Virologic and Immunologic Studies of C-type Virus Particles Isolated from Bovine Leukemia[1,2]

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DISCLAIMER

Summary

C-type virus particles were isolated from leukemic and lymphocytotic cattle in mitogen stimulated lymphocyte cultures, but not in normal cattle. The extracellular C-type virus particles were 95 to 125 nm in diameter in thin sectioning, and 100 to 150 nm in negative staining. Intracytoplasmic A-type virus particles and budding particles were observed. These virus particles were banded in a density of 1.15 to 1.18 g/cm^3 in sucrose gradient. The extracellular C-type virus particles were in a sense cell associated in that the virus particles were attached to extracellular material. Attempts to adapt C-type virus to bovine cell lines were largely unsuccessful, though, limited success was observed in bovine fetal spleen and endocardial cell lines. Immunologic tests failed to demonstrate the presence of antibody in leukemic and lymphocytotic cattle to C-type antigen. Immunodiffusion tests with C-type virus antigen and bovine syncytial virus antiserum were negative. Syncytial virus antigen tested against sera from leukemic and normal cattle also were negative. C-type virus particles were consistently isolated from cattle inoculated with tumor material as fetuses and a placental lymphosarsoma tumor developed in one of these intrafetal inoculations.

Introduction

It is a widely held hypothesis that leukemia of cattle is caused by a virus. Epidemiologic studies initially formed the basis for this hypothesis (3,4,5,17,23,34,37). Previous reports on attempts of virus isolation and transmission studies have been inconclusive and did not confirm a viral etiology (9,10,16,17,18,19,24,29,33,36,39). However, a major breakthrough in the etiology of this disease has recently been reported by two research groups. Miller et al. (25) was the first to report on demonstration of C-type particles from leukemia and preleukemic (persistent lymphocytotic) cattle. This was followed shortly by Dutta et al. (11) on the isolation of similar C-type particles from both groups of cattle, but not from defined normal cattle. In addition, Dutta et al. (11) reported budding forms, which further demonstrated the similarity of these isolates with C-type particles reported in other known animal leukemias.

The objective of this paper is to report further studies on the isolation of C-type virus particles from lymphocytotic, leukemic, and experimentally inoculated cattle. A second objective is to report on virus concentration, cell culture, and immunologic studies utilizing the C-type virus particles isolated from bovine leukemia.

Lymphocytotic cattle were included in this study, because of the evidence that these animals are in the prodromal stage of bovine leukemia (4,13,16,22,30,35,40).

Materials and Methods

Source Animals

Bovine leukemia cases were purchased and transported for study to the University of Minnesota Veterinary Hospital. The diagnosis of leukemias on all cases studied was confirmed by clinical, hematological, and necropsy examinations (41). The cows with persistent lymphocytosis were obtained from high incidence leukemia herds. These cows had exhibited persistent lymphocytosis for over 5 years and had an absolute lymphocyte count of over 10,000 lymphocytes/cmm of blood, but were normal in all other respects.

The 10 normal control adult cows reported here were obtained from herds with a history of no leukemia. The 4 pregnant cows used in the fetal inoculation studies were also from these herds. These cows, as all other cows in the source herds, had lymphocyte counts in the normal range at the time of this study and on periodic examinations the preceeding 3 years.

Lymphocyte Cell Culture

The procedure for separation of lymphocytes from blood was similar to that reported by Joel et al. (20). Blood was collected by jugular venapuncture and was defibrinated by glass beads. The defibrinated blood was filtered through several layers of cotton gauze, and centri-

fuged at 800 x G for 10 minutes. The layer of cells beneath the serum which contains most of the lymphocytes together with a small amount of serum, was carefully removed with a siliconized Pasteur pipette and mixed gently in a siliconized tube. For final separation, 1 part of a silicone mixture of sp. gr. 1.078 (prepared by mixing 1 part of silicone fluid #710 and 4 parts of silicone fluid #550) was placed in siliconized tubes and was carefully overlaid with 3 parts of this leukocyte suspension. The tubes were centrifuged at 1500 x G for 20 minutes. This resulted in separation into 4 layers, i.e., serum, predominantly mononuclear cells with a few red blood cells, silicone fluid and red blood cells with some leukocytes. The top serum layer was discarded and the mononuclear cells were collected with siliconized pipettes. The lymphocytes thus separated were suspended in Eagles MEM containing 20% bovine fetal serum and antibiotics (300 units of penicillin, 150 µg of dihydrostreptomycin, 100 µg of neomycin, and 5 µg of fungizone per ml.) to a concentration of 1 million cells per ml. Mitogen (Phytohemagglutin in 2% or concanavalin 0.06 mg./ml.) was added to the lymphocyte suspension in the tissue culture flasks and incubated at 37C. The cultures were harvested after 72 hours. For electron microscopic (EM) studies, the lymphocyte cultures were centrifuged at 1000 rpm for 10 minutes, pellets were processed and then examined.

Density Gradient Zonal Centrifugation

Density gradient zonal centrifugations were performed by continuous flow banding and batch-type methods. (7,38)

For continuous flow banding zonal centrifugation, a KIII continuous flow zonal centrifuge (Oak Ridge National Laboratory) was used. Eighteen liters of mitogen stimulated lymphocyte culture containing C-type virus particles were stored frozen and then thawed for processing at Oak Ridge National Laboratory, according to the procedure described by Breillatt, et al. (6).

Sucrose gradients prepared in 0.005M Tris buffer, pH adjusted to 7.2, were used. The rotor was loaded with sucrose gradients in the following order: 59.5%-550 ml., 49.8%-600 ml., 40.3%-600 ml., 32%-600 ml., and 21%-600 ml. After loading the gradients, the rotor was accelerated and the acceleration was controlled from 0-500 rpm at two revolutions per second (rps) from 500-2000 rpm at 4 rps. When the speed reached 2000 rpm, loading of virus material was started and the speed was accelerated to 35,000 rpm. After loading the rotor with 18 liters of virus material, it was centrifuged at 35,000 rpm for additional one hour. After the run, the rotor was decelerated to 2,000-500 rpm at 4 rps, 500-0 rpm at 2 rps. When the rotor stopped, the content of the rotor was displaced with 60% sucrose solution through the lower exit, monitored at 280 mμ in ultraviolet analyzer and collected in 100 ml. fractions.

For batch-type zonal centrifugation, TI 15 zonal rotor (Beckman) was used according to the procedure described by Anderson, et al. (1). The rotor was loaded at 3,000 rpm through the wall with 1,200 ml. of sucrose gradient of 10-55% prepared with Beckman high capacity gradient pump, Model No. 141. The remainder of the rotor was filled with 60% sucrose. Two hundred ml. of bovine leukemic lymphocyte culture containing C-type virus particles, which had been frozen at -90°C and thawed, was introduced through the core and then overlaid with 80 ml. of Tris buffer. This material displaced the 60% sucrose through the wall leaving approximately 185 ml. as a cushion. After loading was completed, a small amount of overlay was displaced through the core by loading an equal amount of 60% sucrose through the wall to remove any air bubble trapped in the core. The loaded rotor was then accelerated to 30,000 rpm and centrifuged at 30,000 rpm for 2 hours. Then the rotor was decelerated to 3,000 rpm and the content of the rotor was removed through the core by displacing with 60% sucrose through the wall, monitored at 280 mu in an ultraviolet analyzer and collected in 20 ml. fractions.

Appropriate fractions from the zonal centrifugation methods, after dialyzing against Tris buffer 0.005 M, pH 7.2 for 24 hours, were examined for the presence of virus particles with EM by thin sectioning and negative staining techniques. For thin sectioning the fractions were pelleted at 80,000 g for 90 minutes and the pellets were processed.

Cell Culture

Bovine fetal spleen, endocardial, embryo, kidney, lymph node, lungs, and thymus cell lines in continuous culture were used. Bovine fetal endocardial, spleen, kidney, and thymus cell lines were characterized by epithelial-type cells and bovine fetal lungs, lymph node, and embryo cell were characterized by fibroblast C-type cells. The trypsinized cell suspensions were mixed in tissue culture flasks in a ratio of approximately 1 to 5 with the lymphocyte culture suspension from 72 hour lymphocyte cultures containing abundant C-type virus particles. Also these cells were mixed in the same ratio with the frozen and thawed lymphocyte culture suspensions devoid to viable lymphocytes as monitored by trypan blue staining. They were incubated at 37 C. for 2 to 3 hours after which Eagles MEM containing 20% bovine fetal serum was added to the flasks to dilute to a concentration of approximately 100,000 trypsinized cells per ml. The flasks were incubated at 37 C., on the fourth day the medium was replaced and on the seventh day the first cell passage was done. The cell cultures were examined regularly for any cytological change and were serially passaged on every fifth day. From each passage, floating cells and attached live cells dispersed by trypsinization were pelleted together at 1,000 rpm for 10 minutes, processed for EM and examined for the presence of virus particles.

Serological Methods

Complement fixation, immunodiffusion, immunofluorescence and cytotoxic tests were performed by standard methods. Lymphocyte cultures from leukemic and lymphocytotic cows having abundant C-type virus particles provided the source of the antigen. Sera from leukemic and lymphocytotic cows, positive for C-type virus particles, and hyperimmune guinea pig and rabbit serum were used in the serologic tests. Hyperimmune sera were prepared by 6 weekly subcutaneous inoculations of frozen and thawed preparations of concentrated (50 X) lymphocyte cultures containing abundant C-type virus particles. The bovine sera used were whole sera and sera fractionated in sephadex G 200 columns. Lymphocyte cultures and sera from normal cows were used as controls.

Complement fixation tests were performed in tubes by the standard methods. Lymphocyte cultures containing abundant C-type particles were centrifuged and the cells were resuspended with the supernatant fluid to 1/50 of the original volume. These cell suspensions were then frozen and thawed or sonicated in a Bronson sonifier (model 125) at power setting 7 for 15 seconds and used as antigen. The amount of guinea pig complement used was 1.8 units. Complement fixation tests were also performed by Dr. Sarma from the National Institute of Health by microtechnic methods (31), using the above said antigens and sera.

Immunodiffusion tests were performed as described (24) using the same antigens and sera used in the complement fixation test. In addition to the above antigens and sera, syncytial virus positive antigen and positive sera obtained from Dr. Vander Maaten (National Animal Disease Laboratory, Ames, Iowa) were used.

In immunofluorescence studies, gamma globulin from sera precipitated by 50% saturated ammonium sulfate was conjugated with fluorescein isothiocyanate. The unconjugated fluorescein was removed by passing the conjugate through sephadex G 25 column (8,28). The conjugates were absorbed with bovine liver powder before use. Smears of cell pellet suspensions from the lymphocyte cultures prepared on coverslips were stained with the conjugates.

Cytotoxic tests were performed according to the procedure described (26).

Electron Microscopy

Thin sectioning - The pellets were processed and thin sections were prepared for EM as described (11).

Negative staining - A 200 mesh copper grid with formvar membrane coated with carbon was floated on a drop of sample with the membrane side down. Then it was floated for one minute on a drop of 2% solution of phosphotungstic acid (PTA) of pH7. At times this process was repeated with

another drop of PTA. The grid was then removed, excess of fluid was taken out with a piece of filter paper, dried, and examined.

All specimens were examined by RCA EMU 3 electron microscope.

Experimental Infection Studies

These studies included intrafetal tumor tissue inoculation. The 4 fetuses used for intrafetal inoculation were between 65 to 85 days of gestation. The tumor material used was obtained surgically from tumor masses from an adult bovine leukemia case. The inoculum consisted of 0.5 gm. of tumor tissue homogenate suspended in 3 ml. of Eagles medium containing 20% bovine fetal serum. The fetuses were injected intraperitoneally through the uterine wall which was exteriorized through a lateral abdominal incision. These inoculated fetuses were delivered as apparently normal healthy calves.

For virological studies, blood was collected from these calves at 12 and 24 months of age, and lymphocyte cultures were prepared according to the procedures outlined previously. The cultures were examined by EM for the presence of C-type virus particles.

Results

Isolation of Virus

C-type virus particles were isolated from both leukemic and lymphocytotic cattle in mitogen stimulated lymphocyte cultures. In 10 leukemic cases, C-type virus particles were isolated from all animals.

These cases included one animal with the thymic form of the disease. Ten of ten animals with a persistent lymphocytosis yielded C-type virus particles. Three of the lymphocytotic cows subsequently developed clinical leukemia and were again found to be positive for C-type virus particles. Therefore, a total of 13 clinical cases of leukemia were found to contain C-type virus particles. Virus isolation attempts on all 10 normal control cows failed to reveal similar virus particles using the same methods. A second attempt to isolate virus was made one year following the first attempt on all control to verify the initial negative results.

A number of factors were found to influence the ease with which C-type virus particles could be isolated. Maximum virus production was observed in 72 hour cultures. Concanavalin and PHA were both satisfactory mitogens for virus production, although the latter gave more consistent results. Lymphocyte cultures from leukemic or lymphocytotic cattle having high numbers of immature lymphocytes yielded more virus particles than did those from cases with lower lymphocyte counts. It was difficult to isolate virus from lymphocyte cultures from spleen, lymph nodes or other tumor tissue of leukemia cases.

The mature C-type virus particles were most frequently observed extracellularly, usually in clusters, closely adjacent to viable lymphocytes (fig. 12). The extracellular clusters of virus particles were

always attached to cellular material (fig. 3). The mature virus particles were 95 to 125 nm in diameter with a central dense nucloid surrounded by a single or rarely a double membrane. C-type and A-type virus particles were also seen intracellularly in the cytoplasmic vacuoles (fig. 4,6). The intracellular C-type particles were the same size as the extracellular particles and the A-type virus particles were 70 to 90 nm in diameter. The A-type virus particles were of typical morphology with two shells, giving them the characteristic doughnut shape. Buddings of virus particles from the plasma membrane into the cytoplasmic vacuoles and the cell surfaces were observed (fig. 5,6).

Concentration and Purification Studies

The virus particles in the lymphocyte cultures were partially purified and concentrated by zonal centrifugation. These virus particles were banded in densities ranging from 1.15 to 1.18 g/cm^3 as determined by EM examinations of different fractions (fig. 7). The majority of the virus particles were in the density of 1.17 to 1.18 g/cm^3 and were primarily found to be attached to fragments of cellular material. In the density of 1.15 to 1.16 g/cm^3 most of the virus particles appeared to be free and were not as concentrated. The virus particles which had been concentrated, on thin sectioning EM, appeared idientical to the virus particles observed in the lymphocyte cultures. On negative staining the

virus particles were observed to be 100 to 150 nm in diameter and covered with a membrane.

Cell Culture Studies

Attempts to adapt C-type virus to bovine cell lines were largely unsuccessful. Cytological changes were not observed in inoculated bovine fetal embryo, lymph node, lung, kidney, and thymus cell lines. On EM examination virus particles were not observed in these cell cultures. Limited success was observed in bovine fetal spleen and endocardial cell lines inoculated with viable lymphocytes containing C-type virus particles. Many attempts to infect these two cell lines were made. In a few cases isolated cytological changes with rounding of cells and occasionally syncytial type changes were observed after 3 or 4 serial cell passages. Some of the cells became detached and floated in the medium. On EM no virus was observed in intact live cells. But, in the debris of degenerated cells occasionally there were isolated clusters of virus similar to bovine syncytial virus. In some instances a few isolated C-type virus particles were observed in the debris of degenerated cells. Serial passages of these two cell lines were done in an effort to increase the number of C-type virus particles but was unsuccessful, in fact the number of C-type particles decreased and was lost with subsequent passages.

Similar studies with these two cell lines using frozen and thawed lymphocyte culture suspension were also unsuccessful.

Serological Studies

The objective of the serological studies was to attempt to demonstrate immunologic relationships between virus rich lymphocyte culture antigen and various sera. More specifically to develop a serologic procedure to determine infection with C-type virus particles.

Complement fixation tests with bovine lymphocyte culture viral antigen and sera from leukemic, lymphocytotic, and normal cattle resulted in no reaction. Also, there was no reaction with hyperimmune guinea pig and rabbit sera.

The lymphocyte culture viral antigen and sera from leukemic, lymphocytotic, and normal cattle did not react in immunodiffusion tests, nor did the hyperimmune guinea pig or rabbit sera. Also, there was no reaction between this antigen and serum positive for syncytial virus antibody. Moreover, with the exception of 3 lymphocytotic cows, sera from the 10 leukemic, 7 lymphocytotic, and 10 normal cows studied failed to react with syncytial virus antigen.

There was no immunological reactions evident in the immunofluorescence and cytotoxic tests using the lymphocyte culture viral antigen and sera from leukemia, lymphocytotic, and normal cattle.

Experimental Infection Studies

These attempts to transmit the disease are preliminary in nature and are reported primarily because of the success in isolating C-type

virus particles from inoculated animals.

In the fetal transmission study, all 4 inoculated fetuses were delivered as apparently normal, healthy calves. The calves remained in good health for the duration of the study period. Periodic hematologic examination during the 2 years of study revealed that none of the animals developed a lymphocytosis. All lymphocyte cultures prepared at intervals from these animals revealed the presence of C-type virus particles. The virus particles were identical in morphology to those isolated from adult leukemic and lymphocytotic cows.

A significant incident occurred in one of the experimental inoculations. An extensive lymphosarcomatous tumor was present in the placenta of one of the cows at parturition. It was interesting to note that the calf born from this cow had a high number of virus particles in lymphocyte cultures. Lymphocyte cultures from this cow did not reveal any virus particles - nor did she develop any evidence of leukemia during the period of study.

Discussion

C-type virus particles were isolated consistently from all leukemic and lymphocytotic cattle studied. In addition, A-type and budding virus particles were also observed. Morphologically these virus particles appeared to be identical in characteristics with other known C-type

leukemia viruses of different species. (27) In the density gradient studies the bovine leukemia virus particles were located between density 1.15 to 1.18 g/cm^3. These results also indicated the similarity to the other known leukemia viruses. (21) Unlike the other leukemia viruses, the bovine leukemia C-type virus particles were in a sense cell associated in that the extracellular virus particles were attached to extracellular virus particles were attached to extracellular material.

The failure to demonstrate C-type virus particles in defined normal cattle further substantiates the significance of the viral isolations. The defined normal cattle differed from the leukemic cattle population only in that the herds of origin had 1) no history of leukemia, 2) no contact with leukemic cattle and 3) hematological studies over a 5-year period of time revealed no animals with a persistent lymphocytosis.

The significance of a persistent lymphocytosis in dairy cattle and its relationship to bovine leukemia has been the subject of much controversy over the past decade. This is despite the evidence from numerous studies demonstrating the association of a preclinical lymphocytosis with clinical leukemia (4,13,16,22,30,35,40). The consistent isolation of C-type virus particles from cows with a persistent lymphocytosis which were identical with particles isolated from leukemic animals provides additional confirming evidence of this relationship

In addition, during the 2-year period of study, 3 cows of the 10 lymphocytotic cows studied developed clinical leukemia. C-type virus particles were isolated from these animals during the time they were lymphocytotic and again later when they developed clinical leukemia. In some instances C-type virus was repeatedly isolated from lymphocytotic cows 2 years prior to the time they developed the tumorous phase of leukemia. The results of this study plus the evidence from the other studies supports the hypothesis that leukemia has a prodromal stage characterized by a persistent lymphocytosis.

C-type virus particles were isolated from all 4 of the calves inoculated as fetuses with tumor material. These results suggest that successful transmission of C-type virus occurred. These animals have been studied for a period of 3 years and as yet have not developed any clinical evidence of leukemia. The development of a placental lymphosarcoma in one of the dams, and the presence of C-type virus particles in all of the fetally inoculated calves provides evidence for both a successful transplantation and transmission. Evidence that the source of the transplanted cells and the transmitted C-type virus was the inoculum is demonstrated by the fact that this cow was repeatedly negative for C-type virus particles and did not develop clinical evidence of leukemia.

It was found that the addition of a mitogen was necessary for the production of large numbers of C-type virus particles from lymphocyte cultures. However, a few C-type virus particles were observed in cultures not stimulated by mitogens. Mitogens are known to cause an increase in cell metabolism, increase in nucleic acid and protein synthesis and lymphocyte transformation (2). But, the mechanism of stimulation of lymphocyte culture for virus production is not known. Both concanavalin and phytohemagglutin were effective in stimulating virus production. Thus it appears that these mitogens do not have any specific action. Possibly when the genome of the virus is present in the lymphocytes, the increase in cell metabolism and protein synthesis due to the presence of mitogen in the culture cause activation of the viral genome resulting in virus production.

There was a positive correlation between the count, the degree of immaturity of the lymphocytes and the yield of virus particles. This correlation was observed not only in leukemic and lymphocytotic cows having different levels of lymphocyte counts, but also in the same animals having different lymphocyte counts throughout their period of study.

Even though large numbers of C-type virus particles were isolated from the peripheral blood lymphocyte cultures it was difficult to isolate the virus from the lymphocyte cultures from spleens and lymph nodes of these animals. This suggests a differential virus potential for lymphocytes from these different sources.

Immunologic reactions were not observed between the lymphocyte culture viral antigen and sera from the leukemic and lymphocytotic cows. There have been reports (14,15,32) of immunological relationship between the known leukemia viruses of different species. In our study (12), concentrated bovine C-type virus preparation prepared by zonal centrifugation reacted (titre 1:4) in complement fixation (CF) test with the broad-reacting murine leukemia group-specific antisera prepared in rats by the induction of transplanted murine sarcoma virus-induced tumor (32). The specificity and reproducibility of this CF reaction is under investigation.

A relationship by immunodiffusion tests between the bovine C-type virus and the bovine syncytial virus could not be demonstrated. Thus giving evidence that possibly the syncytial virus is not directly involved in bovine leukemia.

The presence of C-type virus particles in leukemic, lymphocytotic, and experimentally inoculated cattle and their absence in defined normal cattle strongly suggests that these C-type virus particles are the etiological agent of bovine leukemia. Conclusive evidence of the role of these virus isolates in bovine leukemia can only be determined by transmission experiments using cell free virus material. Studies of this type are presently in progress.

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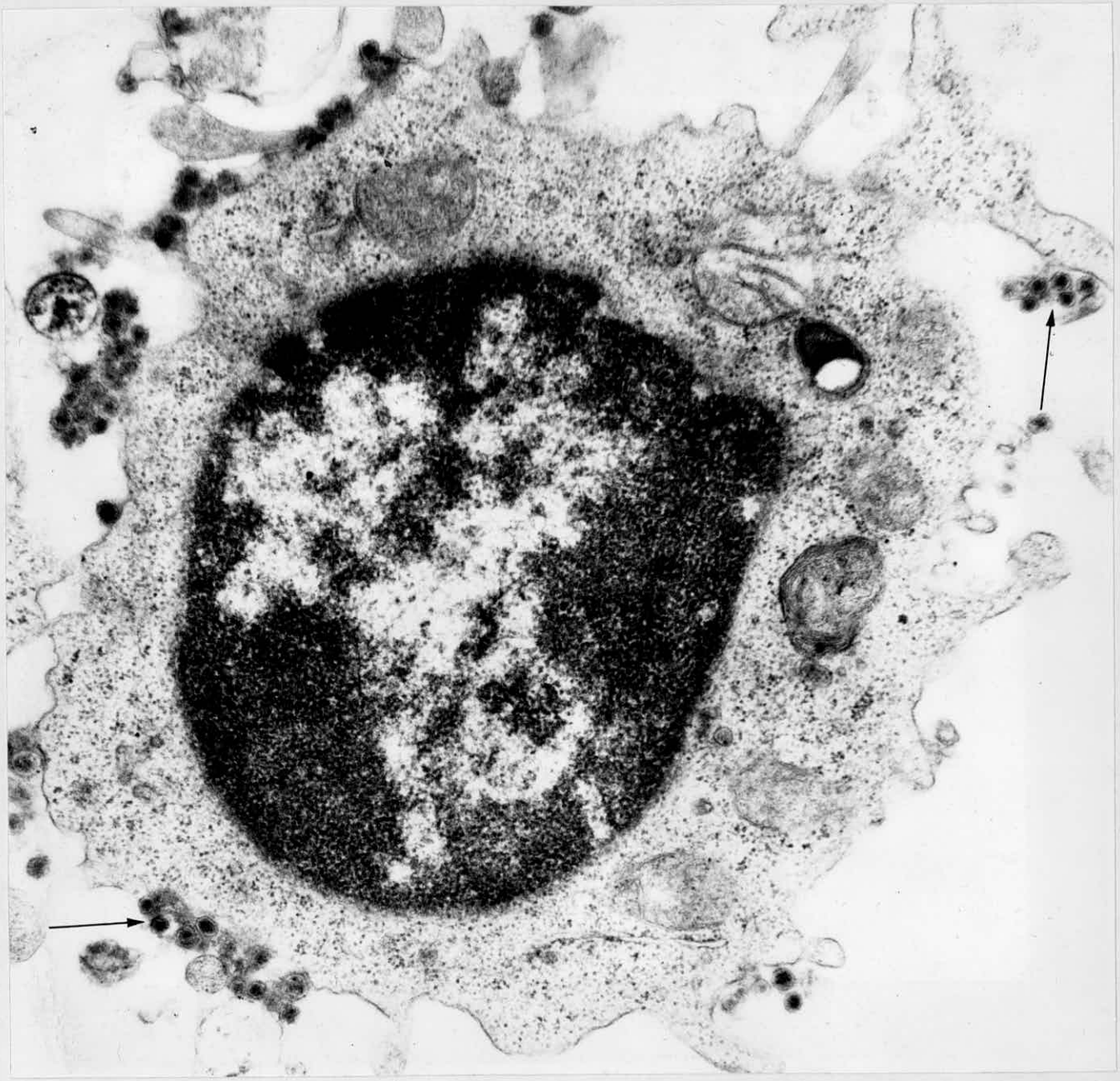

Fig. 1 Lymphocyte from a mitogen stimulated lymphocyte culture from a leukemic cow. Mature C-type virus particles (arrow) in clusters are present extracellularly adjacent to the lymphocyte 30,250x.

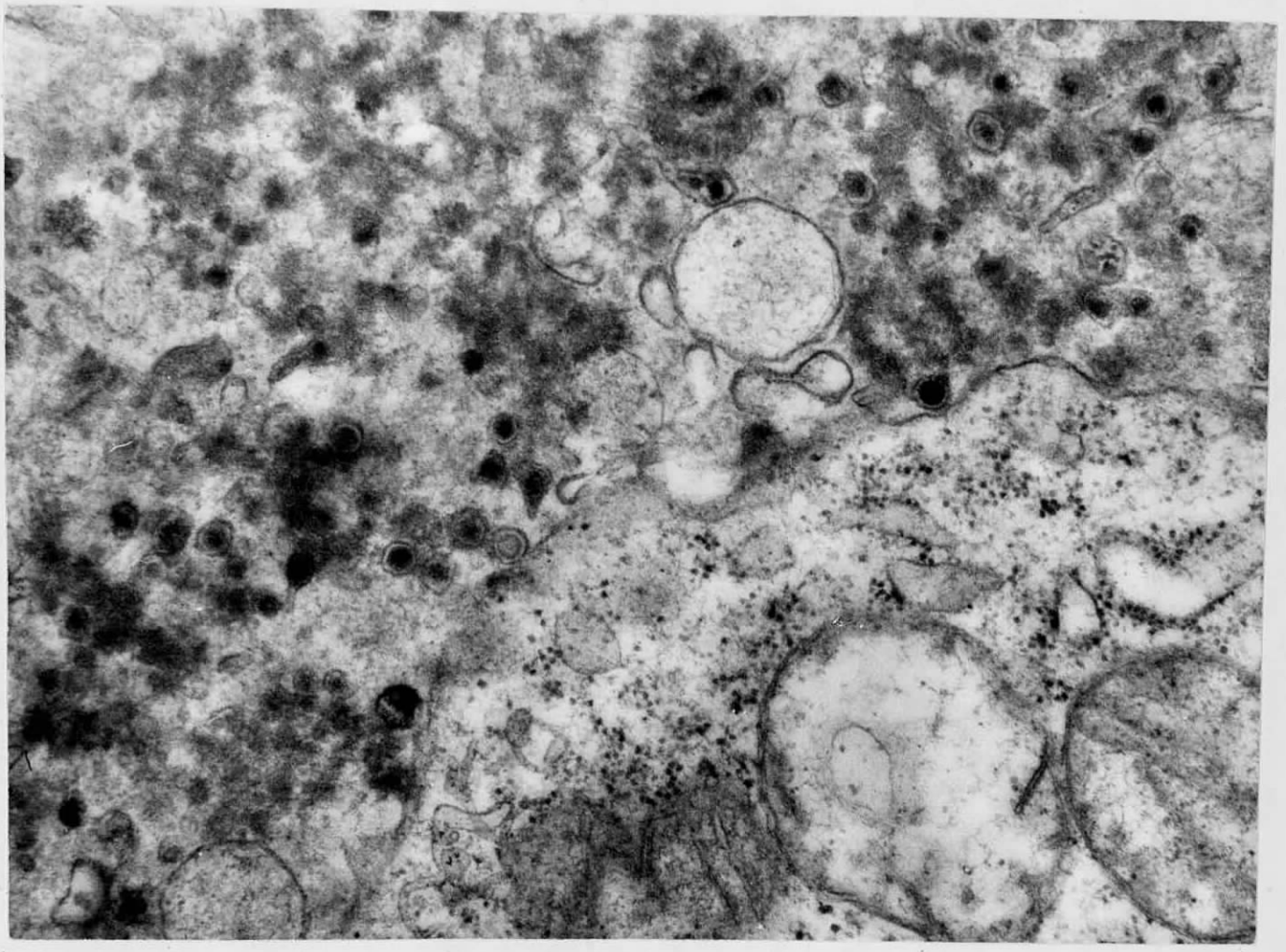

Fig. 2 Portion of a lymphocyte from a mitogen stimulated culture from a lymphocytotic cow. Mature C-type virus particles are present extracellularly attached to cellular material. 50,000x.

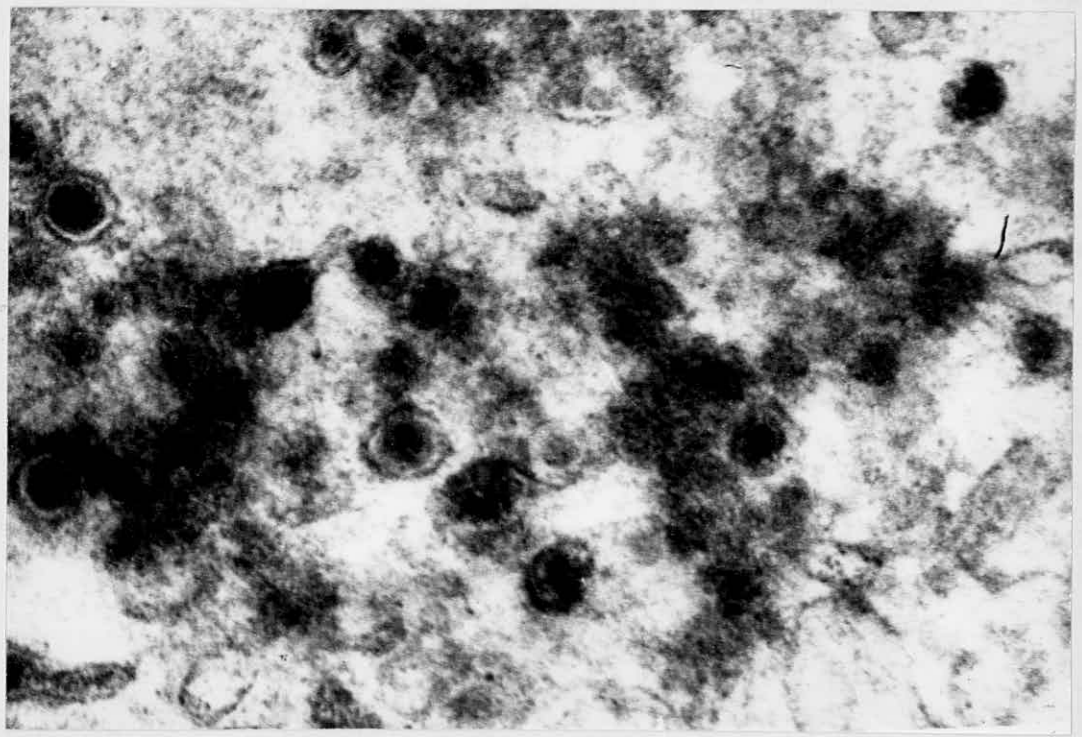

Fig. 3 Extracellular mature C-type virus particles from a mitogen stimulated lymphocute culture from a leukemic cow. Note the virus particles are attached to cellular material. 100,000x.

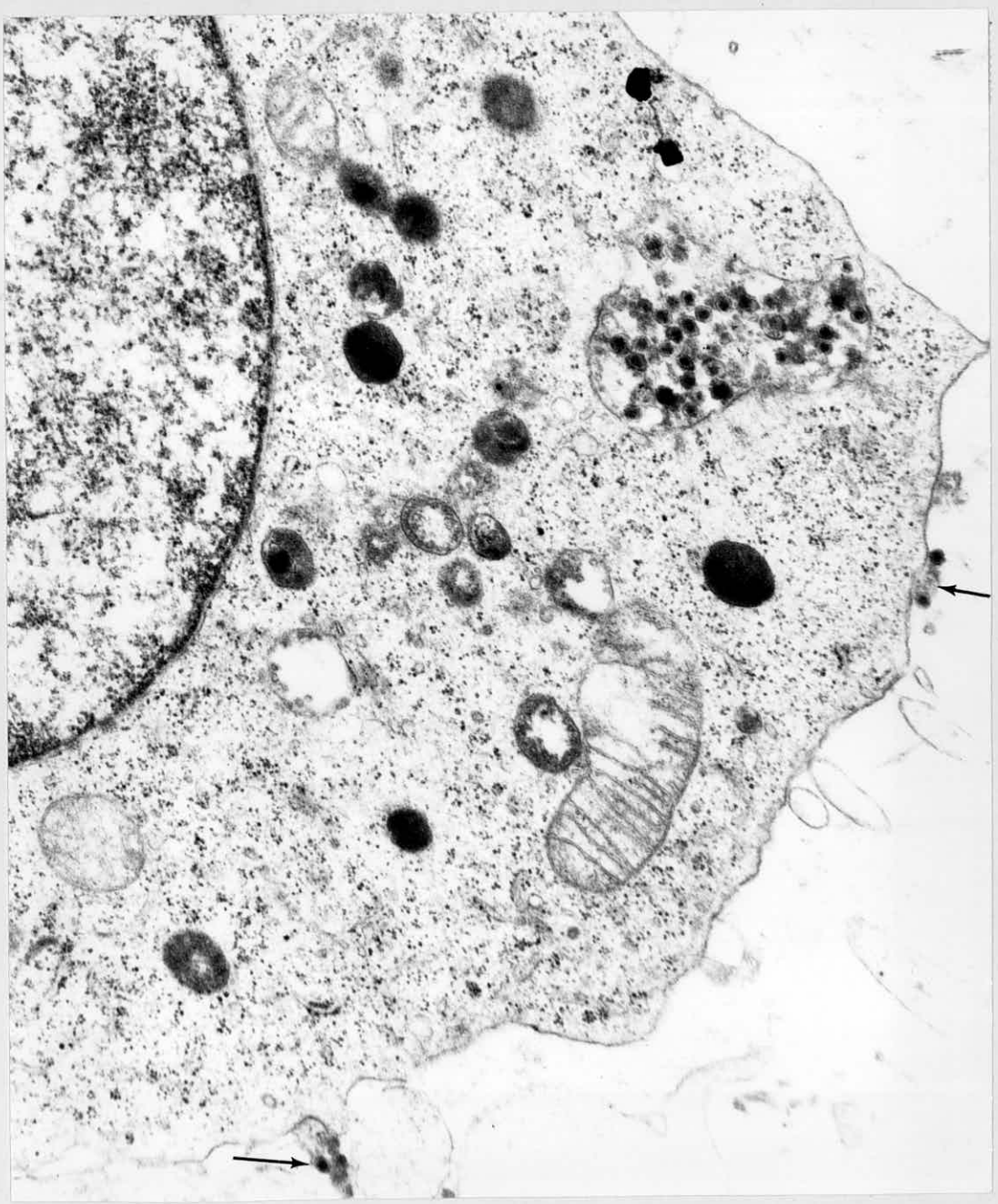

Fig. 4 Lymphocyte from a mitogen stimulated lymphocyte culture from a leukemic cow. C-type virus particles are present intracellularly in a cytoplasmic vacuole. Extracellular C-type virus particles (arrows) are also present. 25,000x.

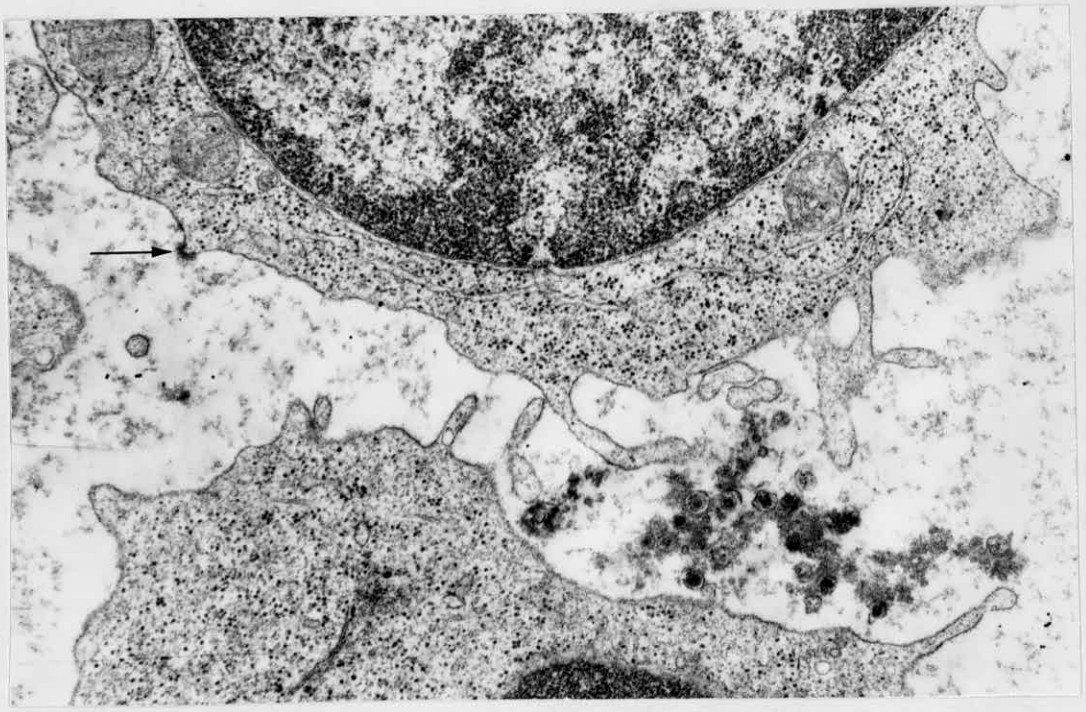

Fig. 5 Lymphocytes from a mitogen stimulated lymphocyte culture from a lymphocytotic cow. Note a surface budding of a virus particle (arrow) and extracellular clusters of C-type virus particles.

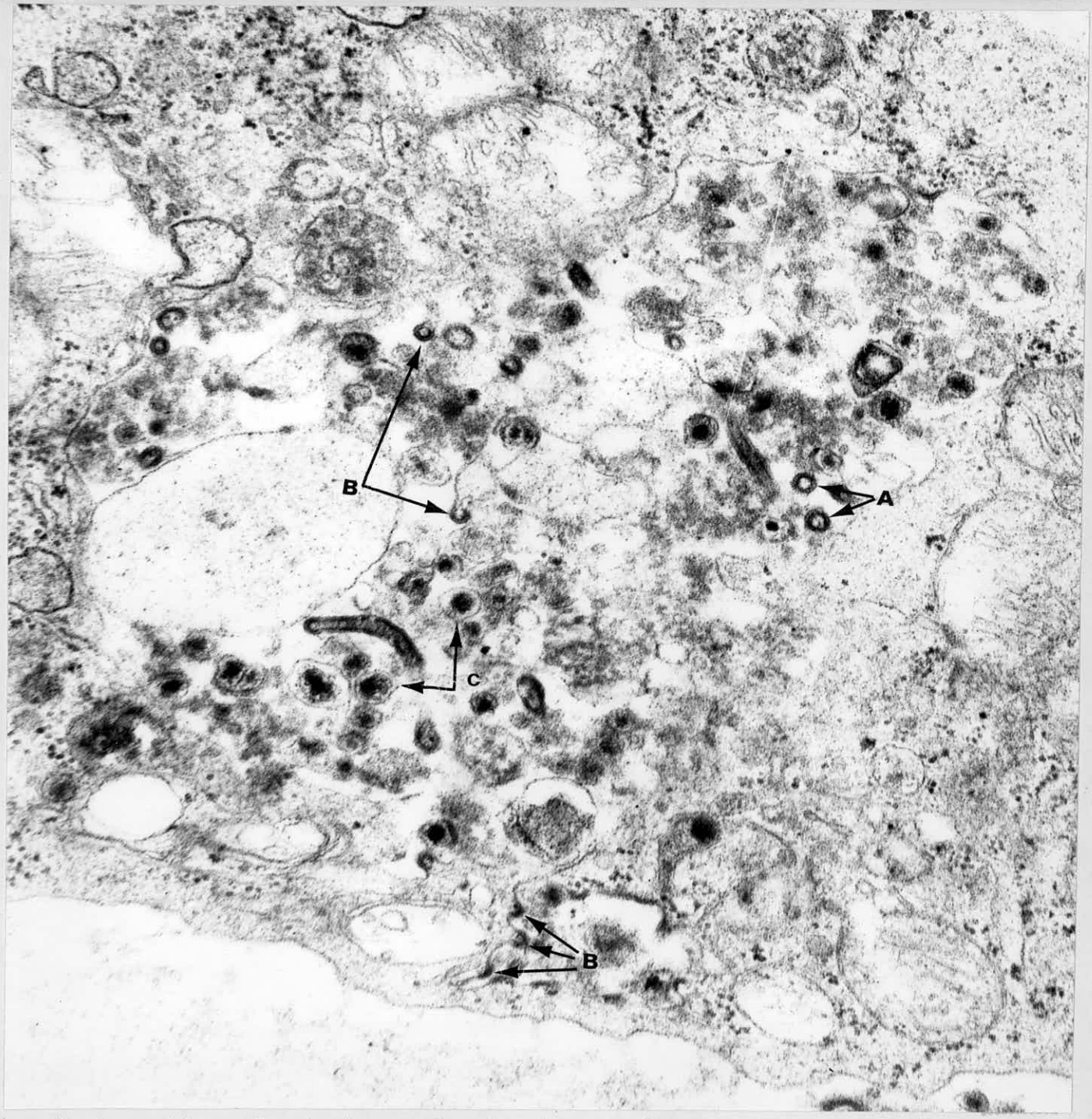

Fig. 6 A portion of cytoplasm of a lymphocyte from a mitogen stimulated lymphocyte culture from a leukemia cow. Note intracellularly the budding virus particles (B), A-type virus particles (A), and C-type virus particle (C). 50,000x.

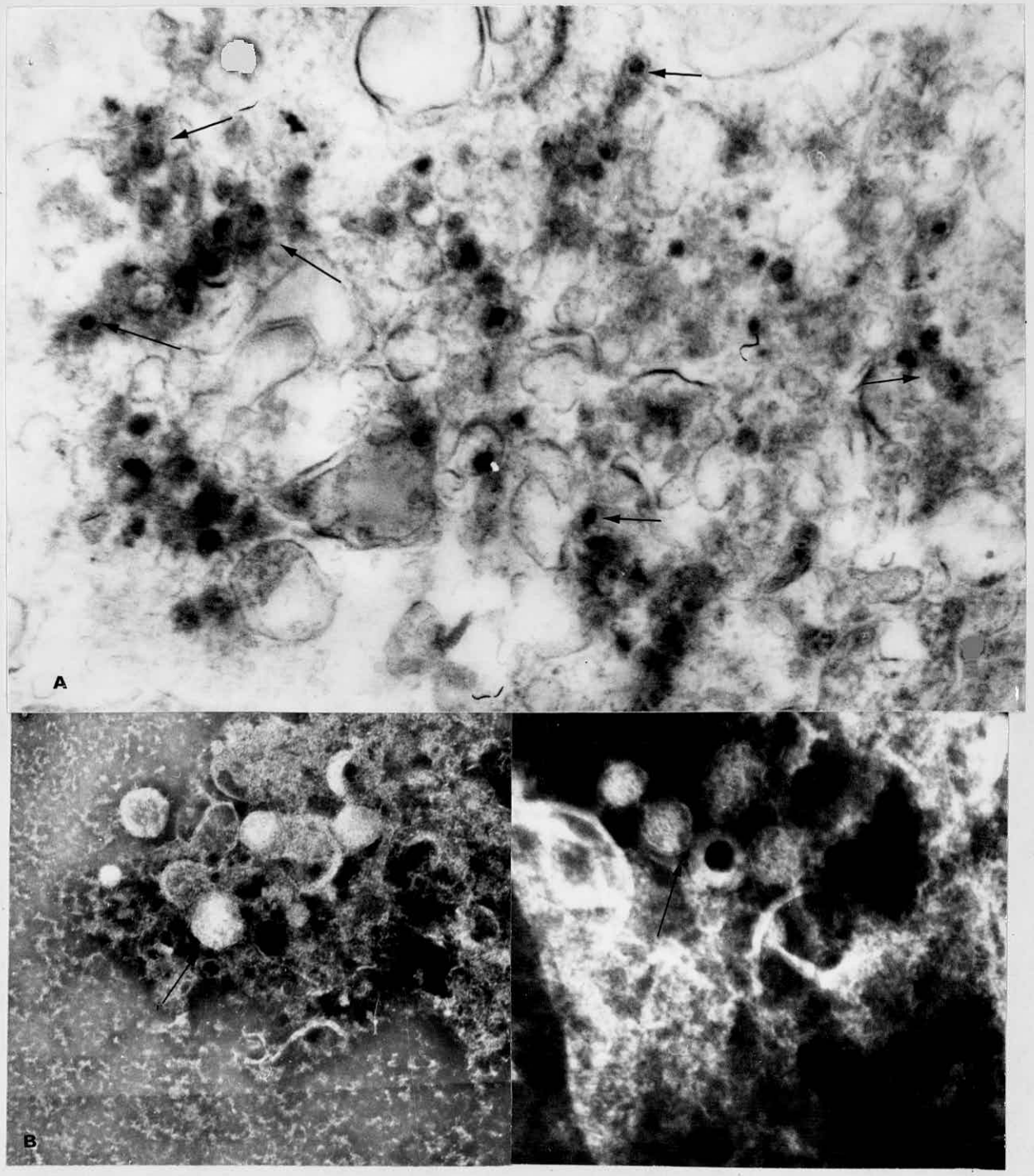

Fig. 7 C-type virus particles (attaching to extracellular material) from zonal centrifugation fractions of density 1.15 to 1.18 g/cm^3 on thin sectioning (A) and on negative staining (B). 50,000x.