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BOVINE LYMPHOCYTIC LEUKEMIA: STUDIES OF OCCURRENCE AND DISTRIBUTION INCLUDING INVESTIGATIONS OF FAMILIAL AND ENVIRONMENTAL FACTORS WITH SUPPORTING CLINICAL, HEMATOLOGIC, PATHOLOGIC, INDUCTION AND TRANSMISSION STUDIES

PROGRESS REPORT NO. 9 TO THE U. S. ATOMIC ENERGY COMMISSION

ON RESEARCH PERFORMED UNDER CONTRACT AT (11-1)-910

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DISCLAIMER

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I. PERIOD COVERED BY THIS REPORT:

This report covers the period from January 1, 1968 to December 31, 1968. In certain sections where work has been concluded, the data are comprehensive. In other sections only data from the past year are presented.

II. BACKGROUND INFORMATION:

A. Background

On June 1, 1960 a contract between the United States Atomic Energy Commission and the University of Minnesota was negotiated. The initial proposal raised these provocative questions: (1) Is the apparent frequency of occurrence of bovine leukemia in Minnesota real? (2) Is there an increasing prevalence of this disease as is implied in Meat Inspection Reports? (3) Is there a marked geographic variation in the frequency of the occurrence of bovine leukemia, and does it correlate with that noted in human leukemia? (4) Do environmental factors influence the occurrence and distribution of bovine leukemia in this region? (5) Is there a familial relationship in the occurrence of bovine leukemia? These questions were raised at a time when the influence of environmental factors (such as radioactive fallout) were of pressing importance.

At this time, there were no reports of studies conducted in the United States to determine the occurrence and distribution of leukemia in cattle, and no organized attempts had been made to study the relationship of environmental or familial factors to the

occurrence of bovine lymphocytic leukemia.

The annual reports of the Meat Inspection Division of the U. S. Department of Agriculture reveal that cattle condemnations attributed to malignant lymphoma showed an apparent increase from 9.7 to 18.1 per 100,000 cattle slaughtered during the years 1952 to 1959. These records further indicate that in 1958 one fifth (18.6 %) of all cattle condemnations in the United States were reported from the federally-inspected slaughtering plants in Minnesota and nearby Eau Claire, Wisconsin and further that South St. Paul Meat Inspection Station in 1958 reported 14.0% (464) of the malignant lymphoma condemnations in the United States while slaughtering less than 5.0% of the cattle. Reports for human mortality attributed to leukemia in the United States reveal that Minnesota had one of the highest mortality rates in the nation. Studies of leukemia in man in Minnesota have shown significant geographic variations in the distribution of mortality rates among counties and groups of counties in different areas of the state. Studies to determine the influences of environmental factors on the occurrence and distribution of leukemia in man in Minnesota were in progress.

Because Minnesota had a high mortality attributed to human leukemia and studies on the influence of environmental factors were in progress, it was prudent to initiate studies on the occurrence and distribution of leukemia in cattle and to study the relationship of environmental or familial factors to its occurrence in the state of Minnesota. This was the only comprehensive study of this type being conducted in the United States.

During 1964 investigators at Michigan State University reported on epizootiologic studies of bovine leukemia. They reported data from 304 laboratory confirmed cases of leukemia obtained from a 19 county study area in Michigan for the years 1962-1965. More limited epizootiologic studies have been reported from Pennsylvania, California and Illinois.

Currently, epidemiologic and/or control eradication programs for bovine leukemia are underway in a number of countries, namely, Great Britain, Denmark, West Germany, Poland, Russia, Czechoslovakia, Yugoslavia, Isreal, East Germany and Brazil.

The criteria of lymphocytosis in herds and on an individual animal basis is the principal parameter of measurement and is being used to diagnose and identify leukemia on a herd basis. Denmark and West Germany have initiated comprehensive control and eradication programs involving all cattle.

Preliminary unpublished data available indicate that lymphocytosis is not a feature of bovine leukemia in Great Britain. However, the adult form of the disease in multiple case herds has not been observed. In other countries, the adult form of leukemia in multiple case herds are recognized. Preliminary unpublished and published reports substantiate the previous reports from European countries that lymphocytosis is a feature of bovine leukemia.

There have been two other studies on the occurrence of leukemia conducted in other countries of the world, the studies by Bendixen of Denmark initiated in 1952 and by Olson in Sweden initiated in 1956. Other data on the distribution and prevalence of bovine leukemia in various countries is limited to meat inspection statistics and

observations by veterinarians in practice.

B. General Information

The approach to the study and the procedures used are described in some detail in Progress Reports 1 and 7 to the U. S. Atomic Energy Commission (see Contract AT (11-1)-910) and will not be repeated in this report. Additional procedures initiated are described in the various sections.

A number of terms need definition in the general introduction as they will be used in various sections of this report.

1) Single case data, submission or reports refer to information on specimens obtained from a case of leukemia in a herd where only one case was reported in the past year.
2) Juvenile leukemia - a consistent clinical form of leukemia occurring in young animals usually under 6 months of age but up to one year.
3) Thymic-lymphosarcoma - a consistent clinical form of leukemia occurring in young animals from 6 months to 2½ years of age, approximately.
4) Adult leukemia - leukemia with variable clinical and pathological manifestations occurring in animals usually over 2 years of age. The adult type typified the type of leukemia found in multiple case herds.
5) Multiple case herd data refers to data obtained from herds where more than one case of leukemia (adult type) has occurred in one year.
6) Lymphocytosis - referred to as a pre-clinical phase of the

tumor form of bovine leukemia. The lymphocytosis is characterized by an elevation of lymphocyte count above 3 standard deviations greater than the normal count for animals for that age. The lymphocytes consist of a morphologically normal population of cells and are found in normal animals in leukemia herds. Counts to 30,000 or more in a number of animals (mean 12.5 %) are usual. The lymphocytosis is not associated with any demonstrable pathologic process or tumor growth and appears somewhat analagous to the early stages of chronic lymphocytic leukemia of man.

C. Scientific Scope and Objectives of the Study Including Modifications

The initial contract to study bovine lymphocytic leukemia was negotiated June 1, 1960. This project proposed to study the occurrence and distribution of bovine lymphocytic leukemia in Minnesota and adjacent areas, with particular reference to the investigation of environmental and familial factors. This project included clinical, hematologic and pathologic studies to provide definitive diagnoses and to characterize and relate the morphological types with the occurrence and distribution of the disease.

The specific aims of these studies (1960) were:

1. To study the frequency of occurrence and the geographic distribution of bovine lymphocytic leukemia in Minnesota and adjacent areas of surrounding states:

   a. to obtain information regarding incidence and prevalence in relation to age, sex, breed, familial relationships

and the movement of animals from one population group (herd) to another;

b. to evaluate within the limitations of reporting any changes in incidence during the next several years, in relation to the "apparent" increase reported by the U. S. Department of Agriculture Meat Inspection Division records and a sample of practicing veterinarians in Minnesota;

c. to determine if there is any statistical relationship between selected environmental factors and the frequency of occurrence and distribution of bovine leukemia, particularly in herds where multiple cases occur, and emphasis will be given to herd management practices and history of previous disease, soil types and available geologic information, and the use of agricultural and other chemicals which may be known or suspected carcinogens.

2. Clinical, hematologic and pathologic studies will be made:

a. to establish a definitive diagnosis for the cases of the disease as they occur and are reported in Minnesota and adjacent study areas;

b. to ascertain whether lymphocytic leukemia can be classified into acute and chronic courses, and if any relationship exists between the course of the disease and the age of the animal;

c. to obtain more definitive and complete information regarding the manifestations of spontaneously occurring

bovine lymphocytic leukemia by detailed study of a limited number of selected herds and cases where multiple cases of the disease occur.

The above were the initial objectives of the research project. Essentially there was no major deviation from these objectives during the first five years of the study. We did however, change our procedures somewhat and broaden our efforts in certain areas. These changes of procedure include the use of the electron microscope in the pathologic studies and the initiation of hematologic studies in selected cattle populations to better define the role of a lymphocytosis in bovine leukemia.

The changes of procedure and the additional studies initiated were (sixth renewal request, 1965):

1. It was proposed to complete the studies on the occurrence and distribution of bovine lymphocytic leukemia in Minnesota and the adjacent areas:
   a. to do limited retrospective studies on socio-economic factors that may influence the reporting of leukemia and effect the data obtained on incidence and rates as determined by age, herd size, breed distribution and geographic distribution.
   b. to obtain data on environmental factors on the selected normal herd for comparison to the single case herds.
   c. to complete the statistical analysis and evaluation of accumulated data on occurrence and distribution and the role of environmental factors in bovine lymphocytic leukemia.

2. It was proposed to continue limited studies on multiple case herds:

   a. to investigate the relationship between lymphocytosis and the occurrence and course of new cases of leukemia in these herds.

   b. to analyze and evaluate the data on the role of genetic factors as they may influence lymphocytosis and or tumor development.

3. It was proposed to obtain additional hematologic data on selected normal herds and single case herds:

   a. to complete base line parameters for the evaluation of single case herds characterized by leukemia occurring in young animals and adult animals.

   b. to complete the comparison studies of the single case herds to multiple case herds.

4. It was proposed to shift the emphasis of the intensive clinical, hematologic, biochemical and pathologic studies on leukemic animals to animals with a marked lymphocytosis (pre-tumor phase).

   a. to determine the relationship of lymphcoytosis to the tumor phase of bovine leukemia.

   b. to determine the eventual natural outcome of animals with a marked lymphocytosis (pre-tumor phase) by intensive clinical and pathologic studies.

   c. to attempt to increase the incidence of tumor formation in lymphocytosis animals by various known promotor factors.

In 1966 our leukemia research team requested a review and critical evaluation by a review team. This evaluation was requested because it was our thought that some of the objectives of our research had been met. This was particularly true of certain aspects of the studies on occurrence and distribution and on the relationship of environmental factors to the occurrence and distribution of leukemia. It seemded apparent that sufficient data had been collected to fulfill the objectives of the epidemiologic studies and that statistical analysis of the data should be completed. Further, it was suggested that we increase our efforts on determining the significance of lymphocytosis and initiate studies on the transmission of bovine leukemia.

The review team was composed of Dr. Donald Anderson, Richard Barnes, Douglas Grahn, Leo Whitehair and Bibbs.

The initial objectives of this study were modified in consultation with the review team. A brief summary of these changes in objectives follows.

1. To complete the studies on the occurrence and distribution of bovine leukemia and the possible influence of environmental factors. Since the Atomic Energy Commission site visit committee posed the question of the role of socio-economic factors on the reporting of bovine leukemia, it was deemed necessary to initiate studies on the role of socio-economic influence on the reporting of bovine leukemia to validate data on the occurrence and distribution.
2. To continue limited studies on multiple case herds to determine the relationship between lymphocytosis and

tumor cases and to evaluate the role of genetic factors.

3. To obtain additional data on selected normal herds and single case herds to complete base line parameters for the evaluation of the single case and multiple case herds.
4. To shift the emphasis of the intensive clinical, hematologic, biochemical and pathologic studies on leukemic animals to animals with a marked lymphocytosis to determine the natural outcome of lymphocytosis animals, to attempt to increase the incidence of tumor formation, to determine the nature and significance of ultrastructural differences in bovine lymphoid cells of normal, preleukemic and leukemic animals.
5. Following suggestions of the Atomic Energy Commission site visit committee to initiate preliminary studies on attempts of transmission of bovine leukemia.

Following are the specific objectives of the proposed research as they appear in the eighth renewal request, 1968.

1. It was proposed to complete the statistical evaluation of data on occurrence and distribution of bovine lymphocytic leukemia in Minnesota and the adjacent area:
   a. to determine the role of selected environmental factors, reporting factors and socio-economic factors on the occurrence and distribution of bovine lymphocytic leukemia.
   b. to draft and submit for publication the results of unpublished epidemiologic data.
2. It was proposed to statistically evaluate and analyze the additional hematologic data on normal, single case and

multiple case herds:

a. to complete the comparison and evaluation of the single case herds with multiple cas herds.

b. to draft and submit for publication the results of the extensive herd studies.

3. It was proposed to continue limited studies on multiple case herds:

a. to provide a source of lymphocytosis and leukemic animals for other studies.

b. to obtain additional data on the natural outcome of lymphocytosis animals.

c. to augment our limited data on cohort groups and the role of genetic factors.

4. It was proposed to study intensively a limited number of animals to determine the relationship of lymphocytosis to the tumor phase of bovine leukemia:

a. to attempt to increase the incidence of tumor growth in lymphocytotic animals by various known promotor factors.

b. to continue ultrastructural studies of bovine lymphocytes to determine aberrations common to leukemia.

c. to continue to study lymphocyte kinetics by isolated lymph node systems in lymphocytotic animals.

d. to continue selected studies on the immuno-responsiveness of normal, lymphocytosis and leukemia animals to iodoacetate treated leukemia cells.

5. It is proposed to continue studies on the transmission of bovine leukemia:

   a. to determine transmissibility with whole cell intra-uterine fetal inoculation.

   b. to determine transmissibility by colostrum and milk with foster nursing technique.

   c. to determine the nature and significance of leukemia virus-like particles found in lymphocytosis animals.

   d. to attempt to prepare a suitable inoculum of virus-rich material for further transmission studies.

   e. to study selected porcine leukemia cases in regards to presence of C-type particles in appropriate cell culture systems.

In 1968 we received a critical evaluation by a U. S. Atomic Energy Commission review team. The review team was composed of Drs. N. P. Page, F. T. Brooks, R. W. Touchberry, R. R. Marshak, and G. H. Theilen.

III. PROGRESS REPORT

A. General Information

The initial objectives to study the occurrence and distribution of bovine leukemia and the possible influence of environmental factors have been completed. The studies on the role of social economic influence on the reporting of bovine leukemia advised by an Atomic Energy Commission Review Committee (1966) has been completed. As will be noted in changes in objectives, emphasis has been placed on other aspects of bovine lymphocytic leukemia. The data will be presented

under several sections as follows:

B. Epidemiologic Studies

C. Multiple Case Herd Studies

D. Hematological Studies On Normal, Single Case And Multiple Case Herds

E. Studies On Animals With A Marked Lymphocytosis

F. Transmission Studies

The studies under section B, Epidemiologic Studies, and section D, Hematological Studies On Normal Single Case and Multiple Case Herds are concluding reports. The studies reported under C, Multiple Case Herd Studies, E, Studies On Animals With A Marked Lymphocytosis, and F, Transmission Studies are reports of work in progress.

B. Epidemiologic Studies

The epidemiologic studies on the occurrence and distribution of bovine leukemia in Minnesota have been concluded with completion of study of the social economic influence on reporting. The latter study was encouraged by the 1966 U. S. Atomic Energy Commission review team. Major aspects of this portion of the contract are presented in two manuscripts attached to this report. These manuscripts are entitled: a) Selected Epidemiological Aspects of Bovine Leukemia in Minnesota, 1961 to 1965, and b) Association of Veterinary Medical Service with Reporting of Bovine Leukemia in Selected Minnesota Dairy Herds.

Reprints of previously submitted manuscripts concerning epidemiologic aspects of bovine leukemia are appended. Additional manuscripts on minor aspects of this portion of the study are in various stages of preparation.

C. Multiple Case Herd Studies

1. General

The general description, criteria for selection, and monitoring procedures for high incidence leukemia herds have been reported in previous progress reports and will not be discussed here.

A total of 33 multiple leukemia case herds have been studied since the initiation of this project in 1960. Twenty of these herds were studied more intensively and for longer periods of time. These 20 herds were selected on the basis of being more desirable for intensive study, i.e. having better records and more interest shown by the owner and the farm veterinarian. Many herds, which had been studied, are no longer under study due to herd dispersals. Several study herds were dispersed during the past year so that presently six herds are under study. These herds have been studied for periods up to 8 years. Four additional purebred herds with good records will be added to this study group in the near future.

All herds studied as multiple case herds have had at least 2 confirmed cases of bovine leukemia. The annual leukemia incidence rates in several of the herds have been 10% of the adult cattle. One herd has had 8 cases over a 4 year period. Fourteen herds have had additional cases occur after their selection for herd study on the basis of at least 2 index cases.

The breed distribution of the intensively studied herds was: 15 Holstein, 2 milking Shorthorn, 1 Guernsey, 1 Jersey and 1 Red Polled. The disproportionate number of Holstein herds is more a

reflection of the breed's predominance in this area than any breed predisposition.

The average number of adult cattle in these herds ranged from 14 to 100 cows. The median herd size was 45 cows.

The age distributions and average adult age in these herds compared favorably with statistics reported by DHIA for Minnesota cattle. Therefore the high leukemia incidences in these herds is apparently not due to increased numbers of aged cattle.

These herds were not characterized nor similar in respect to management practices nor environmental sanitation. The variation between the herds, in these respects, was about what would be expected from a random sample of the herds in Minnesota.

Introduction of new cattle was found to be an important source of leukemia cases in the herds. The reported index leukemia cases in 16 herds were either introduced animals or progeny of introduced animals. In 12 of the herds leukemia cases continued to occur in indigenous animals which were born after the introduced animals had entered the herd. The leukemia cases which were indigenous to these herds were not related to the introduced leukemia cases. Many of the introduced animals originated from herds with a history of leukemia.

2. Hematological Studies

All cattle 2 years of age or older in these herds were examined hematologically on a semiannual basis. Elevated lymphocyte counts were observed on 22% of all blood counts done on these cattle.

Persistent lymphocytosis was noted in 27% of the cattle studied more than one year and which had at least 2 blood examinations.

The percentage of adult cows which exhibited lymphocytosis varied between the herds. In general, the herds with the higher incidences of lymphocytotic cows were those herds where indigenous animals had died of leukemia and the herds which were studied for longer periods of time, i.e. 3 to 4 years, after the first occurrence of leukemia in introduced animals. Therefore it might be concluded that a period of time must elapse between the time that the leukemia agent is introduced and the time at which adult lymphocytotic cows are observed in the herd. In herds where the index cases were purchased animals this time lapse was noted. In these herds lymphocytosis was seen only in animals born after the index case animals were introduced.

3. New Cases

Leukemia cases to be discussed here had preclinical hematological data available and developed in herds under investigation. A total of 25 "new cases" of leukemia developed in 14 of these study herds.

All 25 "new cases" of leukemia had at least one preclinical blood examination and the majority had several examinations during periods up to four years. Blood examinations were usually made semiannually on all animals which are two years of age or older in the study herds. Twenty-four of the 25 "new cases" of leukemia exhibited significant lymphocytosis (significant by Bendixen's Key and Minnesota Key) prior to the appearance of clinical leukemia. The one animal which did not exhibit lymphocytosis had only one blood examination. This examination was made when the cow was 2½ years of age, which was 6 months prior to the appearance of tumors.

These 25 "new cases" represent 2% of the total number of animals studied in the herds. Since 24 of these cases exhibited preclinical lymphocytosis, probably a more appropriate denominator for calculation of the risk of leukemia for these cases would be the total number of animals with persistent lymphocytosis. In which case, the incidence rate for 24 new cases is 9% of those animals with persistent lymphocytosis during the period of study. Since all animals in these herds were under similar observation then the larger group of animals which did not exhibit lymphocytosis should have had many more than one case of leukemia occurring in it if chance alone were governing the occurrence of leukemia. The 9% incidence of leukemia in the animals with persistent lymphocytosis is very significantly different from the 0.1% incidence for the animals with normal lymphocyte counts.

Numerous reports on age specific incidence rates of bovine leukemia have indicated that the incidence of leukemia increases with age. This is especially true for animals over 5 years of age. Of the 25 cases presented here, 21 (88%) of them were 5 years of age or older. It has been noted in study of high incidence leukemia herds in Minnesota that animals with persistent lymphocytosis are "culled" at an average age of 5 years due to normal production practices. Thus, half of these cattle are not kept in the herds until the ages of higher risk of leukemia. The majority of the animals are "culled" because of low milk production or infertility. Under conditions other than those of dairying, these animals would have many years of additional expected longevity during which to develop leukemia.

4. Cohort and Genetic Studies

Analysis of leukemia cases occurring in multiple case herds with respect to date of birth, indicates a high incidence of leukemia case clusters in certain cohort groups.

Studies of animal records in seven high incidence leukemia herds revealed that 2 or more animals which died of leukemia had been born within a four-week period. Four of these herds had two leukemia cases, two herds had three cases and one herd had four leukemia cases born within a four week period. A total of 30 leukemia caseswere seen in these seven herds and (16%) of these cases were born in neonatal cluster groups.

Cohort analysis of data for all adult animals in 11 herds revealed a significant clustering by date of birth of animals with persistent lymphocytosis. This clustering involved animals sharing a common neonatal period with one or more animals which later developed leukemia as an adult (Table VIII-A). Sixty-four (64) adult cows were studied, which were born in 14 neonatal cluster groups (cohort groups). Twenty-five (39%) of these animals developed leukemia and an additional 31 animals (48%) of the animals in these clusters exhibited persistent lymphocytosis during adult life. Each cohort group contained progeny of leukemic dams and/or dams with persistent lymphocytosis. Several of the leukemic dams were in the early clinical stages of leukemia when these progeny were born. Needless to say, these 64 animals represent only a fraction of the entire cohort group since nearly all male animals and some female animals are normally culled before reaching maturity.

It is interesting to speculate on all the possible common exposure experiences of these groups of young calves including direct contact

with each other and common sources of food. All ten farmers did pool fresh colostrum and also it was possible for any young calf, fed milk, to receive some of excess colostrum. Cohort clustering of leukemic and lymphocytotic animals may be related to such practices.

Analysis of hematological data on 310 dam-daughter pairs in high incidence leukemia herds revealed familial similarities (Table VIII-B). Of the dams which developed leukemia or a persistent lymphocytosis, 49% had daughters which exhibited persistent lymphocytosis in contrast to 29% of the daughters of cows with normal lymphocyte counts. Examination of birth dates of the daughters on this 29% group revealed that 88% of them to be members of neonatal clusters with high incidence of lymphocytosis.

In summary, there should be little doubt about the significance of the close proximity of birth in the six clusters of leukemia cases observed in this sample. Neonatal exposure experience with calves which do develop leukemia as adults evidently is also in some way associated with occurrence of persistent lymphocytosis. If the persistent lymphocytosis seen in cattle of leukemia herds is a prodromal stage of leukemia, then these associations have added significance. The association of leukemic or lymphocytotic dams with these clusters is consistent with the concept of a transmissible agent. In view of these data the hypothesis for both vertical and horizontal transmission of bovine leukemia can be explained. Familial similarities of lymphocyte counts in dam-daughter pairs suggest the possibility of vertical transmission in leukemia and lymphocytosis. Dissimilarities between "normal dams" and some of their daughters can largely be explained on the basis of possible horizontal transmission of an

TABLE VIII-A

Relationship of Tumor Cases and/or Lymphocytosis to Time of Birth In Multiple Case Herds

Herd Number	Month of Birth	Number Studied	Number Developing Leukemia	Number Developing Lymphocytosis	Percentage With Leukemia and Lymphocytosis
MH-1	Aug. 1955	7	3	2	71%
	July 1958	9	1	7	89%
MH-2	Sept. 1954	2	2	..	100%
MH-4	Sept. 1956	8	1	6	88%
	Sept. 1957	5	1	3	80%
MH-15a	Oct. 1955	4	1	3	100%
MH-20	Aug. 1960	3	1	1	67%
MH-23	Sept. 1957	6	4	2	100%
MH-25	Aug. 1959	5	1	2	60%
	Nov. 1960	4	1	3	100%
MH-27	Jan. 1958	3	2	1	100%
MH-31	Oct. 1959	2	2	0	100%
MH-32	Oct. 1958	3	3	0	100%
MH-39	Nov. 1961	3	2	1	100%
	Total	64	25	31	

TABLE VIII-B

Analysis of the Difference Between Presence of Lymphocytosis and Leukemia in Dam-daughter Pairs

	Dams		
Daughters	Non-Lymphocytosis	Lymphocytosis and/or Leukemia	Total
Non-lymphocytosis			
Observed	164	41	205
Expected	152	53	
Lymphocytosis or Leukemia			
Observed	66	39	105
Expected	78	27	
Total	230	80	310

$X^2 = 10.84$ 1 d.f. P = .001

NOTE - The cohort and familial data presented in this section has been published. A reprint of this article is enclosed under the title: Epidemiological Studies on the Mechanism of Vertical and Horizontal Transmission of Bovine Leukemia.

agent from affected dams or their progeny during the neonatal period. It is apparent in these studies that some common exposure experience was specific for certain groups of calves during the neonatal period and not for other animals born during the same year.

It will be interesting to see if the cohort clustering of leukemia cases which has been so common in past studies continues as cases develop in the herds to be studied in the future.

Genealogical data is obtained on all animals studied in multiple case herds. Familial clusters of leukemia cases have been observed in a few herds. Overall, the study of pedigree data has not revealed any consistent pattern which would definitely incriminate genetic predisposition as an outstanding requirement for the occurrence of leukemia. These data may prove more valuable in the future when a causative agent for leukemia is demonstrated.

5. Conclusions From Multiple Case Herd Studies

The study of high incidence leukemia herds has resulted in certain recurring patterns in the data which allow the formulation of a hypothesis on the cause and occurrence of bovine leukemia. The occurrence of the multiple case herds or micro-epidemics have the characteristics of an infectious disease in that a disease of the relative rarity of leukemia would not be expected to occur as often in clusters if chance alone were the determining factor. The endemic character of the disease in these herds also is very consistent with a viral theory of causation especially since an environmental factor can not be incriminated.

Under the hypothesis of a viral cause of leukemia it would appear that introduction of infected animals (many of which

develop leukemia) from herds with a history of leukemia served as the source of infection in many of the herds studied. The close temporal-spatial neonatal relationship of cases indicates that horizontal transmission most likely occurs and/or susceptibility is limited to the neonatal period. Familial patterns in the occurrence of leukemia and/or lymphocytosis also demonstrates that vertical transmission does occur.

The association shown between persistent lymphocytosis and the occurrence of tumorous disease indicates that persistent lymphocytosis in cattle in these herds is a prodromal stage of the disease.

D. Hematologic Studies on Normal, Single Case and Multiple Case Herds

1. General

In previous progress reports we outlined in detail, criteria for the selection of several dairy cattle populations to determine hematologic norms for Minnesota cattle and the frequency of lymphocytosis in apparently normal cattle, in single case herd cattle and in multiple case herd cattle. Pertinent population data is depicted in Table D-1.

TABLE D-1

Source of Animals	No. of herds	No. of animals
Steele County (all breeds)	113	2,140
Steele County (all Holstein)	94	1,765
Aitken County (all breeds)	70	1,189
Hubbard County (all breeds)	49	704
Lake County (all breeds)	29	148
Selected Holstein Herds (1965)	36	849
Single Case Holstein Herds (adult type, 1965)	20	553
Single Case Holstein Herds (adult type, 1966)	20	735
Juvenile Leukemia Case Holstein Herds	10	242
Multiple Case Herds (all breeds)	25	987

The Steele, Aitkin and Hubbard County samples are random samples, stratified on the basis of herd size and are of significant size to reflect the status of the entire dairy cattle population consisting of animals 2 years of age and older in the milking herd. The Lake County sample represents the entire population of dairy cattle.

The Holstein herds selected as normal and leukemia-free were first studied in 1963 and bled for normal hematologic parameters in 1965. In 1966, the majority of these herds were rebled, however, the leukemia-free status was not defined.

The single case leukemia herds were all of the Holstein breed and were indexed by a single adult or juvenile type case reported during the study period. The multiple case herds consist of herds under study and are of several breeds.

The statistical treatment of the data is complete and pertinent methods of analysis are described in an appended manuscript entitled "Lymphocyte Counts on Minnesota Dairy Cattle".

2. Results of Hematologic Studies

Our data supported the observation of early European workers and several other investigators in this country and abroad, with exception of Marshak and co-workers in Pennsylvania, that lymphocytosis in apparently normal animals is associated with bovine leukemia.

Lymphocyte values on normal Holstein cattle are age dependent throughout the age groups evaluated, a significant difference from other reports, particularly cattle in the Eastern United States. This difference is most likely related to a low variance for lymphocytes in older animals in these herds. In contrast, single case and multiple case herds are characterized by greater variance in lymphocyte values in older cattle, thus age dependent lymphocyte counts in older animals in these groups are not observed.

Although our accepted norms for lymphocyte counts are based on a selected and defined population of Minnesota dairy cattle, it is highly significant that when the random sample of Steele County animals

are used to define lymphocyte counts for Minnesota cattle, regardless of breed, multiple case herds of the adult type differ and are characterized by a greater frequency of occurrence of lymphocytosis. This can not be expected on the basis of chance alone. This population of Steele County animals does contain known leukemia herds in addition to the four herds that had reported cases during the study period and which are included in the sample. Thus, a greater number of animals outside the 1 percent limits for the selected Holstein population are expected (Table D-2).

The reliability of this method to define lymphocytosis herds (potential leukemia herds) is recognized. The question has been raised as to the persistence of lymphocyte counts and the possibility of movement of animals between lymphocyte groups. In progress report No. 3 we presented data that such movement does occur; however, when exceeding that normally expected, the direction was toward higher counts. Approximately 3 percent of lymphocytotic animals did have counts at subsequent evaluation that would place the animal in a lower lymphocyte count group. This is expected since over 60% of lymphocytotic animals will have lymphocyte counts in the normal range during the period of tumor growth. It must be recognized that the cell of lymphocytotic animals are morphologically normal appearing and may differ markedly from those associated with true leukemia during the tumor phase.

3. Summary of Hematologic Studies

The results of these studies support the hypothesis that lymphocytosis in apparently normal cattle is associated with the tumor form of

bovine leukemia. Further, that at the present time lymphocytosis is the single best criteria for the detection of potential leukemia herds and may provide the best estimate of the natural incidence of this disease in Minnesota cattle.

The voluminous accumulations of data provide the basis for several additional manuscripts which are in various stages of preparation. No further work is anticipated in this area of investigation.

E. Studies On Animals With A Marked Lymphocytosis

This phase of study was initiated in 1967 and has been in progress continually. It was postulated that lymphocytosis is a prodromal phase of bovine lymphocytic leukemia, and that the protracted induction state may be due to a highly competent immune mechanism which controls aberrations as they occur. Further, accepting an Inducer-Promotor theory of leukemogenesis, it was theorized that treatment with carcinogenic factors may shorten the time required for these cattle to develop clinical leukemia. Total body gamma irradiation or prednisolone, a corticosteroid, were chosen to be used as promotor factors.

1. Radiation Studies

Details of the response to sublethal total body irradiation are presented in progress reports No. 7 and 8. The initial exposure to the midline dose of 75r was made in January of 1967. Subsequent exposures to 25r, 25r and 50r were made at intervals. The dosage schedule was discussed with staff members of Brookhaven National Laboratories who offered helpful suggestions on this phase of the study. The initial exposure involved five adult Jersey cows with marked lymphocytosis. Subsequently, three additional animals were irradiated.

One animal died of gangrenous mastitis 9 months following initial irradiation. This animal had a low grade mastitis which was seriously aggravated during the period of neutropenia induced by irradiation. None of the remaining animals have developed tumors.

2. Induction Studies with Adrenal Corticosteroids

In these studies, initiated in 1967, four Holstein cows with persistent lymphocytosis were subjected to two periods of prenisolone treatment at the dose of .16 mg./lb./day for six days followed by four days of diminishing withdrawal. One animal in this group developed the tumor phase of bovine leukemia.

3. Summary and Conclusion of Induction Studies

During the two year period of induction studies one lymphocytotic animal receiving corticosteroids and one control lymphocytotic animal developed the tumor phase of leukemia. These results are not unexpected since in previously reported necropsy studies of lymphocytotic animals, 1 of 14 cows had incipient leukemia.

These cattle are currently aged animals and this phase of work will be discontinued since the results are equivocal.

We do anticipate subjecting other lymphocytotic animals to total body irradiation to study the occurrence of viral particles in plasma and buffy coat cell cultures. As reported in the section on transmission studies, it appears that the lymphoid regenerative phase following total body irradiation is associated with the presence of detectable viral-like particles similar to those associated with murine leukemia.

4. Lymphocyte Kinetics and Immuno-responsiveness of Lymphocytotic Animals

Progress in this area was minimal because of insufficient funding. A manuscript, "Preparation and Partial Characterization of Chronic Prescapular Lymph Node Fistulas in Calves", is appended. One additional manuscript of work partially supported is near completion.

An opportunity to study recent technology and new knowledge in lymphocyte kinetics of cattle was afforded two members of the study group. Dr. Perman completed a quarter leave of absence at the Brookhaven National Laboratory, Medical Research Center sponsored by Dr. E. D. Cronkite. Dr. Stevens is currently on leave at the same laboratory.

5. Ultrastructure Studies

The association of nuclear pockets "blebs" with several forms of animal and human leukemia provided the basis for a similar study of cattle to further support the association and significance of lymphocytosis to bovine leukemia. The various herds under study in the present contract provided source animals for this study. Details of this study supported in part by contract funds are reported in the appended manuscript entitled, "Studies of the Occurrence of Lymphocytic Nuclear Pockets in Normal, Persistent Lymphocytotic, and Leukemic Adult Cattle".

The summary as presented in the paper is as follows:
Studies were conducted concerning the occurrence of nuclear pockets (blebs) in four groups of dairy cattle, namely those with normal hemograms from leukemia free herds, those with normal hemograms and with persistent lymphocytosis (P.L.) from multiple case leukemia herds, and leukemic cattle. Nuclear pockets were found generally to resemble those seen in man and other species, but were limited to the lymphocytes. Statistical evaluation of the frequency of occurrence of nuclear pockets in the four groups showed that: 1) animals with normal hemograms as versus lymphocytotic and leukemic animals,

represented two different populations both with regard to mean nuclear pocket frequency and the distribution of these values; 2) means from control animals and normals of multiple case herds are not statistically different with the sample size used. The variances differ significantly, however; 3) mean and variance comparisons suggest that, for nuclear pocket frequencies, persistently lymphocytic and leukemic animals belong to one common population.

F. Transmission Studies

1. Cell Culture and Viral Studies

To obtain the evidence of virus involvement in bovine leukemia electron microscopic examinations of tissues from leukemic and lymphocytotic cattle were performed. Direct EM examination of tissue was not successful in demonstrating virus or virus-like particles. However, modifications of our procedures, as outlined below were successful in demonstrating virus and virus-like particles and particularly in phytohemagglutinin stimulated lymphocyte cultures it was possible to demonstrate "C" type virus particles.

Direct EM examinations were performed on fresh lymph node tumor tissues, buffy coat cells, cell cultures of tumor tissue and buffy coat cell lines, also pancreatic fluid and plasma pellets obtained from several leukemic and lymphocytotic cattle. In none of the cases was there any evidence of the presence of virus or virus-like particles. Eleven lymph node tumor cell lines were established out of which two were partially resistant to infection with vesicular stomatitis virus.

One cell line has been developed from tumorous lymph node. In the monolayer, some cells appear to have changed morphology. On electron microscopy 3 to 4% of cells possess particles resembling "C" type particles, 80-90 mu in diameter. Superinfection with Rauscher murine leukemia virus resulted in production of 2 types of virus particles. One with a single membrane, is 60-70 mu in diameter and the other, with a double membrane, is 100-105 mu. Superinfection with Moloney murine leukemia virus is less striking, although some viral membrane changes appeared. Superinfection with bovine virus diarrhea virus had no apparent effect.

A cell culture from a placental tumor has been obtained from a cow in which intrafetal inoculation of lymph node cells was performed. Neither the cell culture nor the fresh placental tissue gave any evidence of the presence of virus by electron microscopic examination.

Our modified procedures involve (1) sublethal total body gamma irradiation of lymphocytotic cattle (2) intraperitoneal autotransplantation of lymph node tumor tissues (3) phytohemagglutinin induced transformation of lymphocyte cultures.

Fresh buffy coat and plasma pellet from two lymphocytotic cows following total body gamma irradiation, have yielded virus-like particles. Moreover, the lymphocyte culture from one of these two cows showed particles similar to "C" type particles after superinfection with vesicular stomatitis virus.

In studies on murine leukemia by others at this laboratory, intraperitoneal inoculation with autologous tumor tissue from a spontaneous case of leukemia resulted in the enhancement of isolation of an apparently new murine leukemia virus. This technique has been applied to one clinical case of bovine leukemia. An enlarged lymph node was minced and the cells were inoculated into the animal's own peritoneal cavity (autotransplantation). On the 15th and 30th day postinoculation peritoneal fluids were collected by aspiration. Cells separated from the peritoneal fluid were fixed for electron microscopic examination and also grown in tissue culture. Cells from the peritoneal fluid, cells from the peritoneal fluid grown in tissue culture and the pellet

from the cell free peritoneal fluid were examined by electron microscopy. Cells from the peritoneal fluid and the pellet of the peritoneal fluid were negative for any virus particle, but the cells in tissue culture, from the 30th day peritoneal fluid, reveal on electron microscopic examination virus-like particles and also an evidence of budding.

Lymphocytes from the blood from leukemic and lymphocytotic cattle were separated by the silicone procedure used by Joel et al; (unpublished). These lymphocytes in tissue culture, stimulated with phytohemagglutinin, were pelleted after 72 hours, sectioned and examined by electron microscopy. Two types of virus particles, "A" and "C" types were seen. "A" particles are nonenveloped, 60-90 mu in diameter and intracytoplasmic (Fig. F-1). These particles are seen from the leukemic cases, but due to our limited study of lymphocytotic cattle we have not as yet observed these particles in them. The "C" type particles are more predominant, 55-80 mu in diameter and extracellular. These particles are seen in both leukemic (Fig. F-2,3) and lymphocytotic (Fig. F-4) cattle.

2. Fetal Inoculation Studies

Background

Bovine leukemia transplantation experiments using intrauterine inoculation of fetuses were initiated in 1967 and are still in progress. Transplantation or cellular transmissions were attempted due to a lack of a defined concentrated viral inoculum. A suitable defined concentrated viral inoculum will be available for transmission studies using fetal calves and will be initiated using procedures perfected

under the present transplantation studies.

The experimental animals in these studies are bovine fetuses which have not reached the age of immune competence. Previous progress reports have included reports of studies done by this group to determine the age at which immune competence occurs in the bovine. These studies demonstrated that the bovine fetus is always immunologically tolerant during the first trimester, i.e., the first 90 days of gestation.

Materials and Methods

The fetuses used in this experiment are the progeny of cows and bulls which originated from herds which had no history of leukemia. All adult animals in these source herds were examined hematologically for persistent lymphocytosis. Only animals from lymphocytosis free herds were selected.

The inoculated material was obtained surgically from tumor masses of cases of bovine leukemia (adult type). The inoculum consisted of 0.5 gm. of tumor tissue homogenate suspended in 3 cc. of Eagles media and 20% bovine fetal serum. The fetuses were injected intraperitoneally thorugh the wall of the uterus which was exteriorized through the abdominal incision.

Results

During the past year, 4 calves were born which had been inoculated in utero by the above method. Some information on these animals is listed below.

Animal No.	Sex	Birth Date	Clinical Observations	Lymphocyte Count Last Bleeding
F 254 TS	M	3-19-68	Normal	9,600
F 257 TS	M	4- 5-68	Normal	7,600
F 280 TS	F	4-28-68	Normal	7,900
F 281 TS	F	4- 4-68	*	8,800

* Calf 281 was born 3 weeks premature and was small and very weak. Multiple tumor masses (confirmed lymphoma) were distributed throughout 80% of the placenta, but did not apparently involve the calf.

These 4 calves are presently in apparent good health with no signs of tumors. Lymphocyte counts on these calves have been within normal ranges since birth.

Successful transplantation of neoplastic leukemia cells to the placenta occurred in one case (the dam of F 281 TS). This transplantation must have resulted from seepage of the inoculum from the fetus or accidental injection of the placenta. The placental tumor could not have been of endogenous origin since there were no signs of tumors in the maternal uterus or the calf. This cow and the calf are still in good health with no signs of tumors.

Follow up studies were done with the placental tumor since it had originally been successfully transplanted. Three fetuses were inoculated with tissue culture cells and another fetus with cell free filtrate from the tumors. Of the three fetuses inoculated with the cells; one is unborn, one died and resorbed 50 days post inoculation, and one was born and is presently in apparent good health. The calf which was inoculated, as a fetus, with

cell free material died at birth and was found on necropsy to be free of tumors.

The six calves which remain in these studies will continue to be followed in the future. The present plans for additional intrafetal transmissions will await the availability of a concentrated viral inoculum resulting from studies discussed elsewhere in this report.

The major progress which can be reported from the fetal studies is in the development of intrafetal inoculation techniques and the definition of the period of immune competence to several antigens, and a successful placental transplantation.

3. Foster Nursing Experiments

Background

Our study group, as well as other groups which have intensively studied bovine leukemia, are of the opinion that ultimately bovine leukemia will be demonstrated to be a transmissible viral disease. This concept of a viral etiology is predicated on the knowledge of the disease obtained from epidemiological studies and the similarity to the viral leukemias of mice and chickens. The U. S. Atomic Energy Commission Review Committee which reviewed our contract in 1966 concurred with our opinion of a viral etiology and they strongly encouraged us to initiate transmission experiments. The feeling then, as it is now, was that successful transmission experiments would best be accomplished using a defined and concentrated inoculum. In the absence of a definable inoculum it was decided to initiate preliminary limited foster nursing studies because the epidemiological

data on multiple case herds and in particular the high incidence of leukemia in neonatal cohorts, indicated a strong possibility of horizontal transmission of bovine leukemia via colostrum or milk. The evidence for this hypothesis has been discussed previously in this report under the heading of "Cohort Analysis" and this formed the basis for studies of foster nursing.

These studies were easily and economically initiated because, in part, they were an outgrowth of other studies, i.e., some of the calves and colostrum were from lymphocytotic and/or leukemia animals purchased for virological studies. The study was initiated during 1967 and has not been enlarged during the 1968 contract year.

In view of the expectations obtained from rates of occurrence of leukemia in high incidence herds this experiment could only give meaningful results with much larger numbers of animals followed possibly for 4 to 8 years. Therefore it has been decided that this experiment is economically infeasible under the present contract and is presently being terminated.

Below is a resume of the experimental procedures and the results noted from the study.

Materials and Methods

For these studies, two groups of calves were used: 1) progeny of lymphocytotic cows from high incidence leukemia herds 2) progeny of "normal" cows, i.e., cows with normal lymphocyte counts from herds with no history of leukemia and which had no lymphocytotic cattle. Colostrum and milk used in this experiment were obtained from the dams of the above groups and also milk from clinical cases of bovine

leukemia. This colostrum and milk was either fed shortly after obtained or was stored at -70° C. until newborn calves were available.

Progeny of lymphocytotic cows were removed from their dams immediately after birth and housed separately. These calves were fed only colostrum from "normal" cows for 2 days and thereafter milk replacer until a roughage diet was fed.

Progeny of "normal" cows were removed from their dams after birth and housed separately. These calves were fed either colostrum from lymphocytotic cows or milk from clinic cases of bovine leukemia.

The 2 groups of calves were to be housed separately until they became adults.

Results

The following number of calves were in each experimental group:

1) 5 - progeny of lymphocytotic cows which receive colostrum from "normal" cows.
2) 5 - progeny of "normal" cows which received colostrum from lymphocytotic cows.
3) 5 - progeny of "normal" cows which received milk from clinical cases of bovine leukemia.

The calves in these 3 groups presently range in age from 14 to 19 months and are in apparent good health clinically.

Lymphocyte values are normally quite variable in cattle under two years of age. Therefore, it would be difficult to draw any conclusions on lymphocyte counts obtained on these calves to date. At the present time there is one animal (14 mos. of age), which received milk from a clinical case of leukemia, which has exhibited a persistent lymphocytosis. All other animals have essentially normal lymphocyte counts.

4. Transplantation Studies

The availability of a model system for transmission of bovine leukemia would enhance studies on the etiology and pathogenesis of this disease. In order to explore the possibility of transplantation of leukemia tissue to normal animals studies were conducted at the Brookhaven National Laboratory, Medical Research Center in collaboration with Dr. E. P. Cronkite. Drs. V. Perman and E. A. Usenik conducted the studies while on quarter leave from the University of Minnesota. These studies were not supported by this contract and will not be included in this progress report.

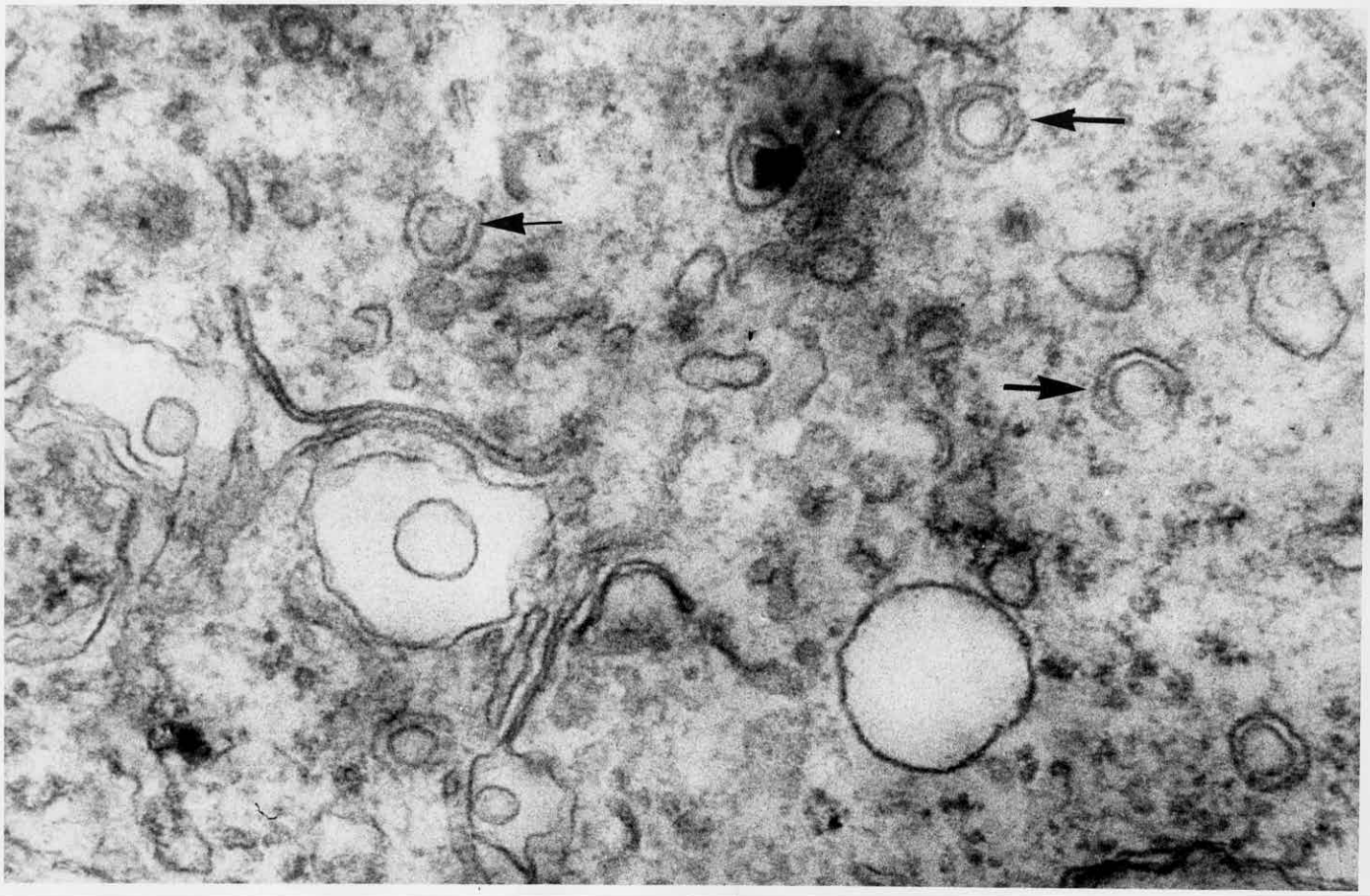

Fig. F-1. Electron micrograph of phytohemagglutinin stimulated culture of lymphocytes from Leukemic cow no. L3398. Intracytoplasmic, nonenveloped, "A" type virus particles (arrow), 60-90 mu in diameter. X 108,000.

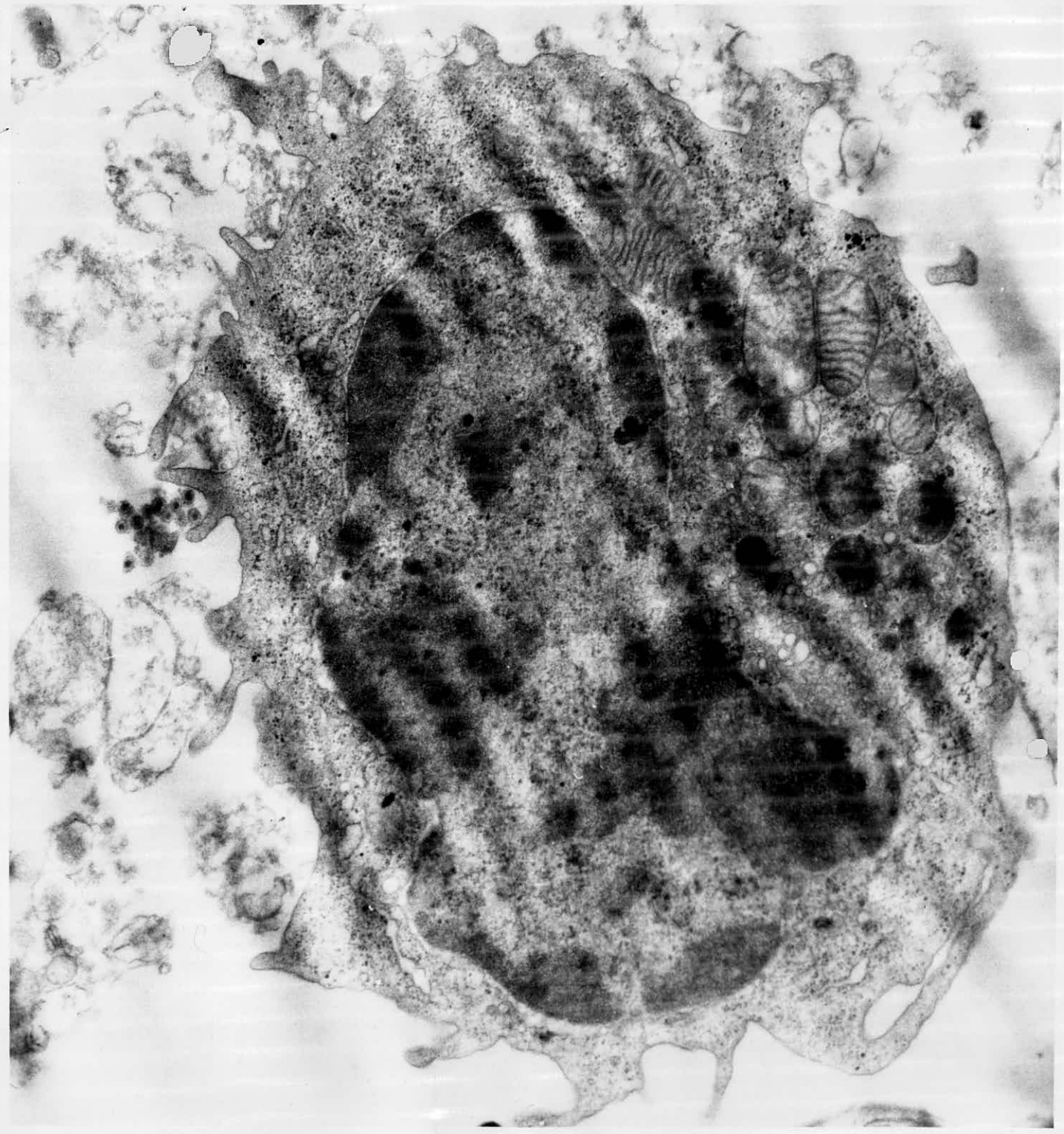

Fig. F-2. Electron micrograph of phytohemagglutinin stimulated culture of lymphocytes from leukemic cow no. L3398. Note the presence of "C" type virus particles adjacent to an agranulocyte. X 30,000.

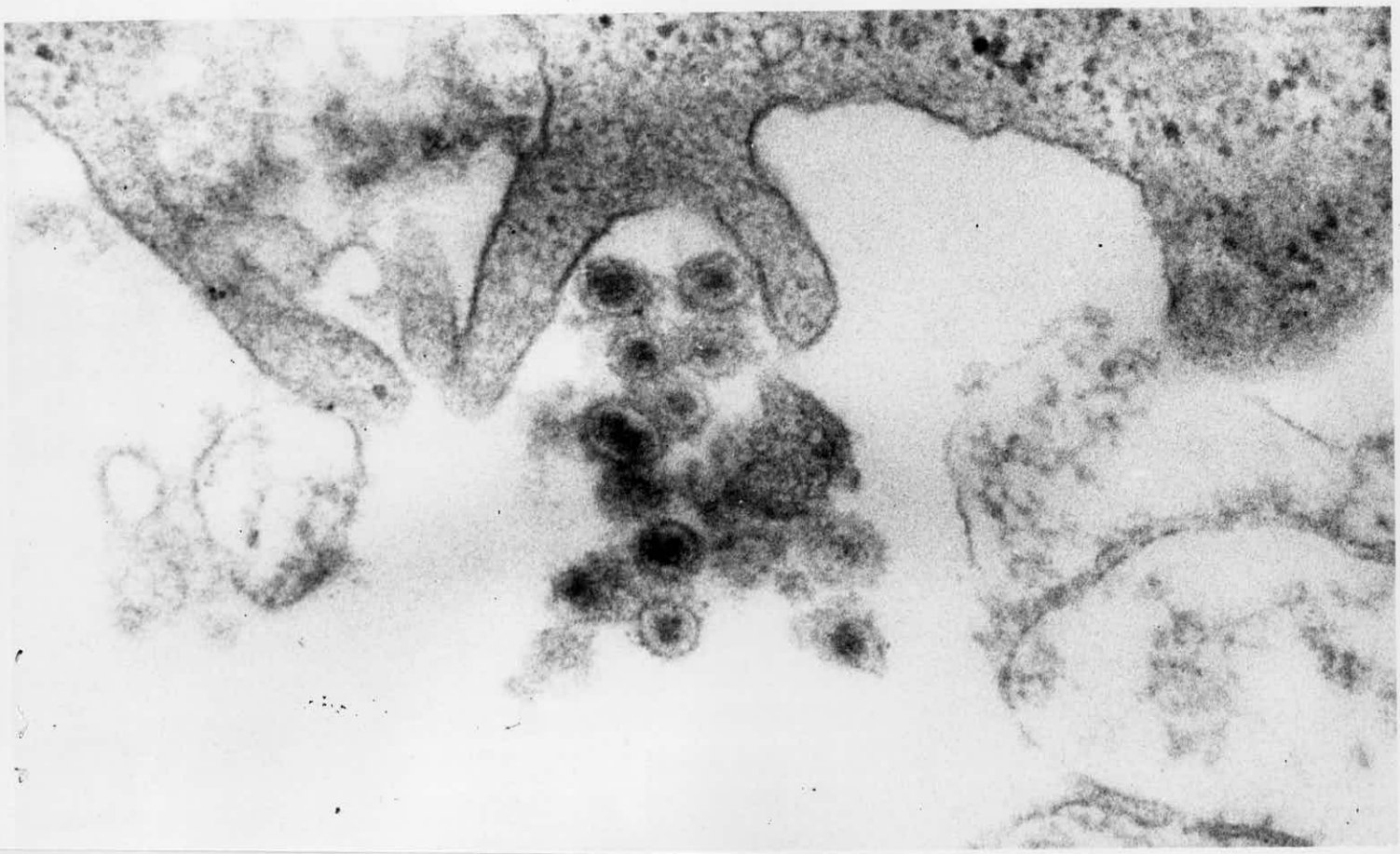

Fig. F-3. Electron micrograph of phytohemagglutinin stimulated culture of lymphocytes from Leukemic cow no. L3398. "C" type virus particles, extracellular, 55-80 mu in diameter (magnified view of virus particles of Fig. 2). X 108,000.

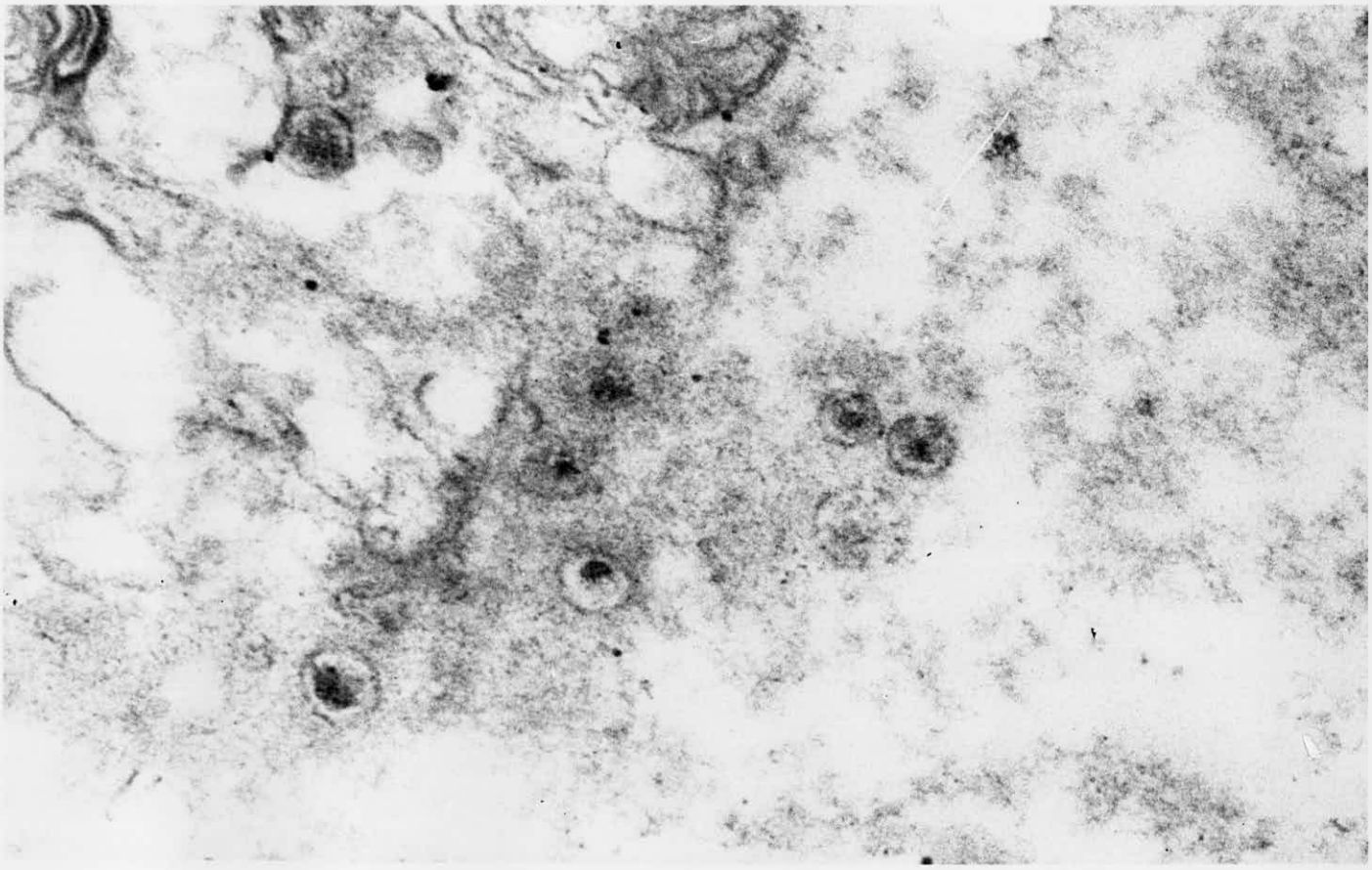

Fig. F-4. Electron micrograph of phytohemagglutinin stimulated culture of lymphocytes from Lymphocytotic cow no. 20. "C" type of virus particles. X 108,000.