Isolation, Immunodiffusion, Immunofluorescence, and Electron Microscopy of a Syncytial Virus of Lymphosarcomatous and Apparently Normal Cattle

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SUMMARY

A viral agent inducing syncytia formation in monolayer cultures of bovine embryonic spleen cells and a rabbit cornea cell line was isolated from cattle with lymphosarcoma. The agent was strongly cell-associated and transmissible with cell-free filtrates at very low dilutions. An antigen prepared from heavily infected cultures produced precipitin lines by immunodiffusion when tested against sera from lymphosarcomatous and apparently normal cattle. Buffy coat cells and cellular elements present in milk from apparently normal cattle whose sera gave positive precipitin reactions contained the virus. Some nonreacting offspring of dams that reacted were also virus carriers. Fluorescent conjugates prepared with precipitating sera stained infected cells specifically while nonprecipitating sera failed to do so. Electron microscopic examination of ultrathin sections from infected cell cultures revealed virus-like particles budding at the cell membranes in a manner resembling the avian and murine leukemia viruses. Morphologically, these virus-like particles bore some resemblance to the mouse mammary tumor virus. Evidence that the virus plays a role, if any, in inducing bovine lymphosarcoma has not been established.

INTRODUCTION

There is considerable agreement among investigators that lymphosarcoma (malignant lymphoma, leukemia, leukosis, lymphomatosis) of cattle is an infectious process and that a virus is the probable etiologic agent. Evidence favoring this thesis is based upon epidemiologic observations (1, 2, 4), reactivity of cell cultures originating from lymphosarcomatous tissue (8-10, 14), and the presence of virus-like particles in milk (6, 7) and sections of lymph nodes of affected cattle examined electron microscopically (22, 24). Transmission experiments using lymphosarcomatous tissue as inoculum have produced suggestive but inconclusive results (12, 20, 23).

Epidemiologic studies in Denmark (2) and the United States (4) favor the hypothesis that transmission of lymphosarcoma is principally vertical, thus indicating genetically determined susceptibility, in utero infection, or transmission via dam's milk. In Sweden there was an increased incidence of lymphosarcoma in cattle following vaccination against piroplasmosis using whole blood from experimentally infected calves (19). This fact suggests that an agent may have been present in calves classified as clinically normal and transmission occurred through blood inoculation.

Cell cultures prepared from affected lymph nodes or cell-free extracts of lymph nodes used as inoculum for cell lines produced syncytia of the type frequently seen with canine distemper or rinderpest virus (8, 10, 14). Lymphocyte adsorption on cells cultured from affected lymph nodes simulated graft rejection reactions (8, 10). On the basis of these observations, Gard (9) suggested that bovine lymphosarcoma is a virus-induced disease in which lymphocytes participate in a violent host versus graft reaction.

The finding of virus-like particles in milk from a herd with a high incidence of leukemia suggests a viral etiology and a possible means of transmission (6, 7). The presence of similar virus-like particles in milk from apparently normal cattle (13, 21) would tend to nullify the significance of the finding unless it could be demonstrated that so-called normal cattle shed biologically active virus into the milk.

Accumulated circumstantial evidence favors the hypothesis of a viral etiology for bovine lymphosarcoma, but there is insufficient proof of any biologic activity associated with a candidate virus. It is our purpose to present evidence that a viral agent is associated with lymphosarcomatous as well as with apparently normal cattle and that it may qualify as an etiologic factor in the induction of lymphosarcoma.

MATERIALS AND METHODS

Experimental Cattle

Lymphosarcomatous cattle were obtained from a packing plant where lymphosarcoma was diagnosed by a veterinary inspector during ante-mortem inspection. Several affected cattle were obtained through practicing veterinarians when the condition was diagnosed in the field. Final diagnosis was made after the affected cattle had been brought to the laboratory and subjected to necropsy and pathologic examination.
Cattle from a closed experimental herd maintained at the National Animal Disease Laboratory (NADL) served as normal controls. Over a period of years no clinical cases of lymphosarcoma had been observed within the herd.

Specific pathogen-free calves were obtained from the NADL experimental herd. The calves were maintained in isolation and fed a synthetic milk diet in place of colostrum and dam’s milk until they could be placed on a diet of pelleted calf feed, alfalfa hay, and water. Calves were inoculated intravenously either 1 or 2 days after birth or at 6 to 12 weeks of age.

Sera

Sera were obtained from a small herd of dairy cattle (Herd No. 1) where 2 cases of lymphosarcoma had occurred in an 18-month period and from a larger herd of beef cattle (Herd No. 2) where there had been 2 cases of lymphosarcoma within a 6-month period. Blood samples were taken from a number of cattle with lymphosarcoma or other neoplastic processes diagnosed at the packing plant but not submitted to the laboratory. Two hundred sera collected at random from samples submitted to the Brucella Testing Laboratory, Animal Health Division, ARS, USDA, Ames, Iowa, were used to determine the incidence of precipitating antibodies in the general cattle population.

For the study of passive transfer of antibodies, sera were collected 2 days after birth and at weekly intervals from calves born in our experimental herd and nursing their dams.

Preparation of Bovine Embryonic Spleen (BESP) Cultures

Fragments of spleens from bovine embryos obtained at a slaughterhouse were finely minced with sharp scissors and placed in Eagle’s minimal essential medium containing antibiotics (penicillin, 100 units/ml; dihydrostreptomycin, 100 μg/ml; kanamycin, 100 μg/ml) and 20% serum. The serum was a mixture of fetal calf serum (90%) obtained from a commercial source (Grand Island Biological Company, Grand Island, N.Y.) or at a commercial slaughterhouse and cow serum (10%) containing neutralizing antibodies against bovine viral diarrhea virus. The latter was incorporated into the medium to eliminate the possible introduction of bovine viral diarrhea virus. The latter was incorporated into the medium to eliminate the possible introduction of bovine viral diarrhea virus. Cultures were implanted in Blake bottles and the medium first changed after 48 to 72 hours. Fibroblastic elements were prevalent in the early growth phase but were gradually replaced by a polygonal cell type. It was sometimes necessary to maintain the cultures for 4 weeks before the desired polygonal cell type predominated. Once established, the BESP cells could be handled as a cell line for months. Routine examinations of cultures microscopically and by fluorescent antibody consistently revealed that the virus under study and other known bovine viruses were not introduced with serum used in the medium. Cultures could be expanded in the ratio of 1:2 or 1:3 at weekly intervals. When they lost vitality, a new cell line was established.

Cell Cultures from Affected and Apparently Normal Cattle

Cultures were prepared from lymph node and spleen of lymphosarcomatous cattle. Lymph node and spleen tissues were treated as described for BESP cells and grown in the same type of medium. Cell suspensions were planted in milk dilution bottles and unattached cells were removed during the first medium change. Cultures were dispensed with 0.02% ethylenediaminetetraacetic acid (EDTA) in Ca++ and Mg++ free phosphate-buffered saline (PBS) and were transferred up to 5 to 6 times at approximately 10-day intervals. Microscopic observations were made daily to detect the formation of syncytia.

Buffy coat cultures from lymphosarcomatous and normal cattle were prepared by collecting 45 ml of venous blood into 5 ml of a 10% sodium citrate solution. The citrated blood was centrifuged at 500 X g for 20 minutes and the plasma aspirated. Buffy coat cells were removed by skimming with a 1.0-ml pipet and placed onto monolayer cultures of BESP cells. Unattached cells and erythrocytes were removed by aspiration after 48 hours’ incubation and replaced with fresh medium. Cultures were subsequently dispersed with EDTA and transferred in the same manner as the spleen and lymph node cultures.

Rabbit Kidney and Cornea Cell Cultures

Kidney cells (RK) were dispensed by trypsinization and grown in Hanks’ balanced salt solution containing 0.5% lactalbumin hydrolysate and 10% specific pathogen-free calf serum. After the cell sheet had grown to confluence, the medium was replaced with Eagle’s minimal essential medium plus 10% of the serum mixture used for BESP cells. Secondary and tertiary cultures were frequently employed as long as they maintained vitality. A rabbit cornea cell line (SIRC) (15) was obtained from the American Type Culture Collection (CCL60), Rockville, Md. It was maintained in Eagle’s minimal essential medium with a 10% serum mixture similar to that used for BESP cells.

Isolation of Virus from Milk

The udders were washed with benzalkonium chloride 1:5000 (Roccal: Winthrop Laboratories, New York, N. Y.) and dried. Milk was drawn directly into 250-ml centrifuge bottles and centrifuged at 500 X g for 20 minutes. The cream and milk were aspirated leaving a deposit of cellular elements. Sedimented cells were resuspended in milk similar to that used for BESP cells, mixed with dispersed BESP cells, and then planted in milk dilution bottles. In addition to the usual antibiotics, nystatin was added to reach a concentration of 100 μg/ml. After cultures had become confluent, they were transferred an additional 3 to 5 times and observed daily for syncytia formation. Milk collected at a dairy was handled similarly. To study the effect of pasteurization, the milk samples were collected from the same bulk supply before and after pasteurization.

Transfer of Virus

Cultures containing numerous syncytia were dispersed and sedimented by centrifugation. A suspension of the infected cells was either added to noninfected BESP cells immediately before planting or seeded onto a cell monolayer. Infected cells from BESP cultures were added to RK or SIRC cells in the same manner.
Maintenance of Infected Cultures

Either BESP or SIRC cell cultures having a large number of syncytia underwent complete cytolysis if transferred serially. To maintain infected cultures, it was necessary to add noninfected cells during the transfer operation.

Preparation of Precipitating Antigen

Suspensions of BESP or SIRC cells were infected by incorporating cells from a heavily infected culture and planting them in Blake bottles. Usually, infected cells from a culture in a milk dilution bottle were added to sufficient noninfected cells to plant 2 Blake bottles. After becoming confluent, the cultures were expanded in the ratio of 1:2. This procedure was continued until most of the cells had formed syncytia. Cells floating in the medium were combined with cells scraped from the Blake bottles and sedimented by centrifugation at 900 Xg for 30 minutes. The supernatant was aspirated until there were equal volumes of packed cells and fluid. The packed cells and fluid were immersed in an ice bath and disrupted with a sonifier, Model LS 75 (Branson Instruments, Inc., Stamford, Conn.) equipped with a micro-tip horn, until the fluid had an opalescent appearance. The antigen was placed in vials and stored at —70°C until used.

Immunodiffusion Test

A 1% agar gel was prepared with lonager #2 (Oxoid) in 0.01 M PBS, pH 7.2—7.5, and preserved with thimerosal 1:10,000. Melted agar was poured into 60-mm plastic Petri dishes. A perspex template was placed over the uncovered Petri dish and a No. 1 cork borer introduced through the holes to cut the agar. The distances from the center well to the outside wells were 2.5 mm. Plates with antigen and sera were incubated at room temperature in a humidified chamber. Final readings were made after 5 to 7 days.

Infected Cultures on Coverslips

Infected BESP or SIRC cells were planted on coverslips in Leighton tubes. These cultures were removed for staining with May-Grunwald-Giemsa or with fluorescein-labeled antibody.

Filtration of Virus

Buffy coat cells from an apparently normal cow (#3892) in the NADL herd were collected and used to infect BESP cells as already described. Medium from an infected culture with over 50% of the monolayer as syncytia was harvested, clarified by centrifugation at 1500 X g for 30 min, and filtered through a 0.22 μ Millipore filter (Millipore Corporation, Bedford, Mass.). Noninfected BESP cells were dispersed and divided into 2 portions, one being implanted with regular medium and the remaining with 100 ml of filtrate. The filter pad was rinsed with PBS, and cell retention was verified by filtering 10 ml of a 24-hour broth culture of Serratia marcesens. The spent filtrate used in implanting one culture was replaced after 72 hours with regular medium. Dispersed cells from confluent cultures implanted with filtrate were transferred to Leighton tubes with cover slips. These cultures were examined after staining with fluorescein-labeled antibody.

Twenty ml of filtrate from the same pool as used for implanting BESP cells were also inoculated intravenously into a 5-week-old colostrum-deprived calf. Previous tests by culturing buffy coat cells had indicated that the calf was not a virus carrier.

Preparation and Application of Fluorescent Antibody

Sera from cattle with lymphosarcoma, normal cattle, or experimentally infected calves were used for the preparation of fluorescent antibody conjugates.

Separation of the γ-globulin fraction and conjugation with fluorescein isothiocyanate was carried out as described by Cherry et al. (3) except that the serum was not held overnight after the first addition of ammonium sulfate but was stirred for 10 min and the precipitate collected by centrifugation. Unconjugated fluorescein was removed by passing the conjugate through a Sephadex G-25 column as described by Dedmon et al. (5). The conjugate was adsorbed with bovine liver powder before use.

Coverslips were rinsed in PBS, pH 7.2, fixed for 15 min in cold acetone, dried, and stained with undiluted conjugate for 30 min at 37°C in a moist chamber. Excess conjugate was removed by placing the coverslips in PBS, pH 7.2, for 15 min. The coverslips were then dipped in distilled water, the upper surface wiped dry, and mounted on slides using 10% glycerin in PBS, pH 7.2, as a mounting medium.

A Leitz Ortholux microscope equipped for fluorescence microscopy was used to examine the slides. Photomicrographs were taken with a 35-mm Leica micro camera attachment and Kodak highspeed Ektachrome daylight-type film (Eastman Kodak, Rochester, N. Y.).

Electron Microscopy

Infected BESP or SIRC cells were propagated and transferred in Blake bottles until over 50% of the monolayers were made up of syncytia. The cells were dispersed with EDTA and lightly sedimented at 120 X g for 5 min. The cells were then resuspended in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer for 30 min, washed in 0.2 M sodium cacodylate buffer overnight, postfixed in osmium tetroxide for 30 min, and then double-rinsed in 0.2 M sodium cacodylate buffer for 30 min. The fixed cells were then pelleted in warm agar by centrifuging at 1300 X g for 10 min. The pellet was cut into 1- to 2-mm cubes, dehydrated through a routine series of alcohols, and embedded in Epon 812 according to the method of Luft (16). Thin sections were cut with an Ultratome (LKB Instruments, Inc., Rockville, Md.) with diamond knife and picked up on 200-mesh uncoated copper grids. The sections were stained with a Reynold's lead citrate stain according to the method of Venable and Coggeshall (25). Sections were examined with a Phillips EM-200 electron microscope at 60 kv.
RESULTS

In 4 of 15 cattle with lymphosarcoma, a viral agent was isolated which induced syncytia formation in BESP cell cultures. Two of the isolates were lost, but the remaining 2, LS-13 isolated from lymph node and milk sediment and LS-18 isolated from spleen, have been maintained for 18 and 13 months respectively. Infected BESP cells formed syncytia (Figs. 1, 2) which were easily differentiated from the polygonal type growth produced by noninfected cells (Fig. 3). Transmission of the bovine syncytial virus was readily accomplished by adding infected cells to noninfected cultures. Serial passage of infected cultures tended to increase the number of syncytia, and complete destruction of the monolayer ensued unless noninfected cells were added periodically.

Buffy coat cells from 5 cows and 1 bull (only 6 cows and 1 bull were investigated) in the NADL herd that did not have clinical lymphosarcoma but reacted in the immunodiffusion test also infected BESP cells. Syncytia in the cultures similar to those produced by isolate LS-13 or LS-18 became evident after the first transfer of the cultures. In addition to isolation from leukocytes, cellular elements from the milk of cow #4699 (one out of 2 lactating cows) infected BESP cells. Leukocytes from a 6th reacting cow, #4739, could not be cultured satisfactorily because of concurrent infection with trypanosomes. However, virus was present in the milk sediment of Cow #4739 and was readily isolated without contamination with trypanosomes.

Buffy coat cells from 11 cows in the NADL herd that did not react in the immunodiffusion test but were offspring of reactors were cultured on monolayers of BESP cells. Two of the 11 were virus carriers as evidenced by the formation of syncytia in BESP cells which also stained specifically with fluorescent antibody. One of the reactors was the offspring of a reacting dam and a reacting sire. A summary of virus isolations from apparently normal cattle is presented in Table 1.

Two samples of raw pooled milk from a dairy were contaminated with fungi so that the cultures could not be maintained long enough for potential virus to produce syncytia. Pasteurized milk obtained from the same milk pools did not induce the development of syncytia in BESP cells during a 6-week observation period.

Infected BESP cells added to RK and SIRC cells resulted in the formation of syncytia of somewhat elongated shape (Fig. 4). Once established in RK or SIRC cells, the infection was readily maintained by adding noninfected cells. SIRC cells appear to be as susceptible to infection as BESP cells, or slightly more so.

Disrupted cells from infected BESP or SIRC cultures having over 50% syncytia in the monolayers contained 3 precipitating antigens (Figs. 5, 6). Some sera reacted only against 1 antigen (Fig. 7), while others reacted against all 3. With some weak antigens, only 1 precipitin line was produced regardless of the serum tested. Lymph node or spleen cultures from lymphosarcomatous cattle in which no viral agent could be demonstrated did not contain detectable precipitating antigen. Results of the immunodiffusion tests with sera from different sources are summarized in Table 2.

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<td>NADL experimental herd (Control)</td>
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Immunodiffusion reactions against bovine syncytial virus antigens in cattle sera.

aOrigin of 2 clinical cases of bovine lymphosarcoma submitted to the laboratory within an 18-month period.

bNo clinical cases of lymphosarcoma have been observed over a period of several years.

The immunodiffusion test proved useful in demonstrating antigenic relationships between virus isolates, duration of passive antibody response, and in screening sera for fluorescein conjugation.

Antigens prepared from 2 different isolates reacted against a heterologous as well as the homologous antisera but not with complete lines of identity (Fig. 8). Antibodies present in the dams were transferred passively to calves via the colos- trum, as indicated by precipitin reactions with sera from cows numbered 3953 and 4739 (Figs. 5, 6), but had largely disappeared 12 weeks after birth. Sera collected from the NADL experimental herd in May 1961 also included specimens from cattle that were virus carriers in 1967. Six of 6 sera from the earlier date reacted in the immunodiffusion test, thus indicating that a carrier state may have existed several years without the development of clinical signs. It is also evident that the
implanted in growth medium of the same composition as the
infected cultures formed syncytia which also stained specific
ly with fluorescent antibody. Extensive syncytial forma-
tions were not evident until after the first transfer. BESP cells
implanted in growth medium of the same composition as the
filter previously used in preparing the filtrate. Cells from a 24-hour broth culture of S. marcescens were re-
tained by the filter previously used in preparing the filtrate.

Buffy coat cells yielded virus when recovered from a calf 21
days after it had been administered 20 ml of cell-free filtrate
from the same pool used to infect BESP cells. However, the
virus was not reisolated fromuffy coat cells at 8 and 14 days
postinoculation.

Two 1-day-old calves inoculated intravenously with centri-
fuged but unfiltered fluids from an infected BESP culture de-
veloped precipitating antibodies and persistent viremia, as
demonstrated by virus isolation. Two 12-week-old calves in-
oculated intravenously with infected BESP cell suspensions re-
sponded similarly. Calves in both age groups have indicated no
evidence of infection during a 6-month period except for de-
velopment of precipitating antibodies and viremic carrier
states.

Fluorescein conjugates prepared from sera which reacted in
the immunodiffusion test also specifically stained cells infec-
ted with bovine syncytial virus whereas nonreacting sera failed
to stain infected cells. There were no differences in staining
activity among conjugates prepared from sera collected from
natural or experimentally infected cattle, nor did a variation in
the number of precipitin lines formed in the immunodiffusion
test have an effect on the staining affinities of the conjugated
sera. Conjugates which stained cells infected with 1 virus iso-
late also stained cells infected with other isolates just as in-
tensely.

Specific staining was observed in both the nuclei and cyto-
plasm of infected BESP cells (Figs. 9, 11). Staining was most
evident in the syncytia formed in infected cultures, but the
same type of staining also was observed in individual infected
cells. Nuclear staining was a constant feature of infected cul-
tures, with individual nuclei varying in appearance. A weak
diffuse fluorescence was seen in some, while others fluoresced
brightly and often appeared somewhat granular. Occasionally
the granular material was localized along the nuclear mem-
brane leaving the center of the nucleus dark (Fig. 10). Because
of these variations there was sometimes a marked difference in
appearance of nuclei within a given syncytium.

There was usually a diffuse dull fluorescence of the cyto-
plasm surrounding the nuclei of syncytia, as well as a more
widely distributed and more intensely stained particulate ma-
terial. The particulate material in some cells resembled small
vacuoles with intensely stained walls (Fig. 9) and in other cells
appeared to be solid granules (Fig. 10). Large granular or
fibrous-appearing structures which stained intensely with con-
jugated sera were also frequently seen (Fig. 11), usually being
found near or among the cluster of nuclei.

Some syncytia which contained brightly fluorescing nuclei
lacked cytoplasmic fluorescence.

Staining of infected SIRC cells revealed changes similar to
those described above, except the cytoplasmic fluorescence
was usually more diffuse and there were reduced quantities of
brightly stained, formed elements in the cytoplasm (Fig. 12).
Conjugates used in these studies did not stain BESP cells
infected with bovine parainfluenza-3 virus or bovine viral diar-
rhea virus, nor did conjugated sera from calves hyperimmu-
nized with either of these viruses stain BESP cells infected
with bovine syncytial virus.

Virus particles were demonstrated in mononucleated cells,
but more often in syncytia formed by infected BESP and
SIRC cells as illustrated by electron microscopy (Figs. 13—15). Relatively few mature virus particles were present in the prepa-

tations, most of them being in various states of maturation.

Virus particles were released by budding at plasma membranes
(Fig. 13, 15) or at membranes around cytoplasmic vacuoles
(Fig. 14), and in this respect they resembled the avian and
murine leukemia viruses. The outer membranes of the bovine
syncytial virus particles were likewise derived from the cell
membranes during the process of budding. Morphologically,
the virus particles somewhat resembled the mammary tumor
virus (17) in that surface projections were prominent (Fig. 13).
Whereas mammary tumor virus tends toward considerable
pleomorphism, the bovine syncytial virus particles are mark-
edly uniform in size and shape. Measurements of maturing
virus particles have indicated the following ranges in dimen-
sions: 35—45 μ as the diameter of the nucleoid; 14—16 μ from
the nucleoid through the outer membrane; 14—18 μ as the
length of the projections; and an overall diameter of
90—115 μ.

In highly infected cultures, virus particles were observed in
about 50% of the cells examined, being most prevalent near the
 plasma membrane of cells with irregular borders or along
membranes of cytoplasmic vacuoles in cells having a promi-
nent Golgi complex. Intracytoplasmic virus particles (Fig. 15)
were essentially spherical with an electronlucent center and
appeared to be migrating toward the cell membrane. Projec-
tions, which were extremely prominent on all budding virus
particles, were first observed when the nucleoid encountered
the cell membrane. The projections were retained on the sur-
face of the outer membrane of the mature virus particle.

DISCUSSION

The foregoing data have established that a viral agent induc-
ing the formation of syncytia in cell cultures is widespread in
the cattle population. Presence of the virus in bovine lympho-
sarcoma does not in itself indicate that it is the etiologic agent
since it also infects a large number of apparently normal cattle.
These are, however, certain facts made available about the
agent which make it a logical candidate virus. Its ubiquitous
distribution only tends to confirm the statement made by
Hansen (11), “Like an iceberg, bovine leukemia hides most of
its mass—and the most dangerous part of it—under the
surface—.”

It is evident from our studies that the virus is difficult to
transmit with cell-free fluid. Whether this characteristic can be
attributed to close cell association or to the production of a
high percentage of defective virus particles has not been deter-
This fact may explain in part why the virus appears strongly cell-associated. There may also be an inefficient process of virus adsorption, thus requiring a large number of virus particles in 1 infective dose. Whatever the reason may be for the low degree of infectivity, it has undoubtedly been responsible for lack of information on the biologic activity associated with the virus.

Using viable buffy coat cells as inoculum for BESPcell monolayers proved to be a simple and apparently efficient procedure for isolating the virus, the only difficulty being the presence of trypanosomes in some of the cultures (18). Fortunately some cattle sera have trypanosome growth-inhibiting properties, and these sera can be incorporated in the cell culture systems at concentrations which will eliminate the parasites in 1 to 3 transfers. Transmission of the virus by transfer of virus-carrying cells, however, makes it impossible to perform the usual types of neutralization tests.

The finding that some apparently normal sires and dams were viremic and that virus was shed in milk presents conditions that make vertical transmission an extreme likelihood. Since we have not observed spontaneous formation of syncytia in BESP cells cultured from numerous fetuses, it is probable that transmission by milk is the more likely event.

The possibility that bovine syncytial virus may be introduced into a culture system where pooled fetal calf serum is used in the medium cannot be definitely excluded. This remains a potential source of contamination whenever bovine serum is used. However, as has been pointed out, transmission of bovine syncytial virus is extremely difficult with cell-free fluid. Wherever cell lines are employed, cultures can also be adequately controlled by employing the fluorescent antibody test. We have never observed spontaneous formation of syncytia or fluorescent cells in BESP or SIRC cell cultures maintained in the type of medium described. Moreover, investigations involving a small number of newborn calves born to viremic dams failed to yield virus from buffy coat cells. In only one instance was bovine syncytial virus isolated from a newborn calf, and this occurred when both the dam and sire were viremic.

Immunodiffusion tests made it possible to detect a number of cattle that were virus carriers or had passively acquired precipitating antibodies. Whether precipitating antibodies are an indication of immunity or a reaction against certain viral precursors or whether they are components without influence on the carrier state is not known. Precipitating antibodies were not produced by all virus carriers nor by all cattle with lymphosarcoma. This fact may be an indication of immunologic unresponsiveness frequently associated with intrauterine or neonatal infections.

Antigenic similarity between isolates of the virus was indicated by cross reactions in immunodiffusion and immunofluorescence. The formation of spurs and incomplete lines of identity in the precipitin patterns, however, suggested strain differences. Whether these precipitin patterns indicate true antigenic differences between isolates or are only artifacts will be the object of future investigations.

The nature of the antigen(s) responsible for immunofluorescence was not determined. Since virus particles mature at cytoplasmic membranes, nuclear fluorescence may be an indication of an early protein synthesis taking place in the nucleus. Intense fluorescence of the membranes surrounding cytoplasmic vacuoles suggests an antibody reaction against maturing virus particles.

Demonstration of virus particles in lymphosarcomatous tissue by electron microscopy according to published reports has been infrequent, and it appears that there are relatively few infected cells in a tumor mass. This finding presents a problem concerning the significance of bovine syncytial virus as an etiologic factor in bovine lymphosarcoma. Morphologically, bovine syncytial virus bears some similarity to mammary tumor virus and undergoes a budding process similar to many of the avian and murine leukemia viruses known to be causative agents of disease. These facts, together with epidemiologic findings, should bring bovine syncytial virus into consideration as an etiologic factor.

We have neither observed infected cells undergoing transformation during prolonged incubation nor have we demonstrated any virus-related antigen(s) in cultures that did not form syncytia. Infected cells containing the antigen(s) may therefore not be truly transformed but would nevertheless be recognized by the host as foreign. Thus, the immune mechanism suggested by Gard (10) may be the actual cause of lymphosarcoma rather than the proliferation of neoplastic cells.

REFERENCES


Figs. 1, 2. Syncytia produced in bovine embryonic spleen cell cultures by a virus present in the cellular elements of milk from a lymphosarcomatous cow. May-Grünwald-Giemsa, × 132 and × 520.

Fig. 3. Noninfected bovine embryonic spleen cell culture propagated in the same type of medium as used for cultures in Figs. 1, 2. Note the uniform size and polygonal shape of the cells. May-Grünwald-Giemsa, × 400.

Fig. 4. Syncytia produced in a rabbit cornea cell line by the same virus as in Figs. 1, 2. The nuclei have a tendency to align themselves parallel to the long axis of the cell. May-Grünwald-Giemsa, × 520.

Figs. 5, 6. Immunodiffusion tests on 2 apparently normal cows, Nos. 3953 and 4739, which were virus carriers, and their offspring 2 days, 2 weeks, 8 weeks, 12 weeks, and 16 weeks after birth are in Wells No. 2, 3, 4, 5, and 6 respectively. There are 3 precipitin lines formed initially which disappear with increasing age of the calf.

Fig. 7. Reacting sera (S) from 2 different animals producing a single precipitin line against antigen (A) produced in bovine syncytial virus-infected spleen cells.

Fig. 8. Antigens A-1 and A-2, derived from 2 different bovine syncytial virus isolates, reacted against their homologous and heterologous antisera S-1 and S-2. Note incomplete lines of identity.

Fig. 9. Bovine embryonic spleen cells infected with bovine syncytial virus. Fluorescence is concentrated in the membranes of cytoplasmic vacuoles. Fluorescent antibody stain, × 520.

Fig. 10. Same cell type and virus as in Fig. 9. There are small fluorescing cytoplasmic granules and variably stained nuclei in a syncytium. Fluorescent antibody stain, × 230.

Fig. 11. Same cell type and virus as in Figs. 9, 10. Nuclei and a large cytoplasmic mass in a syncytium are stained specifically. Fluorescent antibody stain, × 230.

Fig. 12. Culture of a rabbit cornea cell line infected with bovine syncytial virus. There are several syncytia and individual infected cells with brightly stained nuclei. Fluorescent antibody stain, × 132.

Fig. 13. Electron micrograph of bovine embryonic spleen cells infected with the same virus as in Figs. 1, 2. Virus particles are budding at the surface of the cell membrane. Surface projections become prominent when the virus particle first encounters the cell membrane. × 67,250.

Fig. 14. Electron micrograph of cells from a rabbit cornea cell line infected with the same virus as in Fig. 13. Virus particles (arrows) are budding at membranes surrounding cytoplasmic vacuoles. × 54,250.

Fig. 15. Electron micrograph of bovine embryonic spleen cells infected with virus isolated from buffy coat cells of an apparently normal cow (#3892). Virus particles budding at the cell membrane have projections, while nonenveloped particles in the cytoplasm (arrows) have smooth surfaces. × 51,250.
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