different anti-

genicity than the vaccine virus. Isolation of a CE virus from previously vaccinated sheep during the 1978 CE outbreak at the ram-testing station provided a unique opportunity to investigate vaccination failures. Investigations were undertaken to determine if sheep could be protected against the field isolate and cross-immunity experiments were designed to investigate the possible explanations of vaccination failures.

EXPERIMENTAL DESIGN

Contagious Ecthyma Virus Vaccination Experiment

The design of the vaccination experiment is illustrated in Table 19. Five 8 months-old lambs were inoculated in scarified skin of the left axilla with $10^{4.3}$ $CCID_{50}$ of cell culture-passaged ST virus. This virus had been isolated from the 1978 CE outbreak at the Virginia Sheep Breeders Association ram-testing station and had been passed eight times in lamb testis cells prior to inoculation of the lambs. The Cutter Animal Health Laboratories CE vaccine (prepared from infected scab material) was used to inoculate a further five lambs using the same inoculation technique and the inoculum contained $10^{4.0}$ $CCID_{50}$ of virus. Three uninoculated lambs served as controls.

Four weeks later, the 3 groups of lambs were reinoculated in scarified skin of the right axilla with $10^{4.8}$ $CCID_{50}$ of sheep-passaged ST virus. Lambs were examined at 2-3 day intervals following the inoculation and reinoculation.

Cross-Immunity Experiment with Sheep-Passaged CE Viruses

Seventeen 6 weeks-old lambs were assigned to 3 treatment groups. Nine lambs in Group 1 were inoculated in scarified skin of the left groin region with an undiluted and then 1/10 dilution of a sheep-passaged ST virus preparation. Five lambs in group 2 were inoculated in a similar manner with a undiluted sheep-passaged CSL virus preparation as well as 1/10 and 1/100 dilutions of the virus. Three uninoculated lambs (Group 3) served as controls.

Four weeks after the first inoculation the lambs from the 3 groups were reinoculated with an undiluted sheep-passaged CE virus preparation, plus 1/10 and 1/100 dilutions of the same virus in scarified skin of the right groin region. In Group 1, three lambs were reinoculated with ST virus, three with CSL virus and the remaining three with Har/2 virus. Three lambs from Group 2 received CSL virus and two received the ST virus. Each of the 3 viruses was also inoculated into individual control lambs.

The lambs in Groups 1 and 2 received a third inoculation of CE virus 6 weeks after the second inoculation. Inoculations were carried out in scarified skin of the left axilla with some lambs also receiving inoculations in the left groin region or on the muzzle. Lambs in Group 1 received a 1/10 dilution of the sheep-passaged ST virus preparation, while Group 2 lambs received a 1/10 dilution of the sheep-passaged CSL virus preparation.

The undiluted ST, CSL and Har/2 virus preparations contained $10^{4.8}$, $10^{4.7}$ and $10^{4.4}$ $CCID_{50}$/0.05 ml inoculum, respectively. The ST virus had been isolated from sheep during a CE outbreak, 3 months after the

sheep had been vaccinated with Colorado Serum Laboratory (CSL) CE virus vaccine.

The lambs were examined at 2-3 day intervals after the inoculations and blood samples for serologic tests were collected at various times during the study. Lesions were photographed 6 days PI and after completion of the experiment the lesions were graded on a scale of 0-5. A "0" represented no lesion or a lesion less than 2 mm diameter, "1" - one papule (2-3 mm diameter), "2" - two to four papules (2-3 mm diameter), "3" - more than four papules (2-3 mm diameter), "4" - lesion(s) of 4-7 mm diameter and "5" - lesion(s) equal or greater than 8 mm diameter. Biopsy samples were taken from CE lesions 7 days PI for virus isolation and EM examination.

The paired "t" test was used for comparing the period required for healing of lesions after different inoculations and for comparing VN antibody titers.

Cross-Immunity Experiment with Cell Culture-Passaged CE Viruses

Sixteen 6 months-old lambs were assigned to 3 groups. Lambs in Groups 1 and 2 (six lambs/group) were inoculated with an undiluted cell culture-passaged CE virus preparation and with a 1/10 dilution of the same preparation in scarified skin of the left axilla. Group 1 lambs were inoculated with ST virus which had been passed four times in lamb testis cells and the undiluted virus preparation contained $10^{5.6}$ $CCID_{50}$/0.05 ml. Group 2 lambs were inoculated with CSL virus which had been passed three times in lamb testis cells. The undiluted CSL virus preparation contained $10^{5.7}$ $CCID_{50}$/0.05 ml. Four uninoculated lambs in Group 3 served as controls.

The lambs in the 3 groups were reinoculated with an undiluted cell culture- passaged CE virus preparation as well as with 1/10 and 1/100 dilutions of the same preparation. These inoculations were made in scarified skin of the right axilla 4 weeks after the first inoculation. Each of the 3 groups was evenly divided into two subgroups. The lambs from one subgroup in each group received the ST virus as the cell culture CE virus while the remaining animals received the CSL virus.

Three lambs which had received 2 inoculations of cell culture-passaged ST virus were inoculated with a sheep-passaged ST virus. The inoculation was in scarified skin of the left axilla and was 6 weeks after the second inoculation. In a similar manner, three lambs inoculated twice with cell culture-passaged CSL virus were inoculated with sheep-passaged CSL virus.

At regular intervals throughout the study the lambs were examined and blood samples collected. The scoring of lesion sizes and the statistical analysis was as described in the first cross-immunity experiment.

RESULTS

Contagious Ecthyma Virus Vaccination Trial

Following inoculation with cell culture-passaged ST virus the five lambs developed lesions of 2 - 3 mm diameter at the site of inoculation. Lesions were also produced in five lambs after inoculation with Cutter Animal Health Laboratories CE vaccine. Lesions in these animals were 3 - 5 mm in diameter. The results of the reinoculation

of CE virus are presented in Table 19. Lesions of 3 - 7 mm diameter were observed in the three control lambs following inoculation with the sheep-passaged ST isolate, but no lesions ($\geq$2 mm diameter) were observed in the 10 immunized lambs.

Cross-Immunity Experiment with Sheep-Passaged CE Viruses

The lesion scores following the 3 CE virus inoculations are recorded in Table 20. All lambs developed lesions equal or greater than 2 mm diameter following the second CE inoculation. There was no apparent difference in the magnitude of the lesion scores for a given dose of virus after the first and second inoculations. Following the third CE inoculation 8 of the 9 lambs, which had initially received ST virus developed lesions and 2 of the 5 lambs, which had initially received the CSL virus developed lesions. For the lambs which developed lesions, the lesions were smaller than after the first inoculation.

Relatively high concentrations of CE virus were isolated from biopsy samples taken from ST and CSL virus-induced lesions equal or greater than 2 mm diameter and large numbers of parapox virions were seen in EM examination. No virus was isolated and no virions were observed from lesions less than 2 mm diameter.

Heat-inactivated CE virus preparations were inoculated in scarified skin of 7 lambs at the time of the third CE inoculation. No lesions were observed following inoculation, suggesting that lesions observed following inoculation with live CE virus did not result from a hypersensitivity reaction. Inoculation of 4 lambs with live CE virus in scarified skin of the groin, axilla and muzzle revealed that these three areas were equally sensitive for the development of CE lesions.

The period required for healing of the lesions after the 3 CE inoculations is presented in Table 21. The period between virus inoculation to the time when the scabs dropped off was considered the duration of the infection. After the first CE inoculation the mean duration was 25.4 (plus and minus standard error) $\pm$ 1.4 days. Following the second inoculation the mean duration was 14.3 $\pm$ 0.6 days and after the third inoculation the mean was 13.3 $\pm$ 0.8 days. The duration of infection was significantly shorter after the second and third inoculations compared to the first inoculation ($P < 0.001$). The experimental groups were too small to determine if the inoculation of different CE isolates had an effect on the duration of infection.

Serum samples were collected from the lambs at various intervals after the first CE inoculation and the peak VN antibody titers were detected between 2 to 4 weeks PI. The preinoculation and 3 weeks PI antibody titers are presented in Table 22. Combining all groups of lambs, there were significant increases in antibody titers to the 2 viruses (ST and CSL) used in the virus-neutralization test ($P < 0.05$). The increases in antibody titer were very small and the importance of these increases is uncertain.

Cross-Immunity Experiment with Cell Culture-Passaged CE Viruses

Lambs initially inoculated with ST virus did not develop lesions after the second CE inoculation and only 2 of the 6 lambs initially inoculated with CSL virus developed lesions. Following challenge with the sheep-passaged ST or CSL virus, the 6 lambs developed large lesions (6-12 mm diameter). Lesion scores following the 3 CE inoculations are presented in Table 23.

The cell culture-passaged and sheep-passaged viruses used in the 2 cross-immunity experiments had previously been titrated in scarified skin of sheep. One hundred times as much cell culture-passaged virus was required to induce lesions in sheep as compared to the number for the sheep-passaged virus. A further indication of the attenuation of the cell culture-passaged viruses was that the diameter of the lesions induced by these viruses was smaller than for lesions induced by the sheep-passaged viruses. The attenuation of the cell culture-passaged ST virus was particularly interesting since this virus had only been passed four times following isolation from a skin lesion.

Relatively high concentrations of virus were isolated from ST and CSL virus-induced lesions equal or greater than 2 mm diameter. No CE virus could be isolated from lesions less than 2 mm diameter.

The period required for healing of the virus-induced lesions is presented in Table 24. There was no significant difference in the period between virus inoculation and the time when the scabs dropped off for lesions developing after the first, second and third inoculations ($P > 0.05$).

The VN antibody titers prior to CE virus inoculation, 3 weeks PI and 2 weeks after the second CE inoculation are recorded in Table 25. At 3 weeks PI there were significant increases in antibody titers to ST and CSL viruses in sera from ST virus-inoculated lambs and to the CSL virus in sera from CSL virus-inoculated lambs ($P < 0.05$). There were no significant increases in titer to ST or CSL viruses following the second inoculation for either group of lambs ($P > 0.05$).

DISCUSSION AND CONCLUSION

Inoculation of 8 months old lambs with cell culture-passaged ST virus or Cutter Animal Health Laboratories CE viral vaccine protected the lambs from reinoculation with the sheep-passaged ST virus 4 weeks later. However, lambs which were inoculated with sheep-passaged ST or CSL virus at 6 weeks of age were fully susceptible to reinoculation with ST, CSL or Har/2 sheep-passaged virus 4 weeks later. All of the lambs developed lesions after the second CE inoculation and the lesions were of similar size to the lesions induced by the first CE inoculation for a given dose of virus. The rate of healing of the lesions after the second inoculation was faster than after the first inoculation, indicating an immune response was induced, although insufficient to protect the animals from the infection. The majority of the lambs were susceptible to a third inoculation with sheep-passaged CE virus, but the lesions were small and healed rapidly.

Isolation of CE virus and observation of parapox virions by EM examination confirmed that CE virus multiplication had occurred in the lesions. Thus, the development of lesions equal or greater than 2 mm diameter was indicative of active CE infection.

Inoculation of 6 months old lambs with cell culture-passaged ST or CSL virus protected these lambs from reinoculation with cell culture-passaged ST and CSL viruses 4 weeks later. Although the protection was not complete, with 2 lambs developing lesions after challenge, there was cross-protection between ST and CSL viruses. When the immunity of 6 of these lambs was challenged with the more virulent sheep-passaged

ST or CSL virus, all of the lambs were fully susceptible to the sheep-passaged CE viruses.

The immunity of lambs in the vaccination experiment was challenged with the same sheep-passaged ST virus as was used in 2 cross-immunity experiments and it is uncertain why only lambs in the first trial were protected against this virus. Inoculation of control animals in the 3 experiments confirmed the viability of the challenge viruses. Older lambs may have more resistance to CE virus and the lambs in the vaccination trial were older than the lambs in the other experiments. In addition, the vaccination experiment lambs were from a different source than the lambs in the last cross-immunity experiment and some groups of animals may have more natural resistance to CE virus.

Inoculation of sheep-passaged and cell culture-passaged CE viruses induced small increases in serum VN antibody titers and inoculation with the ST viruses induced increases in antibody titer to both viruses (ST and CSL) used in the virus-neutralization tests. The low antibody titers present prior to the CE inoculations may have originated from colostral antibody.

These experiments indicate that it was possible to protect a group of 8 months old lambs against challenge with the sheep-passaged field isolate by immunization with the homologous cell culture-passaged virus or a commercial CE vaccine. Reinoculation with sheep-passaged CE viruses in 2 cross-immunity experiments overcame the immunity of the lambs, whereas the immune response of lambs was sufficient to protect them from reinoculation with the less virulent cell culture-passaged CE viruses. In the cross-immunity experiment with cell culture-passaged

CE viruses, it was revealed that there was cross-protection between the ST and CSL viruses and previously it had shown that there were marked differences between these viruses as seen in cross-neutralization tests and the profiles of their structural polypeptides. Hence the existence of virulent CE isolates may be a more important cause of CE vaccination failures than differences in antigenicity.

GENERAL DISCUSSION AND CONCLUSIONS

Inoculation and subsequent reinoculation of CE virus did not induce high levels of VN antibody or virus-specific lymphocyte transformation which suggested that there was a minimal systemic immune response. The failure of colostral antibody to completely protect lambs from subsequent exposure to CE virus and the difficulty of inducing absolute protection by CE vaccination may be partially explained by the minimal systemic immune response induced following CE vaccination. Higher levels of systemic immunity may be induced by parenterally administering live CE vaccines compared to inducing a localized infection. Replication of CE virus in buffy coat cells suggested that the virus may replicate in macrophages and therefore parenterally adminstered vaccines may be feasible.

The vaccination studies showed that to test the effectiveness of new vaccines and vaccination procedures, sheep should be reexposed to CE virus. The size of the primary vaccination lesions, VN antibody titers and virus-specific peripheral blood lymphocyte stimulation indices cannot be used to predict the degree of protective immunity.

Existence of latent CE infections could not be confirmed, since no virus could be isolated from lesions induced by dexamethasone treatment of previously vaccinated animals. Latent infections if existent, are not likely to be important for the initiation of CE disease outbreaks as no CE lesions developed following inoculation of lambs with suspensions prepared from dexamethasone-induced lesions.

Cross-neutralization and DTH tests could not be used to classify the CE viral isolates. However, analysis of structural polypeptides

revealed differences among the isolates in the position of distinct polypeptide bands in the molecular weight region of 37,000-44,000 daltons, allowing the isolates to be classified in four groups. The polypeptides which varied among the different groups were shown to be located in the surface component of the virion. Unilateral cross-reactions detected in the cross-neutralization tests were found to correlate with classification of the isolates based on the position of the distinct polypeptide bands. Hence this classification might indicate if there is cross-protection between different CE viral isolates.

Cross-immunity tests were performed in sheep using two isolates (ST, CSL) which did not cross-react in the cross-neutralization tests and differences were also detected in the polypeptide profiles of these two isolates. Reinoculation with virulent sheep-passaged CE viruses overcame the immunity of the lambs, whereas there was protection against the less virulent cell culture-passaged CE viruses with cross-protection between the ST and CSL viruses. These results suggested that the existence of virulent CE viral isolates may be the reason for the occurrence of CE in vaccinated animals rather than differences in antigenicity.

Baxby (94) has reported that cowpox and vaccinia viruses have different as well as common antigens on the surface of their virions, but also share some surface components. Contagious ecthyma isolates may share some surface antigens, although their surfaces may not be identical. The absence of bilateral cross-reactivity among isolates, as seen in the cross-neutralization tests, suggested that the antigens

inducing humoral immunity differed among the isolates. A possible explanation for the cross-protection could be that a surface antigen common to many CE isolates may induce CMI and this immune response may determine the resistance to CE infection.

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APPENDIX

Table 1 Virus neutralizing (VN) antibody titers after inoculation and reinoculation of sheep with CE viral isolates

Animal no.	Viral isolate used for first inoculation	VN antibody response on indicated day after first inoculation						Viral isolate used for second inoculation[a]	VN antibody response on indicated day after second inoculation						
		0	7	10	14	21	28		0	4	7	10	14	21	28
2054	Cu	<2[b]	<2	<2	3	4	6	ST	6	2	23	7	140	70	6
2231	Cu	<2	<2	6	11	7	<2	ST	<2	4	4	4	11	9	4
2313	Cu	<2	<2	<2	4	36	14	ST	14	7	36	4	9	4	4
2318	Cu	<2	<2	6	9	18	6	ST	6	NT	4	4	NT	9	6
2309	Cu	sera toxic to cells						ST							
2237	ST	<2	<2	<2	4	9	14	ST	14	18	14	7	6	7	7
2276	ST	<2	<2	7	45	9	11	ST	11	6	9	7	4	4	4
2312	ST	sera toxic to cells						ST							

[a] Second inoculation was 28 days following the first inoculation.

[b] Results expressed as the reciprocal of the serum dilution which neutralized over 50 percent of the test virus as calculated by the Kärber method.

NT Not Tested

Table 2 Whole blood lymphocyte transformation responses of sheep to the Cu isolate of CE virus after inoculation with the homologous virus

Animal no.	Virus-specific lymphocyte transformation response on indicated day after Cu virus inoculation							
	0	2	4	7	10	14	21	28
2054	851[a] (0.6)	527 (1.3)	461 (1.0)	1584 (0.9)	7396 (5.3)	1399 (1.0)	1300 (1.9)	1203 (1.6)
2231	342 (0.7)	305 (1.0)	252 (1.0)	369 (1.2)	1233 (2.7)	703 (1.3)	1066 (2.5)	3220 (2.1)
2309	632 (1.2)	338 (0.9)	407 (0.7)	417 (0.9)	901 (1.3)	565 (1.6)	684 (3.0)	910 (1.3)
2313	350 (0.9)	327 (0.7)	362 (1.0)	368 (1.0)	2987 (10.3)	1505 (6.1)	1162 (8.5)	2999 (5.1)
2318	869 (0.9)	555 (1.2)	398 (0.9)	1177 (1.43)	6069 (5.3)	2667 (3.1)	1820 (5.9)	4916 (3.5)

[a] Numbers represent mean counts/min. obtained with Cu antigen; numbers in parentheses indicate the stimulation index.

Stimulation indices of lymphocytes with PHA ranged from 10-190 for the individual sheep in the study.

Table 3 Whole blood lymphocyte transformation responses of sheep to ST isolate of CE virus after inoculation with the homologous virus

Animal no.	Virus-specific lymphocyte transformation response on indicated day after ST virus inoculation							
	0	2	4	7	10	14	21	28
2237	406[a] (1.0)	380 (0.7)	288 (1.1)	470 (0.9)	237 (1.3)	857 (2.4)	540 (1.1)	644 (1.3)
2276	291 (0.9)	380 (0.8)	180 (1.0)	813 (1.1)	401 (0.8)	796 (3.8)	693 (1.6)	423 (1.2)
2312	402 (0.7)	564 (0.9)	234 (1.1)	749 (1.4)	1035 (1.1)	2320 (4.7)	1951 (1.4)	778 (2.4)

[a] Numbers represent mean counts/min. obtained with ST antigen; numbers in parentheses indicate the stimulation index.

Stimulation indices of lymphocytes with PHA ranged from 49-271 for the individual sheep in the study.

Table 4 Whole blood lymphocyte transformation responses of uninoculated sheep to Cu and ST viral isolates

Animal no.	Viral Antigen tested	Virus-specific lymphocyte transformation response on indicated sampling day							
		0	2	4	7	10	14	21	28
2005	Cu	1697[a] (0.6)	889 (0.9)	2372 (0.8)	2483 (1.0)	4581 (0.8)	2261 (0.9)	5116 (1.1)	2237 (1.0)
	ST	2075 (0.8)	644 (.06)	2292 (0.7)	3003 (1.2)	4902 (0.9)	2238 (0.9)	3255 (0.7)	1983 (0.9)
2007	Cu	1314 (1.6)	392 (0.8)	1042 (0.7)	1354 (0.7)	1764 (0.7)	1867 (0.9)	1530 (0.9)	622 (0.8)
	ST	1056 (1.3)	944 (1.9)	1510 (1.0)	1541 (0.8)	2469 (1.0)	1388 (0.7)	1361 (0.8)	700 (0.9)

[a] Numbers represent mean counts/min. obtained with ST antigen; numbers in parentheses indicate the stimulation index.

Stimulation indices of lymphocytes with PHA ranged from 11-78 for the individual sheep in the study.

Table 5 Whole blood lymphocyte transformation responses of sheep to Cu and ST viral isolates after reinoculation with a CE virus[a]

Animal no.	Viral Antigen tested	Stimulation index on indicated day after CE virus reinoculation							
		0	2	4	7	10	14	21	28
2054	Cu	1.6	3.7	7.7	1.8	1.8	2.2	1.5	1.2
	ST	1.1	1.7	1.1	1.2	1.3	9.1	1.1	1.0
Mean cpm for control antigen (2054)		765	660	483	2391	4703	681	1298	3890
2231	Cu	2.1	1.1	9.4	3.7	2.7	1.4	1.8	2.4
	ST	0.9	0.7	1.0	1.3	0.7	4.2	1.0	1.1
Mean cpm for control antigen (2231)		1515	1160	1173	1353	4071	729	1250	1429
2309	Cu	1.3	3.1	1.4	2.0	2.3	1.0	1.8	2.7
	ST	0.9	1.2	1.0	0.7	1.3	3.6	0.7	1.4
Mean cpm for control antigen (2309)		707	615	480	756	1688	291	813	688
2313	Cu	5.1	2.6	8.3	2.0	4.7	2.0	1.6	2.1
	ST	1.2	1.6	1.3	1.3	1.5	3.4	1.3	1.7
Mean cpm for control antigen (2313)		587	345	342	1035	1231	330	769	797
2318	Cu	3.5	4.3	6.5	2.8	2.6	2.6	7.2	2.8
	ST	1.5	1.6	2.1	0.9	1.5	6.5	4.3	1.5
Mean cpm for control antigen (2318)		1395	1334	311	746	4646	118	737	1262

[a]Sheep were initially inoculated with Cu viral isolate and 28 days later inoculated with ST viral isolate.

Stimulation indices of lymphocytes with PHA ranged from 18-235 for the individual sheep in the study.

Table 6 Levels of VN antibody in sera of lambs and their dams and the clinical lesions which developed after subsequent natural infection with CE virus

Lamb no.	VN antibody titers			Lesions from a natural CE virus infection
	1-2 days old	5-7 weeks old	Dam[a]	
2062	7[c]	<2	6	-
2079	<2	2	3	-
2102	2	NT	5	+ (lips) [b]
2116	9	3	9	+ (muzzle)
2130	4	2	3	+ (lips)
2135	<2	2	5	+ (lips)
2225	<2	2	NT	+ (lips)
901	<2	NT	NCI	++ (lips, muzzle)
902	3	<2	5	+ (lips)
903	<2	<2	6	+ (lips)
904	<2	<2	2	++ (lips, muzzle)

[a] Blood sample collected from dam when lambs 1-2 days old.

[b] Location of lesion(s)

[c] Reciprocal of the serum dilution which neutralized 50 percent of the test virus.

NT Not tested

NCI No colostrum ingested

- No lesions

+ Represents 1-2 lesions ($\leq$ 10 mm diameter)

++ Represents multiple lesions (10-20 mm diameter)

2062-2225 were from vaccinated ewes, 901-904 were from non-vaccinated ewes. Significant positive correlation between antibody titers in sera of lambs (1-2 days old) and of their dams ($P < 0.05$).

Table 7 Levels of serum VN antibody in lambs and their dams

Lamb no.	VN antibody titers	
	1-2 days old	Dam[a]
905	7	] 57
906	5	
907	<4	] 9
908	<4	
909	<4	] 5
910	<4	
911	9	23
912	<4	] 11
913	<4	
917	5	] 9
919	<4	

[a] Blood sample collected from dam when lambs 1-2 days old. All the ewes were vaccinated with CE virus 6 months prior to parturition.

Significant positive correlation between antibody in the sera of lambs (1-2 days old) and their dams ($p < 0.01$).

Table 8 Recrudescence of CE lesions following treatment with corticosteroids

Experiment no.	No. of sheep: CE-inoculated + c/steroid	No. of sheep: CE-inoculated	No. of sheep: c/steroid[a]	CE isolate used for inoculation	Interval between CE inoculation and c/steroid (days)	No. of sheep with lesions at CE inoculation site
A	3			ST	65	2
		2		ST		0
			2			0
B	2			ST	283	0
C	3			Cu	84	0
			2			0
D	4			Unknown[b]	76	0
		2		Unknown		0
			2			0
E	7			ST	75	3
		2		ST		0

[a] Corticosteroid treatment (0.15 mg/Kg dexamethasone was administered parenterally on 5 consecutive days; additionally, in experiments A , C and E 0.5 percent hydrocortisone cream was applied topically to CE inoculation sites daily for 5 days.

[b] Sheep were infected with a CE virus of unknown origin in a natural CE disease outbreak.

Table 9 Effect of corticosteroid treatment on the leukocyte count of sheep

Animal no.	Leukocyte count on indicated day after start of corticosteroid treatment							
	0	2	4	7	10	13	17	24
2237[a]	6,028[d] (11)	8,990 (38)	7,942 (37)	7,822 (19)	6,011 (16)	7,852 (22)	5,809 (12)	6,916 (6)
2312[a]	6,615 (14)	8,302 (36)	7,587 (33)	5,274 (18)	6,189 (19)	4,058 (17)	6,406 (25)	5,745 (4)
2313[a]	6,261 (24)	8,038 (29)	7,879 (29)	6,733 (25)	4,880 (20)	3,440 (13)	5,702 (21)	4,906 (16)
2005[b]	14,200 (23)	16,500 (37)	15,100 (36)	11,900 (27)	13,600 (20)	9,179 (20)	12,700 (51)	11,500 (36)
2007[b]	8,820 (30)	12,500 (59)	12,100 (44)	8,388 (41)	8,564 (33)	7,329 (36)	6,550 (8)	5,465 (27)
2231[c]	6,172 (18)	6,846 (16)	5,987 (10)	7,759 (33)	5,838 (25)	7,139 (18)	8,008 (24)	7,219 (20)
2276[c]	6,379 (14)	6,598 (16)	6,064 (13)	8,898 (12)	5,225 (16)	5,884 (7)	6,677 (7)	8,095 (11)

[a] CE-inoculated sheep treated with corticosteroids

[b] Uninoculated sheep treated with corticosteroids

[c] CE-inoculated sheep not treated with corticosteroids

[d] Numbers represent total leukocyte count/mm^3; numbers in parentheses represent percent neutrophils.

Table 10 Distribution of sizes of lesion after inoculation of CE virus in CE-vaccinated and non-vaccinated sheep (1979 study)

CE vaccination[a] history (no. of sheep)	Percent with lesion[b] of indicated diameter (mm)			
	≤ 1	2-3	4-9	≥ 10
Vaccinated (50)	6 (3)[c]	44 (22)	36 (18)	14 (7)
Non-vaccinated (100)	0 (0)	4 (4)	39 (39)	57 (57)

[a] CE vaccination on farm of origin was 4-8 months prior to CE inoculation at the research station

[b] Measured at 9 days PI.

[c] Number of sheep

Sheep with prior CE vaccination had significantly smaller lesions compared to non-vaccinated sheep ($P < 0.001$). Lesion sizes ≤ 1 and 2-3 mm were grouped together for the Chi-squared test.

Table 11 Distribution of sizes of lesion after inoculation of CE virus in CE-vaccinated and non-vaccinated sheep (1980) study

CE vaccination[a] history (no. of sheep)	Percent with lesion[b] of indicated diameter (mm)				Mean size (±SE) of lesion (mm)
	≤ 1	2-3	4-9	≥ 10	
Vaccinated (66)	8 (5)[c]	11 (7)	45 (30)	36 (24)	7.4 (0.5)
Non-vaccinated (64)	2 (1)	5 (3)	39 (25)	54 (35)	10.5 (0.8)

[a] CE vaccination on farm of origin was 4-8 months prior to CE inoculation at the research station

[b] Measured at 9 days PI.

[c] Number of sheep

Sheep with prior CE vaccination had significantly smaller lesions compared to non-vaccinated sheep ($P < 0.05$). Lesion sizes ≤ 1 and 2-3 mm were grouped together for the Chi-squared test.

Table 12 Relation of size of the primary lesion to production of a lesion after reinoculation of CE virus (1979 study)

Diameter of lesions after reinoculation[a] (mm)	No. of sheep with a lesion produced after reinoculation of CE virus/ No. with indicated response to primary inoculation			
	Diameter of primary lesion (mm)			
	≤ 1	2-3	4-9	≥ 10
2-3	1/3	2/26	8/57	4/64
4-10	0/3	0/26	3/57	3/64
Total	1/3	2/26	11/57	7/64

[a] Lesions less than 2 mm in diameter could not be shown to result specifically from a CE viral infection.

Diameter of the primary lesion was not related to the production of a lesion after CE reinoculation ($P > 0.05$). Lesion sizes ≤ 1 and 2-3 mm were grouped together for the Chi-squared test.

Table 13 Relation of size of the primary lesion to production of a lesion after reinoculation of CE virus (1980 study)

Diameter of lesions after reinoculation[a] (mm)	No. of sheep with a lesion produced after reinoculation of CE virus/ No. with indicated response to primary inoculation Diameter of primary lesion (mm)			
	≤ 1	2-3	4-9	≥ 10
2-3	1/6	0/10	11/55	7/59
4-10	1/6	1/10	5/55	2/59
Total	2/6	1/10	16/55	9/59

[a] Lesions less than 2 mm in diameter could not be shown to result specifically from a CE viral infection.

Diameter of the primary lesion was not related to the production of a lesion after CE reinoculation ($P > 0.05$). Lesion sizes ≤ 1 and 2-3 mm were grouped together for the Chi-squared test.

Table 14 Size of lesions after CE inoculation and reinoculation compared to lymphocyte transformation response and antibody response following the primary CE inoculation

Animal no.	Diameter of lesions (mm)		Lymphocyte transformation response			VN antibody titers		
	Primary inoculation[a]	Reinoculation[b]	Days after primary inoculation 0	9	13	Days after primary inoculation 0	16	23
118	2	<2	1043[c](1.2)[d]	3074 (0.9)	1443 (1.2)	<2	6	8
43	3	<2	793 (1.1)	743 (0.4)	917 (0.7)	2	11	4
90	4	10	2344 (1.1)	1389 (1.1)	2749 (0.8)	<2	11	2
44	5	3	314 (1.6)	2066 (1.1)	2067 (1.5)	3	8	4
82	5	5	680 (2.6)	4602 (1.1)	2299 (0.9)	3	8	3
83	5	<2	NT	NT	NT	6	<2	11
114	6	<2	834 (0.9)	1859 (0.9)	4199 (2.5)	<2	2	11
86	6	<2	NT	NT	NT	<2	8	16
107	8	<2	11136 (1.2)	7043 (2.7)	5189 (1.9)	<2	11	4
112	8	5	NT	NT	NT	<2	6	6
45	10	3	1231 (0.5)	5361 (1.0)	3322 (1.1)	3	3	4
57	10	<2	856 (0.6)	9218 (5.6)	2080 (1.6)	<2	6	4
84	10	<2	NT	NT	NT	<2	8	4
110	10	<2	NT	NT	NT	2	11	4
116	10	<2	6458 (1.4)	3794 (1.5)	1508 (1.1)	<2	6	4
54	15	<2	NT	NT	NT	<2	11	2

[a] Measured on day 9 PI.

[b] Measured on day 14 after reinoculation (35 days between inoculation and reinoculation).

[c] Mean cpm to CE antigen.

[d] Stimulation index with CE antigen.

Table 15 The effect of heat-inactivation of CE virus antiserum and addition of complement on VN antibody titers.

Virus	VN antibody titers[a]			
	Serum[b]	Inact. serum[c]	Inact. serum + 5% C´[d]	C´titration
CSL isolate	18	<4	11	<4

	Titration of virus preparation	Virus dilution for VN test contained[e]
No C´	$10^{7 \cdot 42}$ ($CCID_{50}$/ml)	26 ($CCID_{50}$/well)
5% C´	$10^{7 \cdot 05}$	10

[a] VN antibody titer is the reciprocal of the serum dilution which neutralized 50 percent of the test virus as calculated by the Karber method.

[b] Antiserum.

[c] Heat-inactivated antiserum (56°C; 30 minutes).

[d] Five percent complement (guinea-pig serum) final volume.

[e] In the VN test 0·05 ml of a $10^{4 \cdot 7}$ dilution of the viral preparation was used.

Table 16 Cross-neutralization reactions with 4 isolates of CE virus.

Serum	Virus			
	ST	CSL	Har/2	AM
	VN antibody titers			
ST	1280[a]	20	20	80
CSL	10	160	5	10
Har/2	320	20	60	20
Am	5	20	5	50

Relationships between viruses.

ST	1 (1)[b]	0·015 (0·03)	0·015 (0·29)	0·06 (0·081)
CSL	0·06 (0·03)	1 (1)	0·03 (0·10)	0·06 (0·16)
Har/2	5·33 (0·29)	0·33 (0·10)	1 (1)	0·33 (0·18)
Am	0·10 (0·08)	0·40 (0·16)	0·10 (0·18)	1 (1)

[a] Reciprocal of the serum dilution which neutralized 50 percent of the test virus as calculated by the Kärber method.

[b] Numbers represent the titer ratio which equals heterologous titer/homologous titer (unilateral relationship); numbers in parentheses represent the bilateral relationship which is the geometric mean of the 2 titer ratios.

Table 17 Skin reactions in a CE-infected sheep[a] following inoculations of varying concentrations of CE viral antigens.

Test antigen (dilution)	Skin thickness ratio[b]	Diameter of erythema[c] (mm)
CE virus[d]	3·7	20
CE virus $(\frac{1}{10})$	2·1	13
CE virus $(\frac{1}{100})$	1·7	10
Control[e]	1·0	0
Control $(\frac{1}{10})$	0·9	0
Control $(\frac{1}{100})$	1·0	0

[a] Sheep was infected with CE virus (Cu) 6 months prior to DTH test.

[b] Represents skin thickness 48 hours PI/skin thickness prior to inoculation.

[c] Diameters of erythema measured 24 hours PI.

[d] Contained $10^{6·5}$ $CCID_{50}$/ml of Cu virus prior to heat inactivation.

[e] Lamb testis cell extract.

Table 18 Comparative DTH skin tests to CE viral antigens.

Immunizing virus	No. of sheep	Skin thickness ratio[a] CE viral antigens tested. ST	CSL	Har/2	Cu	Am	Control antigen[b]
ST	3	3·0[c]	2·6	3·0	3·0	2·8	1·1
CSL	3	2·9	2·9	2·5	2·9	2·6	1·1
Har/2	1	3·4	4·1	3·2	3·2	3·0	1·2
Control[d]	3	1·1	1·1	1·1	1·0	1·1	1·0

[a] Represents the skin thickness at 48 hours PI/skin thickness prior to inoculation.

[b] Lamb testis cell extract.

[c] Mean skin thickness ratio.

[d] Not immunized.

Table 19 Contagious ecthyma virus vaccination experiment

Initial inoculation of CE virus	No. of lambs	Second inoculation[a] of CE virus	No. of lambs with lesions
ST (c)[b]	5	ST (s)[c]	0
Cu (s)[d]	5	ST (s)	0
Control[e]	3	ST (s)	3

[a] Second CE inoculation was 4 weeks after the initial CE inoculation.

[b] ST virus passaged 8 times in lamb testis cells.

[c] ST virus prepared from scab material.

[d] Cutter Animal Health Laboratories CE vaccine prepared from scab material.

[e] Control group not immunized.

Table 20 Severity of lesions produced in sheep after inoculation and reinoculation of sheep-passaged CE viruses

Animal no.	Lesion Scores[a]																	
	Initial inoculation					First reinoculation[b]									Second reinoculation[c]			
	ST (s)[d]		CSL (s)			ST (s)			CSL (s)			Har/2 (s)			ST (s)	CSL (s)		
	4·8[e]	3·8	4·7	3·7	2·7	4·8	3·8	2·8	4·7	3·7	2·7	4·4	3·4	2.4	3·8	3·7		
922	5	5				4	4	3							3			
923	5	3				4	2	0							0			
924	5	5				4	4	3							3			
907	4	3							3	4	2				3			
908	4	3							5	3	0				3			
925	5	4							4	4	3				2			
905	4	4										4	0	0	3			
906	4	4										4	4	0	3			
911	5	4										5	4	0	4			
912			4	3	0				5	5	0					1		
913			5	4	0				3	2	0					3		
920			5	5	3				5	5	2					0		
919			5	5	2	5	3	0								0		
921			5	4	0	5	5	3								0		
302						4	4	3										
910									5	4	3							
909												5	3	0				

[a] Size of lesion scored on a scale 0-5 as described in Experimental Design.

[b] First reinoculation was 4 weeks after initial inoculation.

[c] Second reinoculation was 6 weeks after first reinoculation.

[d] (s) Sheep-passaged virus.

[e] $CCID_{50}$/0·05 ml inoculation.

Table 21 Time required for healing of lesions resulting from inoculation and reinoculation of sheep-passaged CE viruses.

Animal no.	Duration of infection[a]						
	Initial inoculation		First reinoculation			Second reinoculation	
	ST (s)[b]	CSL (s)	ST (s)	CSL (s)	Har/2 (s)	ST (s)	CSL (s)
922	32		12			14	
923	37		13			-[c]	
924	32		13			14	
907	21			12		12	
908	21			12		17	
925	35			13		12	
905	32				12	12	
906	21				19	14	
911	21				16	17	
912		24		18			12
913		21		18			9
920		21		14			-
919		21	14				-
921		24	14				-
302			24				
910				21			
909					24		

[a] Period between virus inoculation and time when all scabs have dropped off (days).

[b] (s) Sheep-passaged virus.

[c] No lesion developed.

Table 22 Antibody response in lambs after initial inoculation of sheep-passaged CE virus.

Animal no.	Virus used for inoculation	VN antibody titers to indicated virus[a]			
		ST		CSL	
		Preinoculation	3 wks. PI[b]	Preinoculation	3 wks. PI
905	ST	5	11	7	9
906	ST	5	11	7	14
907	ST	<4	5	7	5
908	ST	<4	7	7	14
911	ST	<4	7	7	11
922	ST	11	23	5	18
923	ST	9	36	14	14
924	ST	9	9	7	14
925	ST	9	18	9	11
912	CSL	14	18	14	14
913	CSL	9	27	9	11
919	CSL	9	5	5	11
920	CSL	5	5	5	5
921	CSL	5	9	5	11

[a] Virus represents the virus used in the virus-neutralization test.

[b] Post-inoculation.

Table 23 Severity of lesions produced in sheep after inoculation and reinoculation of CE viruses.

Animal no.	Lesion Scores[a]													
	Initial inoculation				First reinoculation[b]						Second reinoculation[c]			
	ST (c)[d]		CSL (c)		ST (c)			CSL (c)			ST (s)[e]		CSL (s)	
	5·6[f]	4·6	5·7	4·7	5·6	4·6	3·6	5·7	4·7	3·7	3·8		3·7	
10	3	0			0	0	0				5			
44	3	2			0	0	0				5			
958	5	4			0	0	0				5			
935	4	3						0	0	0				
957	3	2						0	0	0				
968	3	3						0	0	0				
777			3	0				0	0	0			5	
824			5	4				0	0	0			5	
953			3	2				3	2	0			4	
952			3	2	2	0	0							
954			1	0	0	0	0							
955			4	0	0	0	0							
951					3	0	0							
983					2	0	0							
956								4	3	0				
154								4	2	0				

[a] Size of lesion scored on a scale 0-5 as described in Experimental Design.
[b] First reinoculation was 4 weeks after initial inoculation.
[c] Second reinoculation was 6 weeks after first reinoculation.
[d] (c) Cell culture-passaged virus.
[e] (s) Sheep-passaged virus.
[f] $CCID_{50}$/0·05 ml inoculum.

Table 24 Time required for healing of lesions resulting from inoculation and reinoculation of CE viruses.

Animal no.	Duration of infection[a]					
	Initial inoculation		First reinoculation		Second reinoculation	
	ST (c)[b]	CSL (c)	ST (c)	CSL (c)	ST (s)[c]	CSL (s)
10	23		-[d]		28	
44	23		-		28	
958	27		-		18	
935	32			-		
957	24			-		
968	28			-		
777		20		-		28
824		37		-		32
953		24		21		18
952		21	28			
954		24	-			
955		21	-			
951			32			
983			22			
956				32		
154				22		

[a] Period between virus inoculation and time when all scabs have dropped of (days).

[b] (c) Cell culture-passaged virus.

[c] (s) Sheep-passaged virus.

[d] No lesion developed

Table 25 Antibody response in lambs after inoculation and reinoculation of cell culture-passaged CE viruses.

Animal no.	Virus: Initial inoculation	Virus: Re-inoculation	VN antibody titers to indicated virus[a]: ST: Preinoculation	ST: 3 wks post-inoculation	ST: 2 weeks post-reinoculation	CSL: Preinoculation	CSL: 3 wks post-inoculation	CSL: 2 wks post-reinoculation
10	ST	ST	<7	7	14	7	11	14
44	ST	ST	11	9	9	9	27	18
958	ST	ST	7	57	73	11	18	9
935	ST	CSL	<7	27	36	<7	7	7
957	ST	CSL	18	18	23	14	11	14
968	ST	CSL	11	57	23	9	27	18
777	CSL	CSL	11	9	7	9	14	9
824	CSL	CSL	9	18	14	<7	11	14
953	CSL	CSL	7	18	11	<7	9	9
952	CSL	ST	11	57	18	7	9	14
954	CSL	ST	9	9	7	<7	14	14
955	CSL	ST	<7	7	9	7	18	9
951	Control[b]	ST	9	-	36	7	-	18
983	Control	ST	7	-	18	9	-	14
956	Control	CSL	14	-	23	11	-	14
154	Control	CSL	9	-	11	9	-	23

[a] Virus represents the virus used in the virus-neutralization test.

[b] Not inoculated.

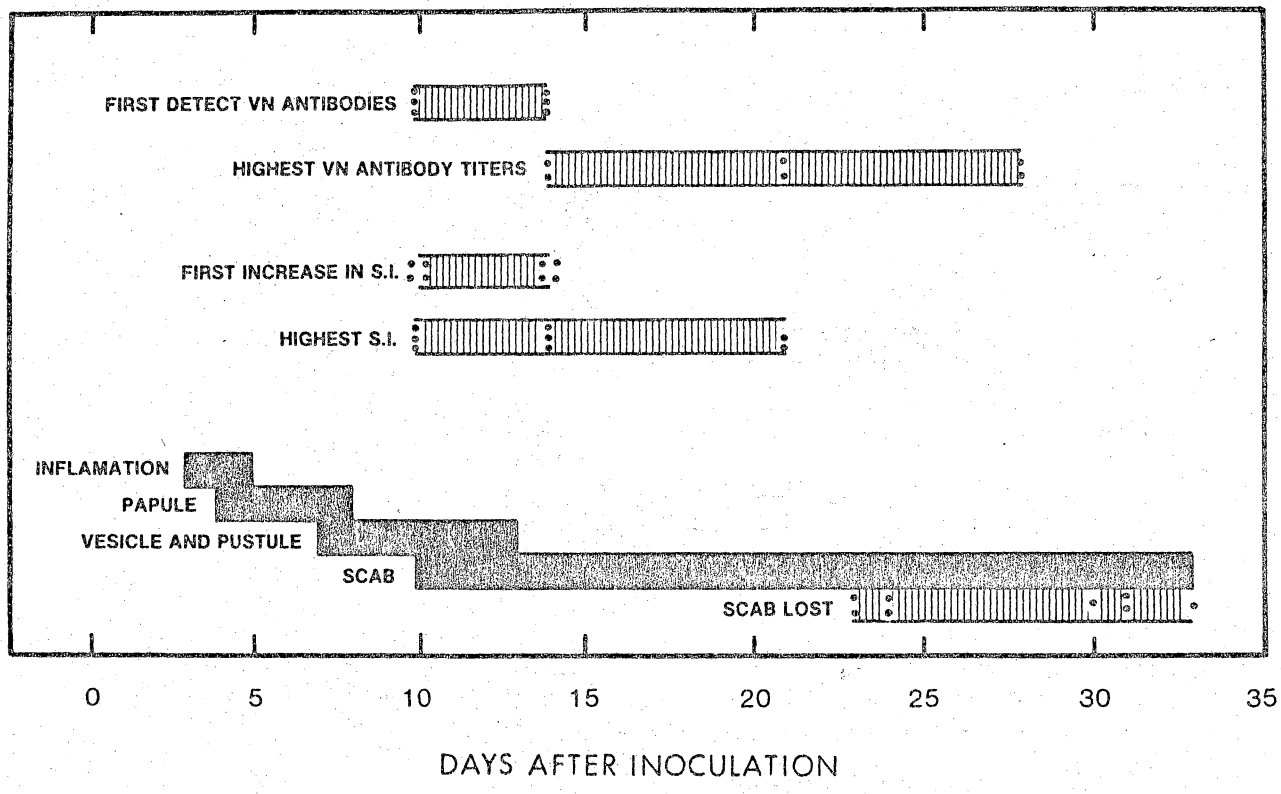


Fig. 1 Time of appearance of CE lesions, increase in whole blood lymphocyte stimulation indices to CE antigen and virus neutralizing (VN) antibody response following inoculation of CE virus. Solid bars represent the duration of clinical lesions in sheep; striped bars represent the range for the indicated response; (·) represent the response of each individual sheep. Development of CE lesions and lymphocyte transformation (n=8); VN antibody response (n=6).

Fig. 2 Papules in the axilla of a sheep 6 days post inoculation (PI) with CE virus.

Fig. 3 Pustules in the axilla of a sheep 10 days PI with CE virus.

Fig. 4 Scabs in the axilla of a sheep 15 days PI with CE virus.

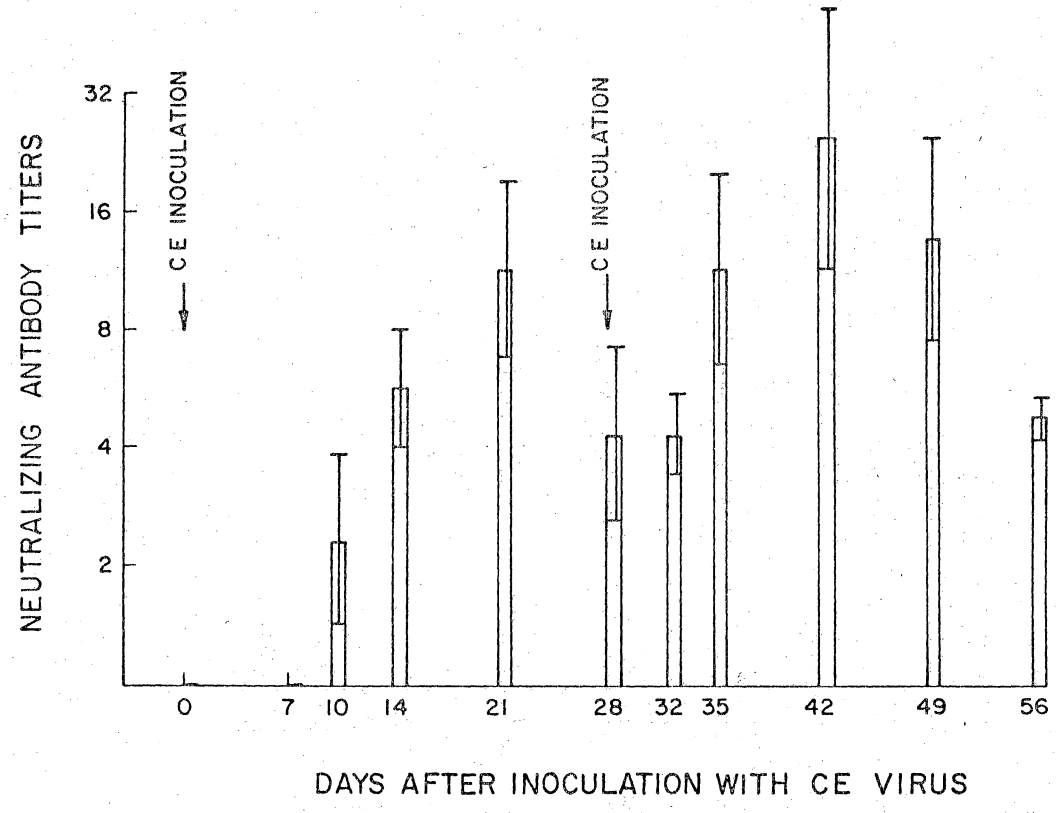


Fig. 5 Neutralizing antibody response to CE virus in sheep after CE inoculation and reinoculation. Data are given as means (bars) ± standard error (brackets). Bars represent CE-inoculated sheep (n=6). Antibody titers are expressed as the reciprocal of the serum dilution which neutralized 50 percent of the test virus as calculated by the Kärber method.

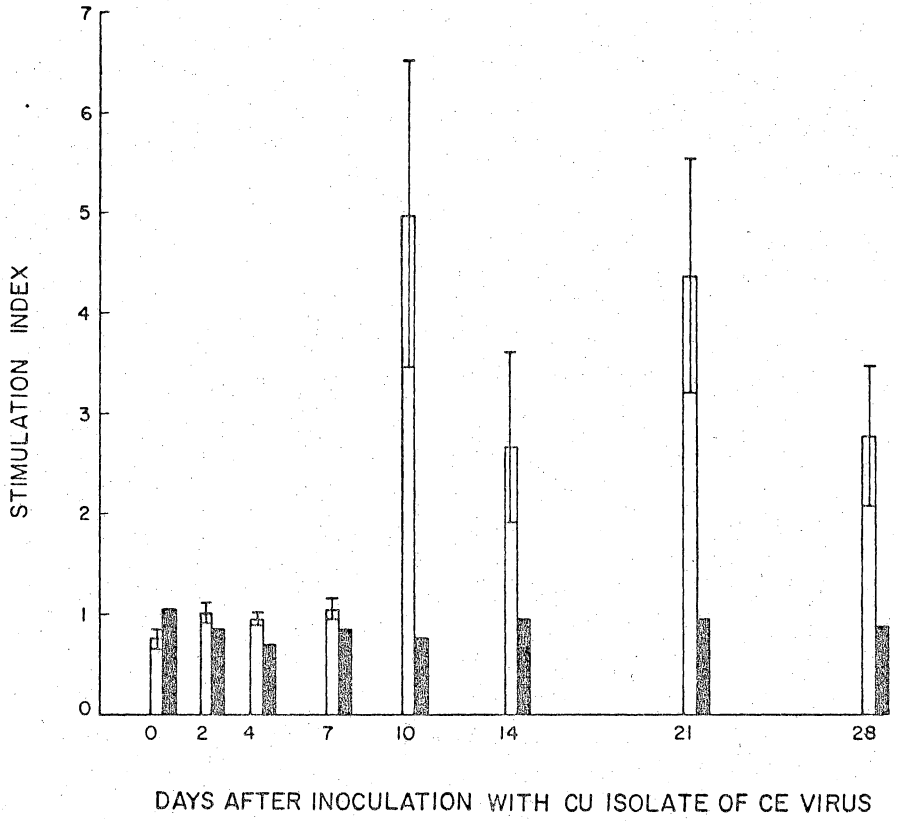


Fig. 6 Whole blood lymphocyte transformation response to Cu viral isolate in sheep after inoculation with homologous virus. Transformation is expressed as a stimulation index which is derived by dividing the mean counts per minute (cpm) obtained in the presence of viral antigen by the mean cpm obtained in the absence of virus. Data are given as means (bars) ± standard error (brackets). Open bars represent Cu virus-inoculated sheep (n=5); solid bars represent uninoculated sheep (n=2).

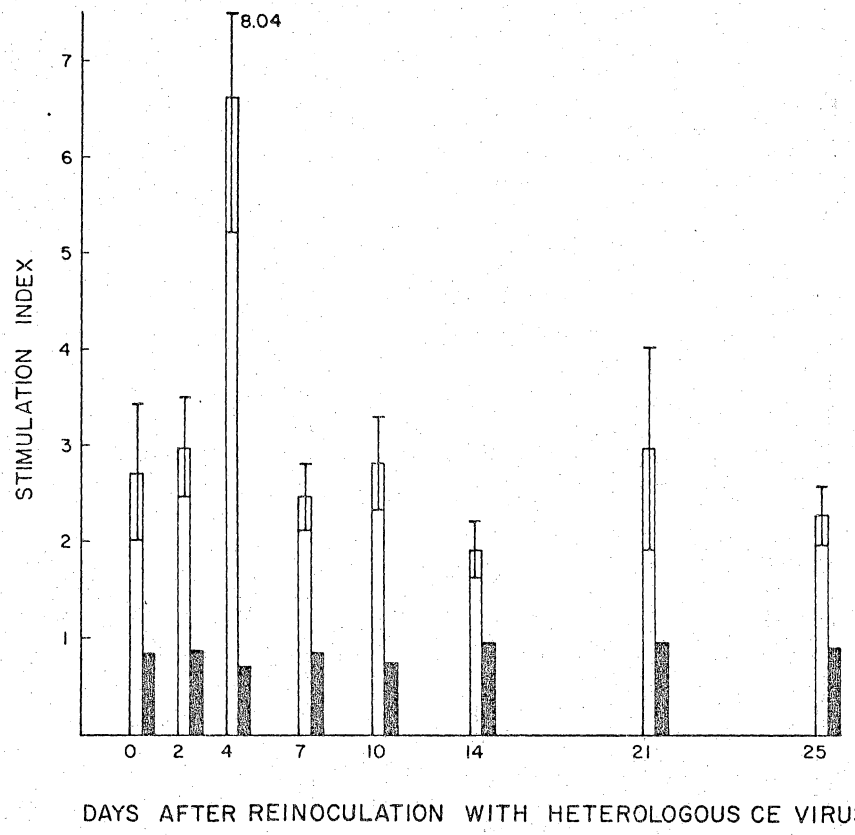


Fig. 7 Whole blood lymphocyte transformation response to Cu viral isolate in sheep after reinoculation with a CE virus. Sheep were initially inoculated with Cu virus and 28 days later inoculated with ST viral isolate. Transformation is expressed as a stimulation index. Data are given as means (bars) ± standard error (brackets). Open bars represent CE-inoculated sheep (n=5); solid bars represent uninoculated sheep (n=2).

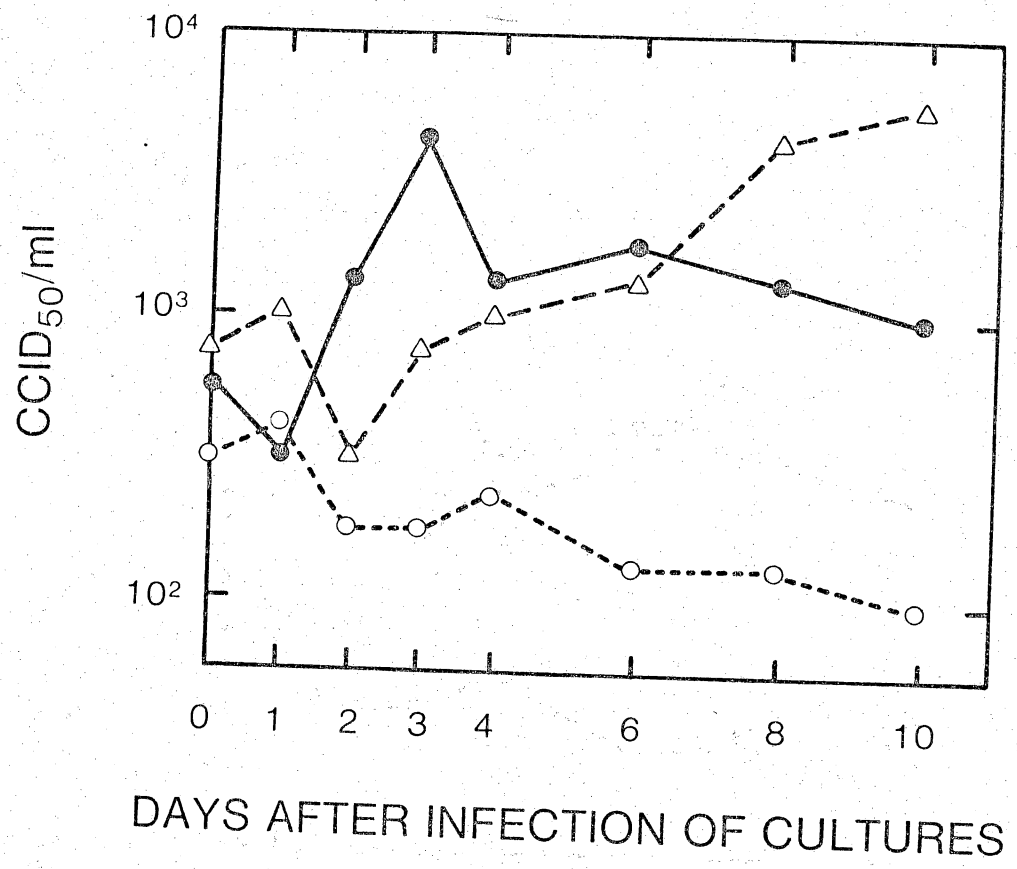


Fig. 8 Replication of CE virus in sheep buffy coat cells. Replication in cells from uninoculated sheep (●), cells from sheep inoculated with CE virus 35 days previously (△) and in the absence of cells (○).

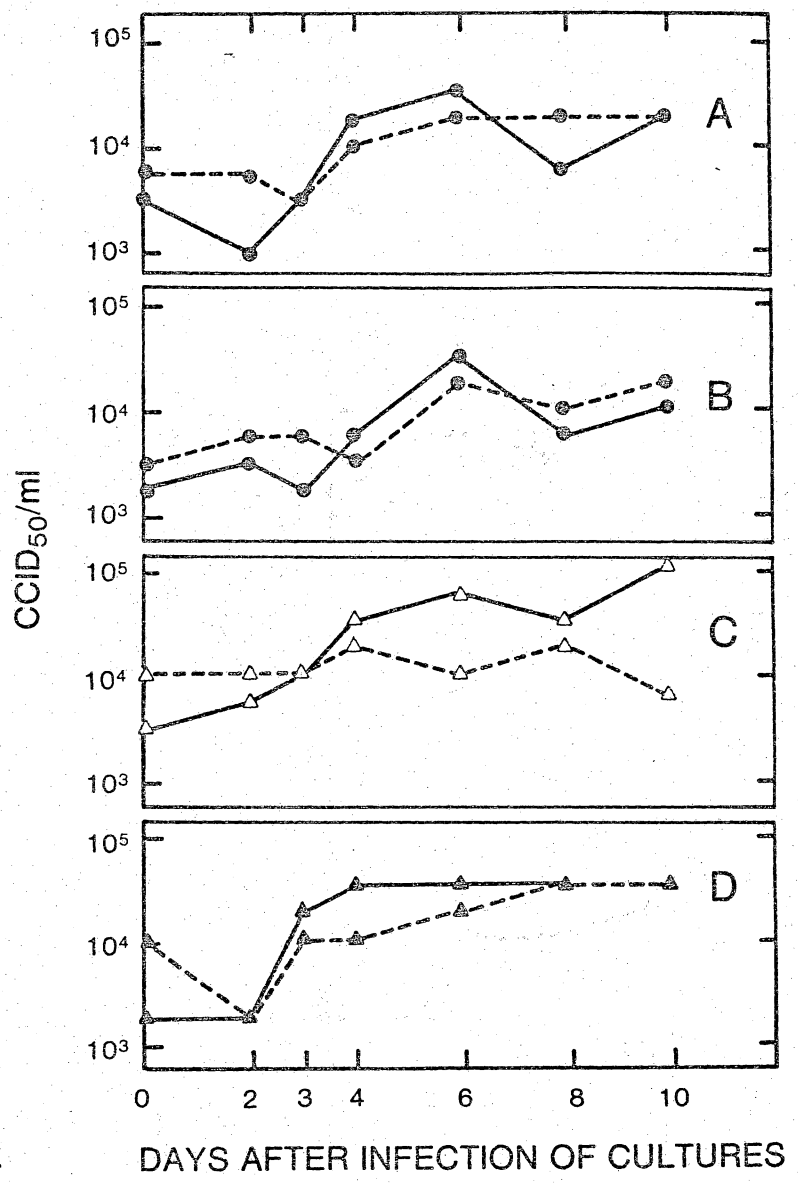


Fig. 9 Replication of CE virus in sheep buffy coat cells. (A and B) replication in cells from non-infected sheep (●——● , ●——●) cells collected day 0, (●---● , ●---●) cells collected 14 days later. (C and D) replication in cells from CE-inoculated sheep (△——△ , ▲——▲) cells collected prior to CE inoculation (△---△ , ▲---▲) cells collected 14 days after inoculation.

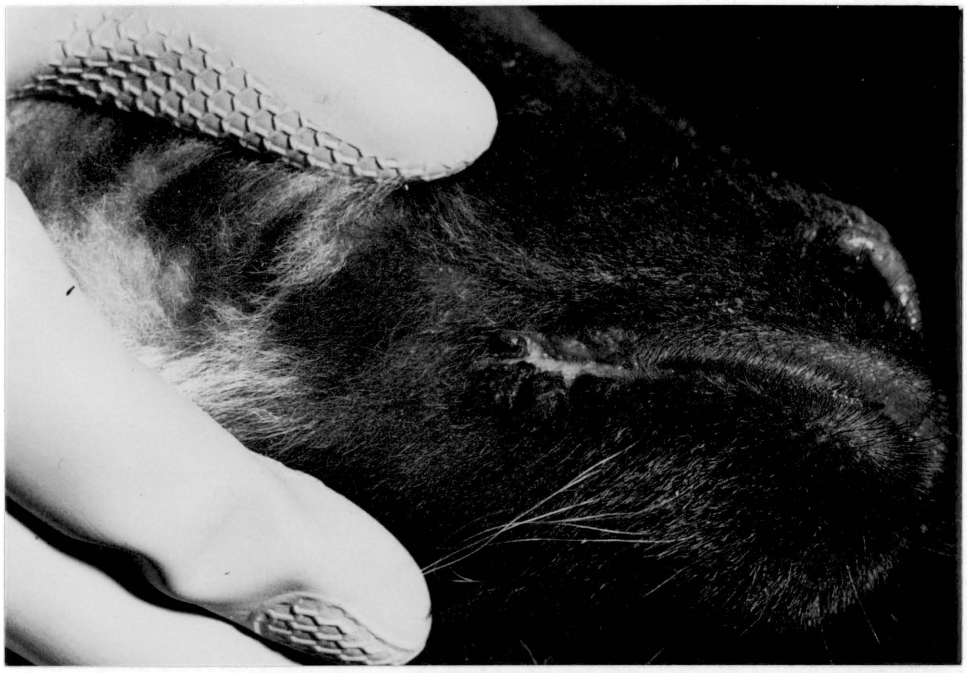


Fig. 13 Contagious ecthyma lesions in the axilla of a sheep 6 days PI.

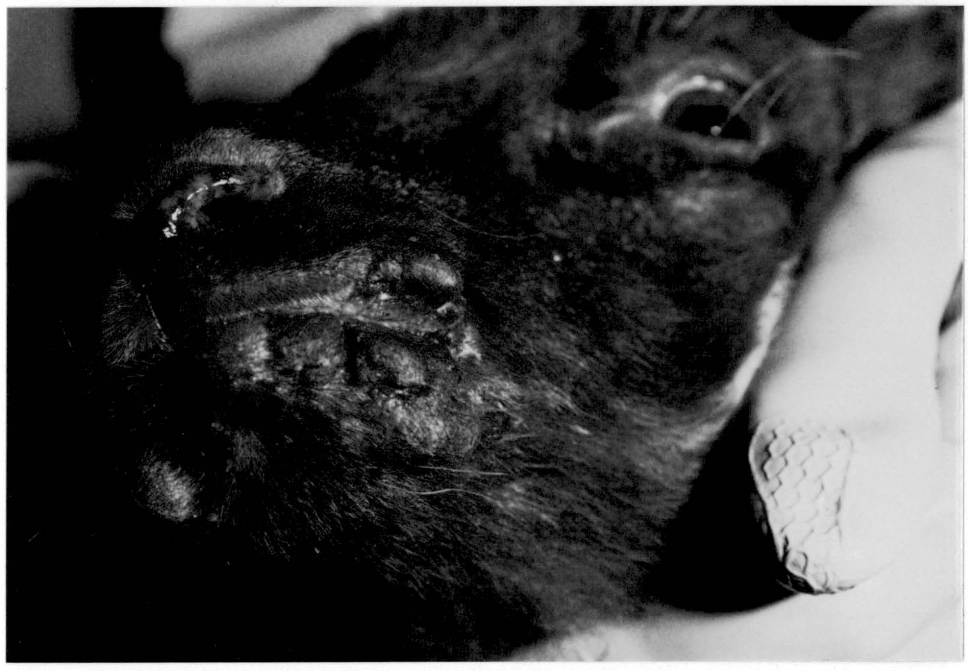


Fig. 14 Lesions in the axilla of a sheep 13 days after the start of the corticosteroid treatment (76 days after CE inoculation).The lesions developed at the site of the initial lesions seen in Fig. 13.

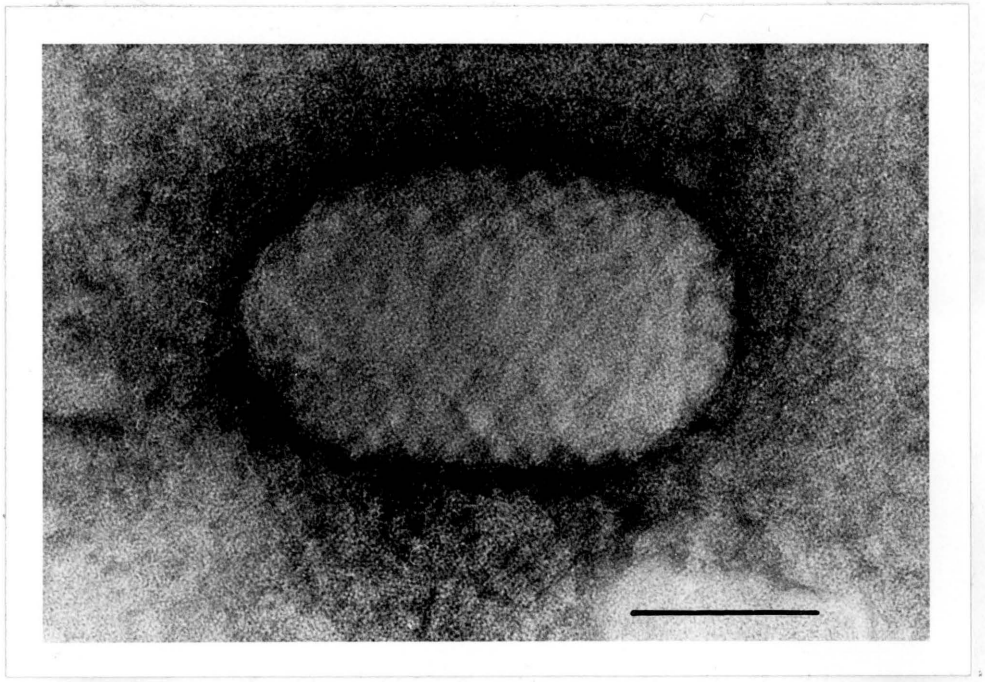


Fig. 12 Contagious ecthyma virion observed by electron microscopy;bar represents 100 nm.

Fig. 13 Contagious ecthyma lesions in the axilla of a sheep 6 days PI.

Fig. 14 Lesions in the axilla of a sheep 13 days after the start of the corticosteroid treatment (76 days after CE inoculation).The lesions developed at the site of the initial lesions seen in Fig. 13.

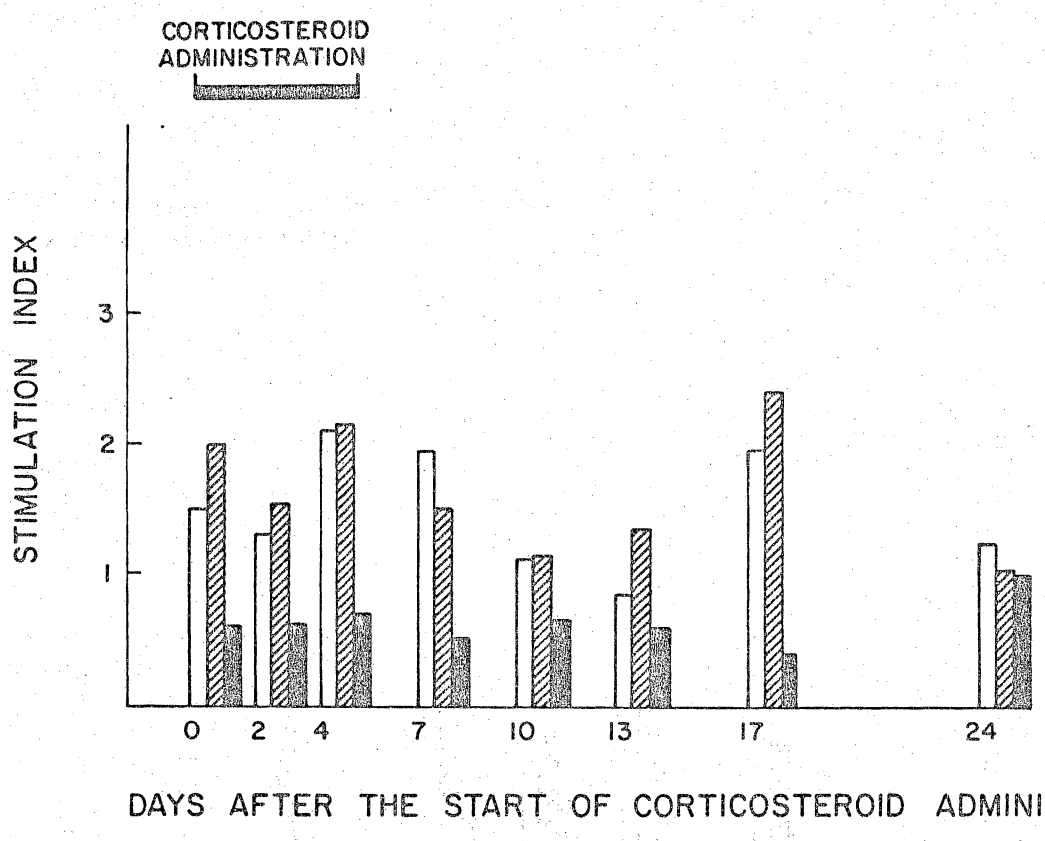


Fig. 15 Whole blood lymphocyte transformation response to CE viral antigen in sheep after the administration of corticosteroids. Dexamethasone 0.15 mg/kg was administered intramuscularly and 0.5% hydrocortisone cream was applied topically daily for 5 consecutive days. Data are given as means (bars). Open bars represent CE-inoculated sheep treated with corticosteroids (n=3); striped bars represent CE-inoculated sheep not treated with corticosteroids (n=2); solid bars represent uninoculated sheep treated with corticosteroids (n=2).

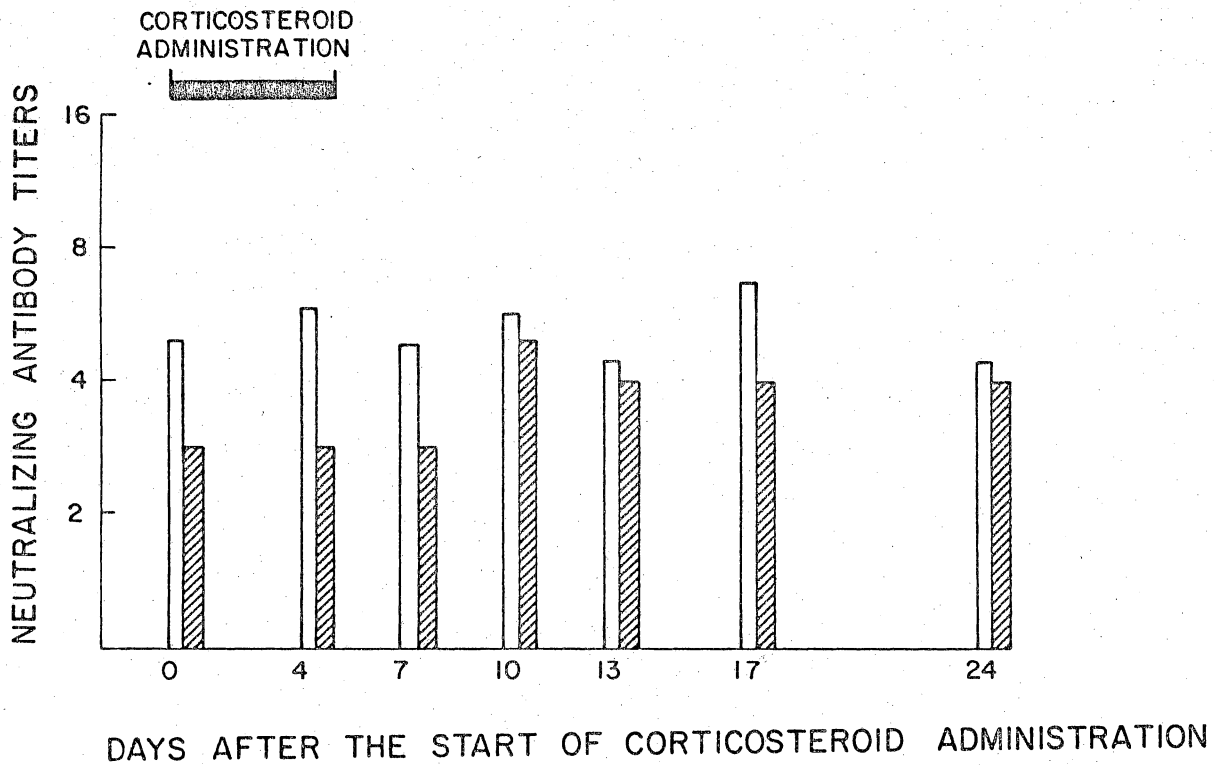


Fig. 16 Neutralizing antibody response to CE virus in sheep after the administration of corticosteroids. Data are given as means (bars). Open bars represent CE-inoculated sheep treated with corticosteroids (n=3); striped bars represent CE-inoculated sheep not treated with corticosteroids (n=2).

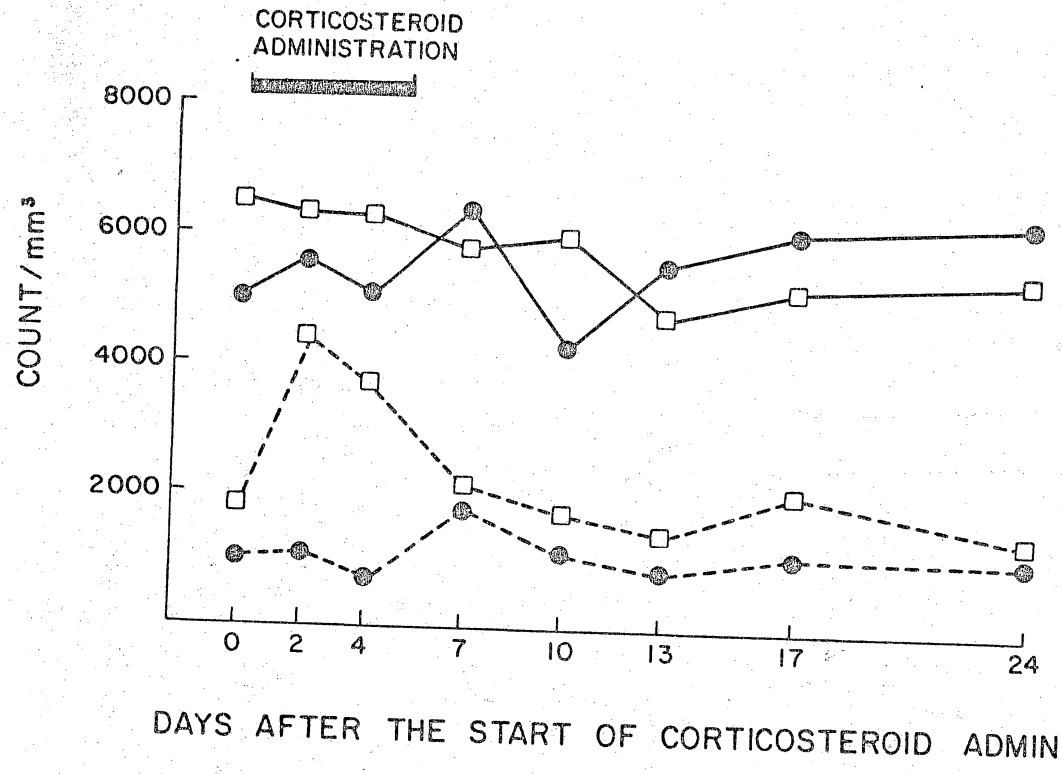


Fig. 17 Changes in leukocyte count in sheep after administration of corticosteroids. Data are given as means. (□) Sheep treated with corticosteroids (n=5); (●) sheep not treated with corticosteroids (n=2); (□——□ , ●——●) absolute lymphocyte count; (□---□ , ●---●) absolute neutrophil count.

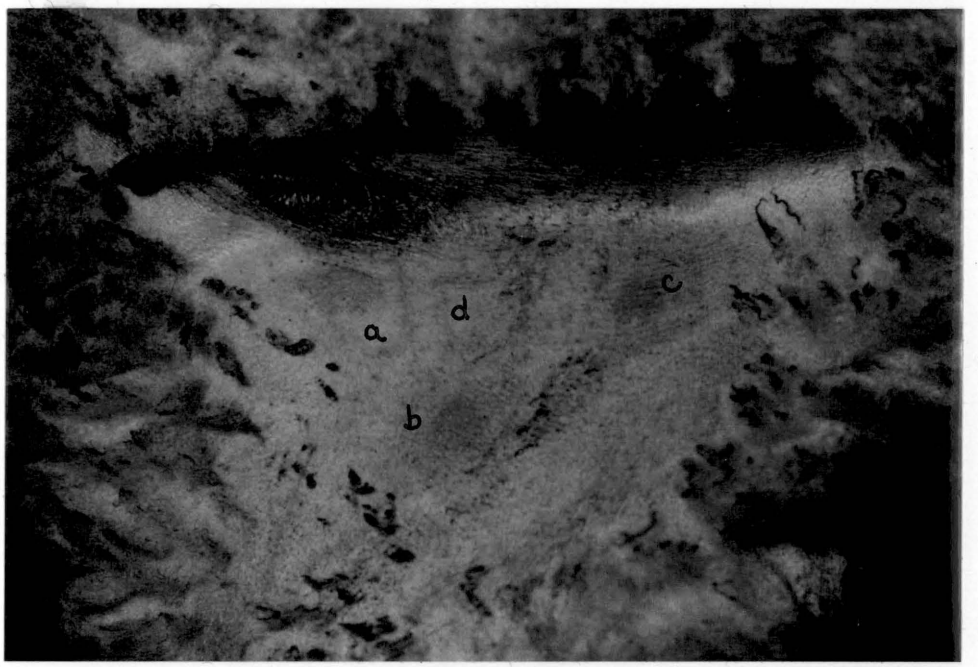


Fig. 18 Comparative contagious ecthyma DTH test performed on a sheep inoculated with CSL isolate of CE virus 35 days previously. Photograph was taken 24 hours after antigen inoculation.The following antigens were inoculated at the four sites : (a) CSL virus, (b) ST virus, (c) Har/2 virus and (d) control.

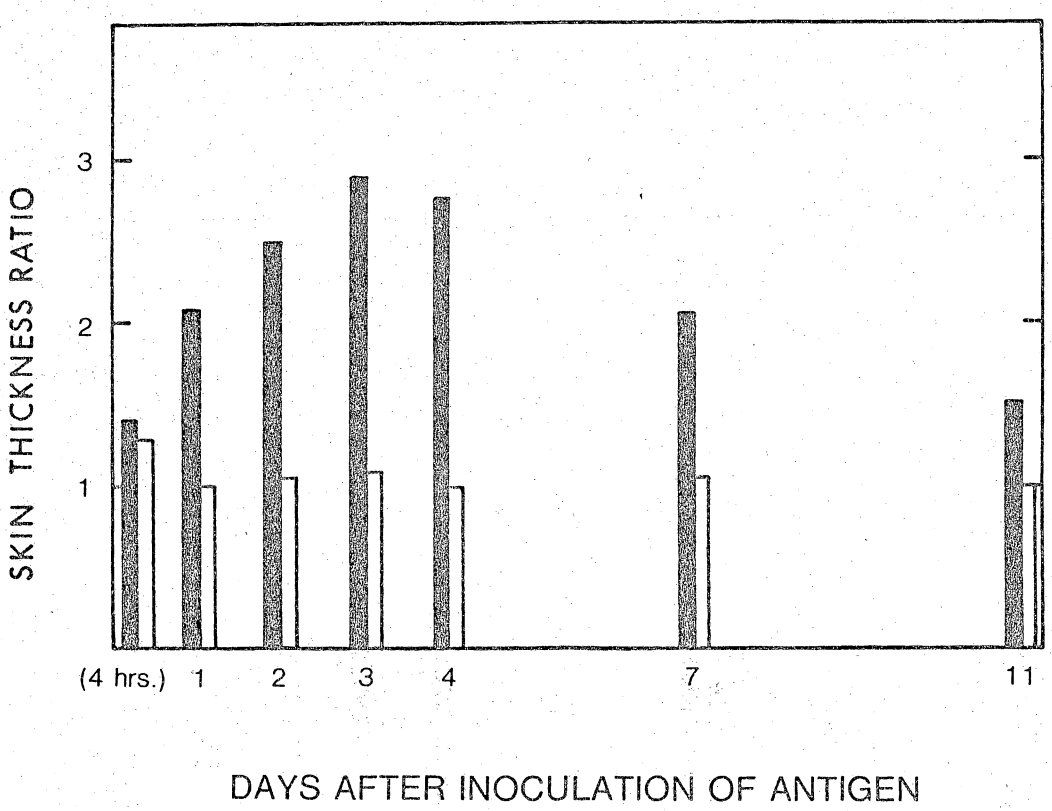
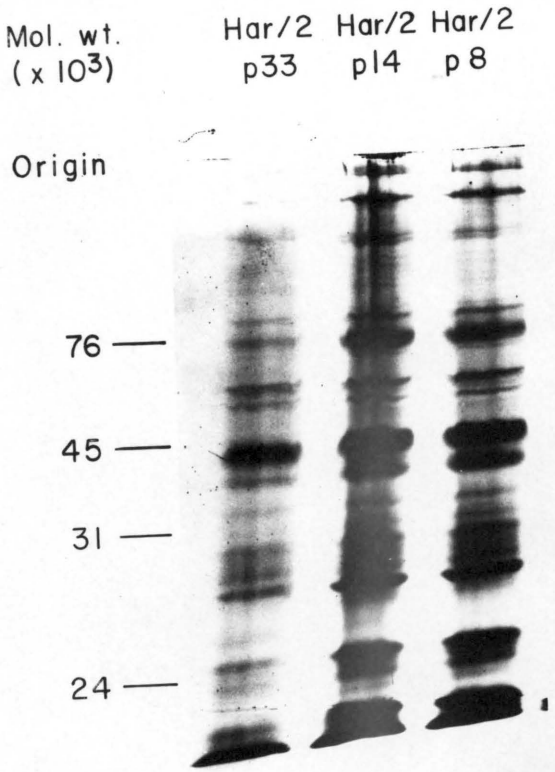


Fig. 19 Time of appearance of the increase in skin thickness following inoculation of CE viral and control antigens. The DTH test was performed 40 days after the sheep had been inoculated with CE virus. Skin thickness ratio represents skin thickness at indicated time after inoculation of the antigen divided by skin thickness prior to inoculation.

Fig. 21 Electrophoretogram of CE virion polypeptides of Cu isolate comparing viruses at different cell culture passage levels (12 percent polyacrylamide gel). Virus had been passed 8 or 32 times in lamb testis cells.

Fig. 20 Electrophoretogram of CE virion polypeptides of Har/2 isolate comparing viruses at different cell culture passage levels (12 percent polyacrylamide gel). Virus had been passed 8, 14 or 33 times in lamb testis cells.


Mol. wt.
(x 10³)
Har/2
p33
Har/2
p14
Har/2
p 8
Origin
76
45
31
24

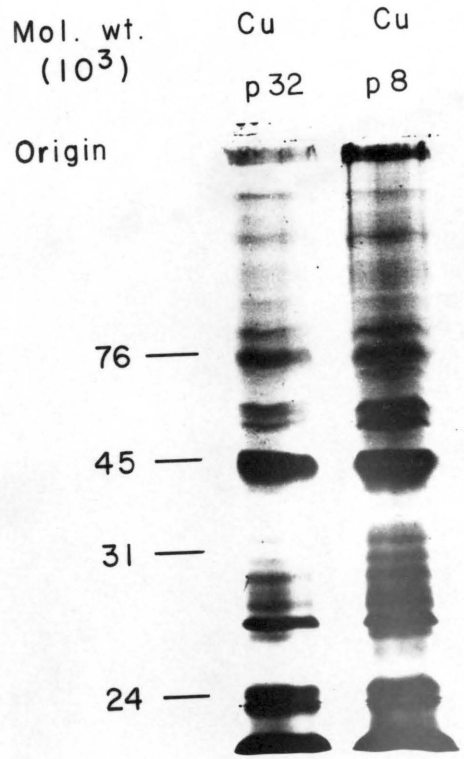

Mol. wt.
(10³)
Cu
p 32
Cu
p 8
Origin
76
45
31
24

Fig. 22 Electrophoretogram of CE virion polypeptides (12 percent polyacrylamide gel). Eleven different isolates of CE virus are shown in columns 1 - 11. The vertical bar on the left indicates the region in which variations among the isolates were detected.

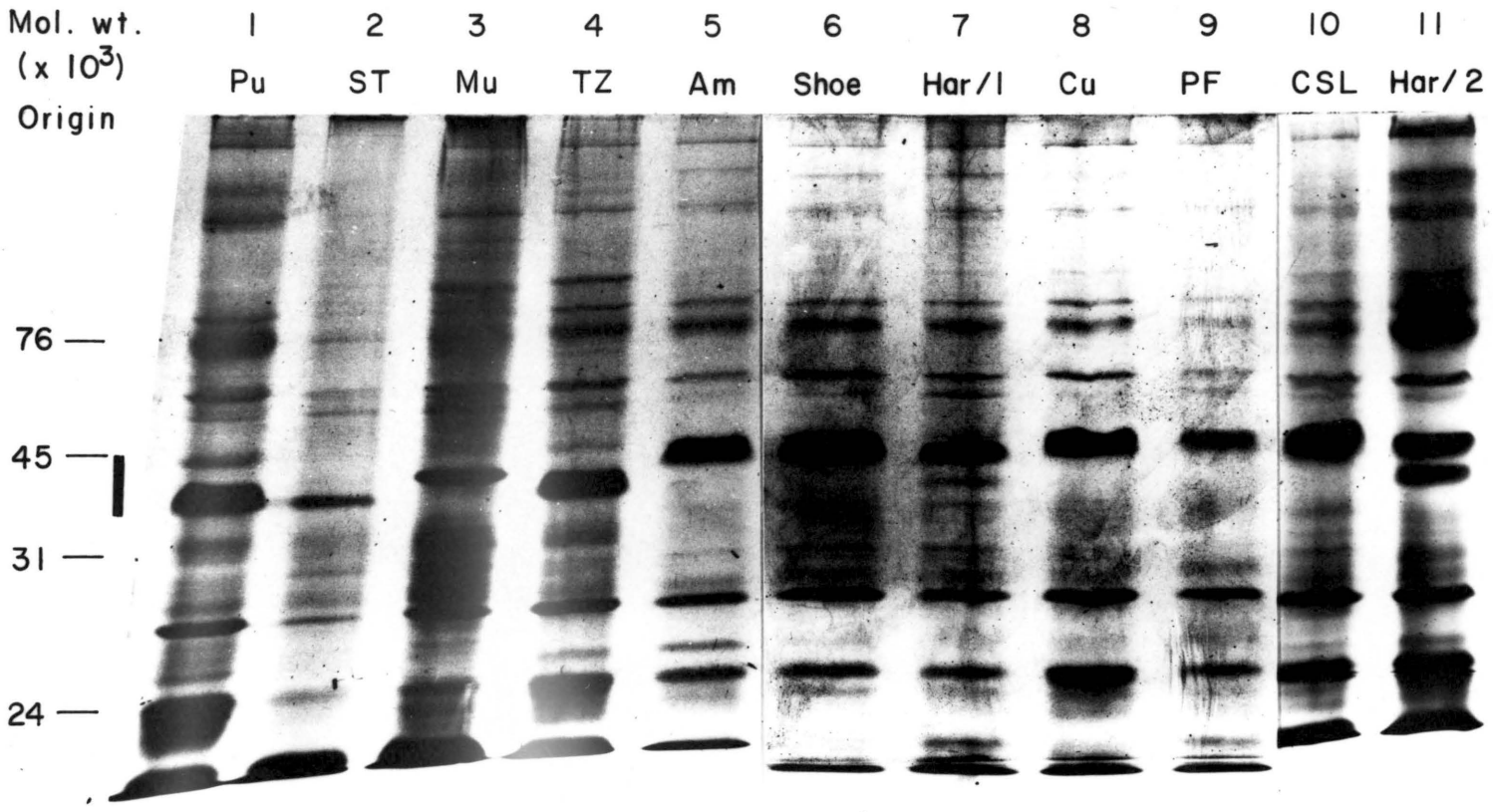

Mol. wt.
(x 10³)
Origin
1
Pu
2
ST
3
Mu
4
TZ
5
Am
6
Shoe
7
Har/1
8
Cu
9
PF
10
CSL
11
Har/2
76
45
31
24

Fig. 23 Electron micrographs of untreated CE virus and virus treated with Nonidet P-40 and 2-ME. (A) CE virions (negative stain). Treatment of CE virus with Nonidet P-40 and 2-ME: (B) viral core is seen still partially associated with surface components; (C) membranous surface structures organized into flat sheets (arrows); (D) partially purified surface components, arrow indicates structure resembling surface tubule. Marker in each panel represents 100 nm.

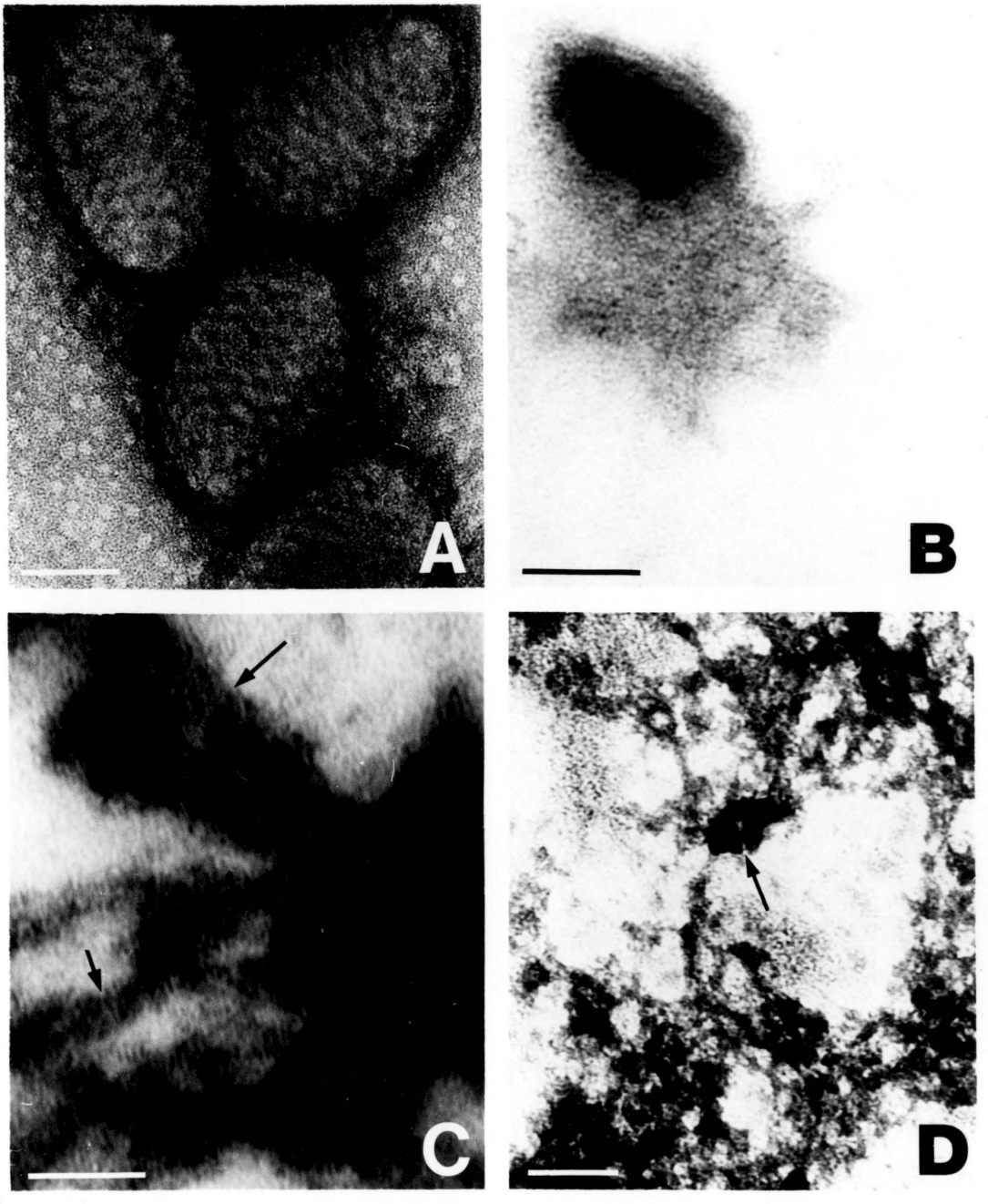
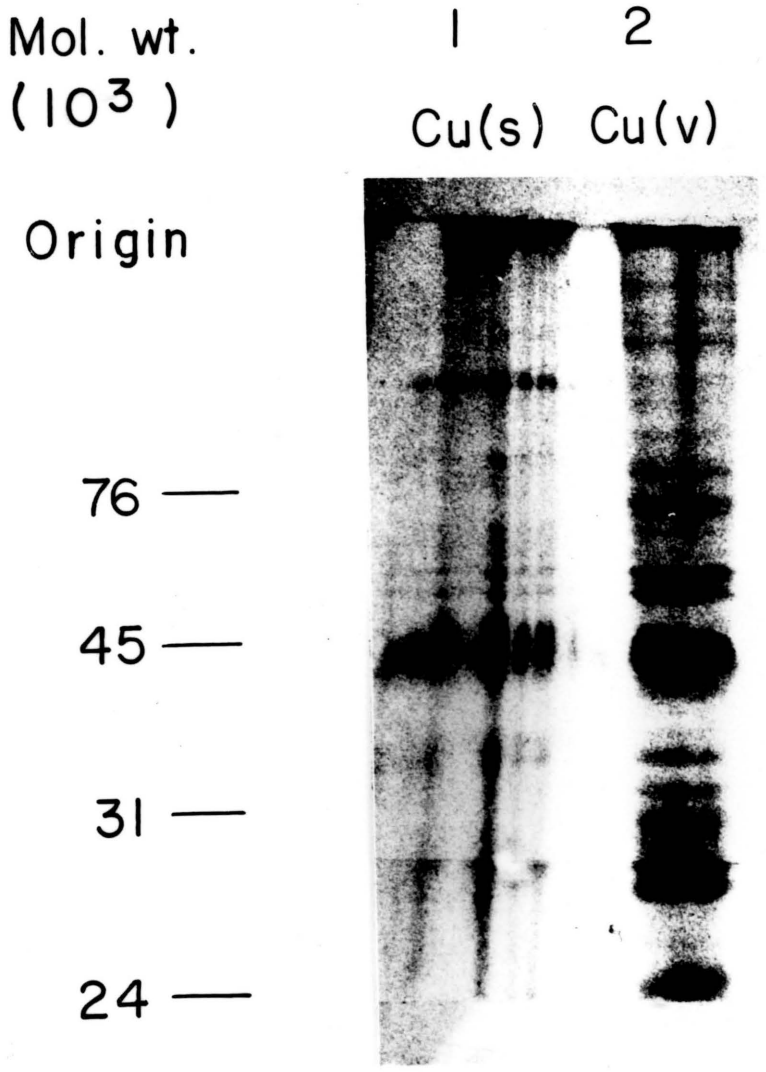

A
B
C
D

Fig. 24 Electrophoretogram of virion polypeptides of Cu virus and polypeptides released from the Cu virus treated with Nonidet P-40 and 2-ME (12 percent polyacrylamide gel). Column 1 - polypeptides released after treatment with Nonidet P-40 and 2-ME; column 2 - polypeptides of Cu virus.


Mol. wt.
(10³)
1
2
Cu(s)
Cu(v)
Origin
76
45
31
24

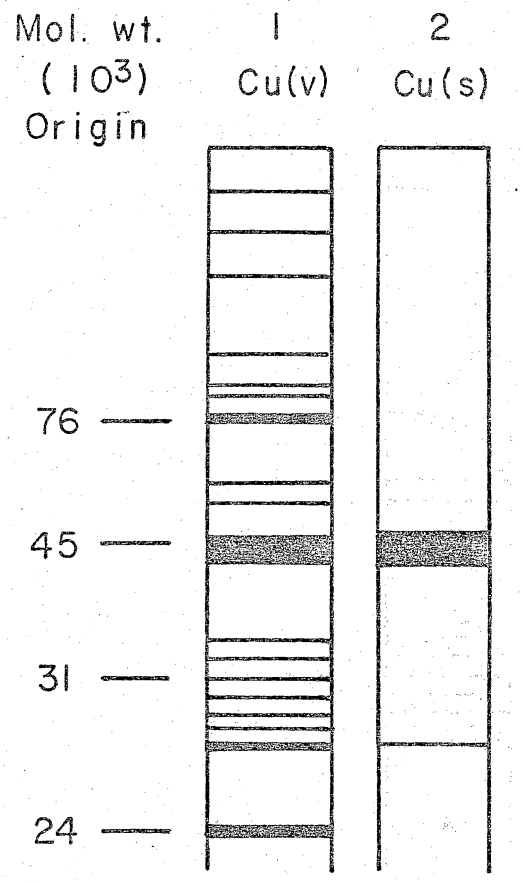
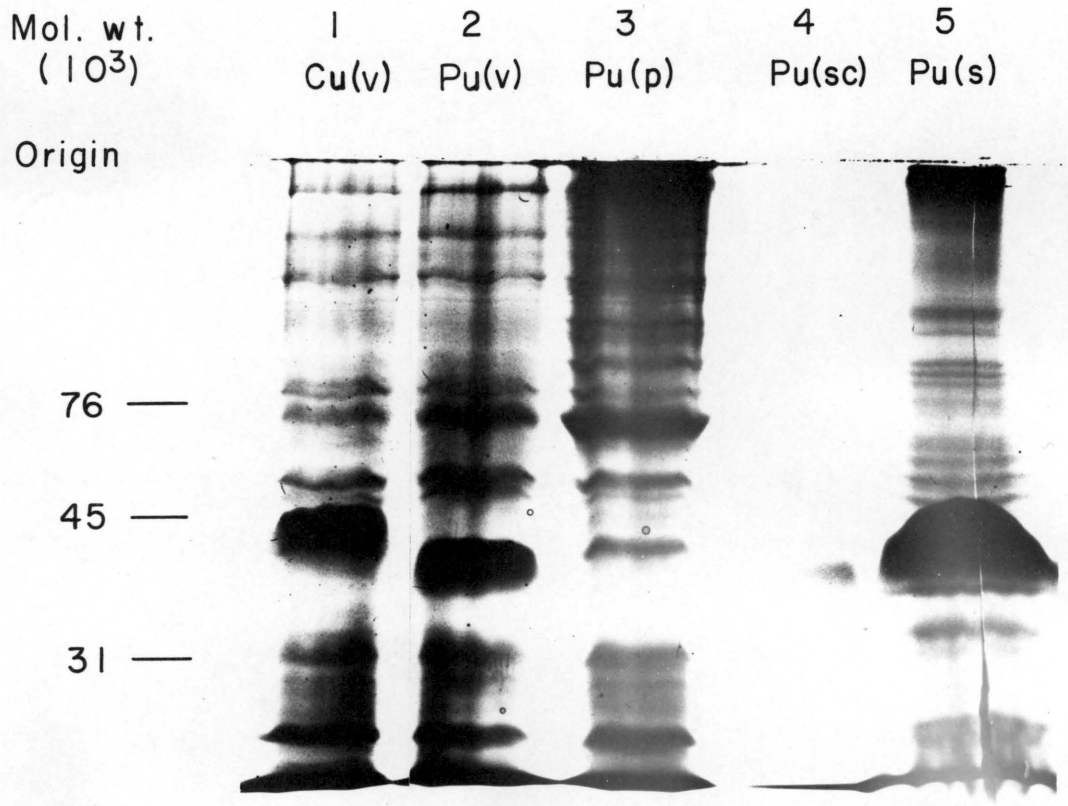


Fig. 25 Schematic diagram of Cu virus polypeptides and polypeptides released from Cu virus treated with Nonidet P-40 and 2-ME and then fractionated by centrifugation (12 percent polyacrylamide gel). Column 1 - polypeptides of Cu virus; column 2 - polypeptides released after fractionation.

Fig. 26 Electrophoretogram of virion polypeptides of Cu virus, Pu virus and polypeptides released from Pu virus treated with Nonidet P-40 and 2-ME (12 percent polyacrylamide gel). Column 1 - polypeptides of Cu virus; column 2 - polypeptides of Pu virus; column 3 - pellet fraction which resulted from centrifugation of the Nonidet P-40 and 2-ME treated Pu virus; column 4 - supernatant fraction from centrifugation described above after passage through a DEAE A-50 column; column 5 - supernatant fraction, polypeptides released after treatment with Nonidet P-40 and 2-ME.


Mol. wt.
(10³)
1
Cu(v)
2
Pu(v)
3
Pu(p)
4
Pu(sc)
5
Pu(s)
Origin
76
45
31

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CONTAGIOUS ECTHYMA VIRUS INFECTION OF SHEEP: VIROLOGIC AND IMMUNOLOGIC INVESTIGATIONS

by

Bryce Malcolm Buddle

(ABSTRACT)

Outbreaks of contagious ecthyma (CE) have been reported in vaccinated sheep and studies were undertaken to investigate the causes of these vaccination failures. The vaccination procedure was very effective in inducing a lesion at the site of vaccination, but a proportion of sheep (17 percent) were not fully protected when reinoculated with CE virus 4 weeks later. The size of the primary vaccination lesions, virus neutralizing antibody titers and virus-specific lymphocyte stimulation indices could not be used to predict the degree of protective immunity.

Measurement of the neutralizing antibody and virus-specific lymphocyte transformation responses suggested that there was a minimal systemic immune response following CE virus inoculation. Higher levels of systemic immunity may be induced by parenteral administration of live CE vaccines compared to the current procedure of inducing a localized skin infection. Replication of CE virus in buffy coat cells in vitro suggested that the virus may replicate in macrophages and therefore parenteral administration of vaccines may be feasible.

Occurrence of CE in vaccinated sheep raised questions about possible variation of antigenic types of CE virus. It was found that cross-neutralization and delayed-type hypersensitivity tests

could not be used to classify the CE viral isolates. However, analysis of the structural polypeptides of CE virions revealed differences among the isolates in the position of distinct polypeptide bands in the molecular weight region of 37,000-44,000 daltons, allowing the isolates to be classified into four groups. The polypeptides which varied among the different groups were shown to be located in the surface component of the virion. Unilateral cross-reactions detected in cross-neutralization tests were found to correlate with classification of the isolates based on the position of the distinct polypeptide bands.

Cross-immunity tests were performed in lambs using two isolates which did not cross-react in the cross-neutralization tests and in which differences in the polypeptide profiles were detected. Re-inoculation with virulent sheep-passaged CE viruses overcame the immunity of the lambs. By contrast, there was protection against the less virulent cell culture-passaged CE viruses with cross-protection between the two isolates. These results suggest that virulent CE viral isolates may be responsible for the occurrence of CE in vaccinated animals rather than differences in antigenicity.

Two epidemiological aspects of CE infection of sheep were also studied. Latent CE infections were investigated by treating CE-inoculated sheep with corticosteroids. Treatment induced recrudescence of lesions at sites of previous CE virus inoculation, but virus could not be isolated from these lesions. Hence, the existence of latent infections could not be confirmed, but it is unlikely that latent

infections are important for the initiation of CE disease outbreaks.

Importance of colostral immunity was investigated with ewes vaccinated 6 months prior to parturition. This vaccination did not result in sufficient colostral immunity to protect lambs from subsequent exposure to CE virus, however, the severity of the CE lesions may have been reduced.