**BOVINE LYMPHOCYTIC LEUKEMIA: STUDIES OF
ETIOLOGY, PATHOGENESIS AND MODE OF TRANSMISSION**

PROGRESS REPORT NO. 14 TO THE U.S. ATOMIC ENERGY COMMISSION

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COVERING THE ONE YEAR PERIOD - JULY, 1973 - JULY, 1974

MASTER

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PROJECT ABSTRACT 1974-1975

We have consistently demonstrated viral like particles in mitogen stimulated lymphocyte cultures from leukemic cows and cows with a persistent lymphocytosis. Our electron microscopic studies of these particles have shown them to be similar to the known oncogenic RNA viruses in size, plasma membrane budding, and in their C-type morphology. We have concentrated these bovine C-type particles approximately 1,000 fold by continuous flow ultracentrifugation.

Our investigations employing these concentrated particles have indicated that they are RNA containing, possess an RNA directed DNA polymerase, and band in a sucrose density gradient at a density of 1.17 gm/cm^3

The goal of our current study is to conclusively prove the viral etiology of bovine leukemia by transmitting the disease using these concentrated C-type particles. In conjunction with this goal we hope to obtain a detailed description of the events occurring early in the pathogenesis of bovine leukemia by extensively monitoring all successfully inoculated animals.

The monitoring studies are designed to detect early precancerous changes in the lymphocytes of the inoculated animals. Each animal will be examined once each month to detect changes in the following: a) the complete blood count, b) the percentage of lymphocytes showing nuclear pockets, c) the percentage of cultured lymphocytes with C-type particles adherent to their surface, d) the percentage of B-lymphocytes in the peripheral blood. In addition we propose to study: a) the responsiveness of peripheral blood lymphocytes to certain mitogens, b) the level of IgG and IgM in the serum as the disease develops, c) the appearance of antibodies directed against the bovine C-type particles.

From our extensive monitoring study we will have a complete record of all changes in the parameters discussed above. We will then be able to detail these changes and place them in their proper sequence. Thus, if a single animal develops leukemia, its records will contribute greatly to the knowledge of the early events in the pathogenesis of this disease. We hope that a detailed description of the events occurring early in the pathogenesis of bovine leukemia may serve as a model for understanding and investigating early events in the pathogenesis of human leukemias.

PERIOD COVERED BY THIS REPORT

This report covers the period from April 30, 1973 to April 30, 1974.

SCIENTIFIC BACKGROUND

It is widely hypothesized that the proposed infectious agent of bovine leukemia is viral in nature. Major support for this hypothesis comes from the conclusive demonstration of the role of C-type viruses in the etiology of transmissible lymphoid neoplasms in the chicken (1), mouse (2), cat (3), and guinea pig (4). Reports on the observation of virus like particles in the milk and lymphoid tissues of leukemic cows and cows from high incidence herds (5, 6) provide additional support for a viral etiology. However, these investigations are not conclusive because the electron micrographs leave much doubt that the observed particles are similar to the C-type viruses known to cause leukemia in other species.

Much more convincing evidence for a viral involvement in lymphocytotic and leukemic cattle has been provided by the examination of cultured lymphocytes. In December of 1969 Miller et al. (7) reported a very significant observation. In many short-term (24 to 72 hour) phytohemagglutinin stimulated cultures of lymphocytes, obtained from the peripheral blood of (A) lymphosarcoma cases, (B) cows from high incidence herds, and (C) cattle that had been inoculated with lymphosarcoma extracts, they found abundant virus like particles. Unlike the particles observed earlier in milk and tissues, these particles clearly resemble the mature C-type viruses known to cause leukemia in the murine (2) and feline (3) species. This important finding was immediately confirmed by our research group at Minnesota (8). Since that time we have consistently demonstrated C-type particles in mitogen stimulated lymphocyte cultures from leukemic cows and cows with a persistent lymphocytosis. Our studies

of these particles have shown them to be similar to known RNA oncogenic viruses in that they: a) bud from the plasma membrane, b) contain RNA, c) band at 1.17 gm/cm³ in a sucrose density gradient, d) show typical C-type size and morphology.

The consistent finding of particles similar to known oncogenic RNA viruses in lymphocyte cultures obtained from leukemia cases and cows with a persistent lymphocytosis, plus the conclusive demonstration of the role of C-type viruses in the etiology of lymphoid neoplasms in other species (1, 2, 3, 4) strongly suggests, but does not prove, a viral etiology for bovine leukemia. This proof awaits a conclusive demonstration of transmission by means of a cell free filtrate. A few investigators have reported on successful cell free transmission (9, 10). However, because of poor experimental designs and the very small number of animals actually developing lymphosarcoma, their studies are not convincing and cannot be considered positive proof of a viral etiology.

Recently, Olson et al. (11) inoculated neonatal sheep with lymphocytes cultured from a case of bovine lymphosarcoma. After a latent period of over two years, 5 of 13 sheep receiving such inoculums developed lymphosarcoma and 11 of the 13 had lymphocytes producing C-type particles morphologically identical to those observed in the original inoculum. Since lymphosarcoma is rarely found as a natural disease in sheep, these five cases are highly significant and represent the successful transmission of lymphosarcoma from cattle to sheep. Thus, the oncogenic potential of the bovine C-type particles has been established but actual transmission of lymphosarcoma from bovine to bovine has, as of yet, not been conclusively demonstrated.

A primary objective of our current study is to conclusively prove the viral

etiology of bovine leukemia by successfully transmitting the disease using a cell free inoculum containing concentrated, partially purified, bovine C-type particles.

In our transmission studies we are also interested in those events preceeding the development of leukemia. Several such pre-cancerous changes have been reported. They include: a) The development of a persistent lymphocytosis (12, 13, 14); b) The occurrence of increased numbers of lymphocytic nuclear pockets (15, 16, 17, 18); c) The production of C-type particles by mitogen stimulated lymphocytes (7, 19, 20); d) A shift in lymphocyte subpopulations to a preponderance of B-cells (21); and e) The appearance in the serum of antibodies directed against bovine C-type particles (22,23). We are currently monitoring our inoculated animals to detect changes in these and other parameters.

PROGRESS REPORT

This report summarizes the proposals made for the 1973-1974 contract period and describes our progress through April of 1974. The rationalization behind these proposals and a brief history of our prior successes and failures in fetal transmission studies is contained in the accompanying renewal request. To avoid repetition, the materials, methods and animals used in all of our bovine leukemia studies are described only in the renewal request. Consequently, it is advised that the accompanying renewal request be read prior to this progress report.

PROPOSAL NUMBER 1: CELL FREE TRANSMISSION STUDIES INVOLVING FETUSES INOCULATED IN UTERO

For the contract year 1973-1974 we proposed to successfully inoculate seven fetuses in utero. A maximum of 10 pregnant cows would be purchased to accomplish

this goal. An inoculation was considered successful if a living calf was born.

PROGRESS ON PROPOSAL NUMBER 1

Six pregnant cows were purchased and the fetuses inoculated. To date five calves have been born. They were born 3, 6, 26, 35, and 42 days after inoculation. All of these calves are currently healthy. Abortion of the sixth fetus is not likely as it was inoculated over 45 days ago. The seventh proposed fetus was not inoculated because the \$5,000 cut in our requested budget did not allow us to purchase and maintain the additional pregnant cow.

Combined with the inoculations of last year, a total of 12 fetuses have now been successfully inoculated. The current fate of these animals is summarized below.

Two animals (653 & 654) have died. The similarity in the course of events leading to death in these two calves was striking. Both calves were inoculated approximately $3\frac{1}{2}$ weeks prior to birth. They remained healthy for two weeks then progressively developed scours, persistent fever, petechial hemorrhages, and rectal bleeding. Both calves died approximately one month after birth. Hematological studies during the last two weeks of life showed a rapid decline in platelets accompanied by a slower decrease in leukocytes and red blood cells. Both animals died with white cell counts below 3,000/mm³ and a differential count showing 98 - 100% lymphocytes. A bone marrow biopsy taken shortly before death showed a complete absence of cells in the myeloid and erythroid series. The gross and microscopic pathology for these two calves was identical establishing beyond a doubt that both calves died from a severe aplastic anemia. In both cases very low

numbers of C-type particles were observed in cultured lymphocytes obtained prior to death. Tissues obtained at post-mortem were examined for the presence of C-type particles, however, because of post-mortem autolysis none could be positively identified.

It is our conclusion that bovine C-type particles, under certain as yet undefined conditions, can initiate changes in the bone marrow resulting in aplastic anemia. We are currently preparing for publication a paper including further details and discussion on these two interesting aplastic anemia cases.

Two other fetuses inoculated last year (650 and 651) are definitely infected. Pre-cancerous changes in their lymphocytes including the production of C-type particles is consistently observed. These calves have not developed a persistent lymphocytosis but they are still less than one year old.

The remaining fetuses inoculated in 1973 (652 and 655) both show nuclear pockets and viral production, but these indicators of infection have been inconsistent or below the level we consider highly significant.

Monitoring is just beginning on the inoculated animals born this year. Already, nuclear pockets and C-type particles have been observed. It is, however, judgment is best made after 1½ years of continuous monitoring.

PROPOSAL NUMBER 2: EXTENSIVE MONITORING TO DETECT EARLY SIGNS OF LEUKEMIA IN INOCULATED CALVES AND FETUSES

For the contract year 1973-1974 we proposed to continue testing every calf and fetus inoculated during the present and previous AEC contract periods. Animals to be monitored included: a) Every inoculated animal under 1½ years of age. b) Those inoculated animals over 1½ years of age judged to be infected. The proposed testing frequency was once each month starting shortly after birth. We proposed to monitor and record changes in the following parameters: a) the complete blood count, b) the percentage of lymphocytes showing nuclear pockets, and c) the percentage of cultured lymphocytes with C-type particles adherent to their surface.

It has been our hypothesis that reliable indications of leukemia appear prior to the development of a persistent lymphocytosis. More specifically, we have postulated that the series of events preceding the development of lymphosarcoma are as follows: a) The percentage of lymphocytes with nuclear pockets increases. b) C-type particles appear when approximately 0.5 percent of the lymphocytes show nuclear pockets. c) The percentage of lymphocytes with closely associated C-type particles increases. d) A persistent lymphocytosis develops.

PROGRESS ON PROPOSAL NUMBER 2

The proposed monitoring has been carried out. Detailed results will be published when our studies are complete. Our progress in terms of infecting the animals is summarized in Table I. We consider our inoculated animals infected when two consecutive monthly samples show a nuclear pocket frequency greater

than 0.5% and C-type viral production by more than 5.0% of the PHA stimulated lymphocytes. It is evident from Table I that four animals (303, 338, 650, and 651) are definitely infected at this time. One animal (652) may soon meet our criteria for infection. Calves 656 - 661, inoculated as fetuses this year, are still too young for meaningful monitoring results.

PROPOSAL NUMBER 3: STUDIES ON THE PHA RESPONSIVENESS OF LEUKEMIC,
LYMPHOCYTOTIC AND NORMAL BOVINE LYMPHOCYTES

We proposed that cattle with leukemia would show immunological deficiencies and specifically that their lymphocytes would be less responsive to PHA than those obtained from normal cows. It was our purpose to demonstrate that a decrease in PHA responsiveness, indicating a decreased functioning of the thymic-dependent immunological system, is a consistent feature in the pathogenesis of bovine leukemia.

TABLE I

Cow Number	Age in Months	Percent of Cells with Nuclear Pockets*	Percent of Cells with C-type Particles*
303*	25	1.5	20.0
336*	4	0.3	2.0
338*	4	2.5	7.0
650	9	1.4	7.0
651	9	3.0	16.0
652	9	0.9	2.0
653	Died-Aplastic Anemia		
654	Died-Aplastic Anemia		
655	8	0.3	0.0
656-661	All less than two months old		

* Average of two most recent samples

** Inoculated as day old calves

PROGRESS ON PROPOSAL NUMBER 3

We have completed our studies on the PHA responsiveness of leukemic, lymphocytotic and normal bovine lymphocytes. In addition we studied the mitogenic activity of pokeweed mitogen (PWM) on peripheral blood lymphocytes obtained from these same animals. Our results are reported in a journal article entitled "CHARACTERISTICS OF LYMPHOCYTE RESPONSES TO PHYTOMITOGENS: COMPARISON OF RESPONSES OF LYMPHOCYTES FROM NORMAL AND LYMPHOCYTOTIC COWS" which is currently in press (Am. J. Vet. Res.). The preprint of this article included at the end of this report should be considered as our progress report for proposal number 3.

PROPOSAL NUMBER 4: STUDIES ON BOVINE LYMPHOCYTE SURFACE IMMUNOGLOBULINS

We have repeatedly argued that bovine leukemia is an excellent model for human chronic lymphocytic leukemia and that laboratory findings in one species are likely to apply to the other. Therefore, when we learned that the percentage of B-cells in the peripheral blood increases in human cases of chronic lymphocytic leukemia, we proposed to study this parameter in the bovine. We specifically proposed to determine the B-cell percentages in normal cows, cows with a persistent lymphocytosis, and cows with lymphosarcoma. The finding of a similar shift in lymphocyte subpopulations in bovine leukemia would add support to our hypothesis that bovine leukemia and human chronic lymphocytic leukemia are very comparable diseases.

PROGRESS ON PROPOSAL NUMBER 4

We have completed our studies on the frequency of surface immunoglobulins

on peripheral blood lymphocytes obtained from normal cows and cows with a persistent lymphocytosis. Our results are reported in a journal article entitled "LYMPHOCYTE SURFACE IMMUNOGLOBULIN: FREQUENCY IN NORMAL AND LYMPHOCYTOTIC CATTLE," (Am. J. Vet. Res. 35:593-595, 1974). The reprint of this article included at the end of this report should be considered as our progress report for proposal number 4.

PROPOSAL NUMBER 5: CONTINUED STUDIES OF HIGH INCIDENCE AND CONTROL HERDS

The study of multiple case herds was reduced during the past years due to budget reductions on the contract. These studies were judged to have a lower priority than the transmission studies which are considered our primary objective. However, since the high incidence and control herds are essential as sources of animals for transmission experiments, it was necessary to study a small number of these herds.

The multiple case herd study presently consists of the study of four herds. These herds have been studied for periods up to thirteen years. Unfortunately, several herds studied in previous years are no longer available for study due to herd dispersals. The present herds have had at least two confirmed cases of bovine leukemia.

RESULTS OF MULTIPLE CASE HERD STUDIES:

As in previous years additional "new cases" of leukemia have occurred in herds which had been under study. Almost all (97%) of these cases have exhibited persistent lymphocytosis for periods up to five years prior to the development of tumorous clinical leukemia.

Hematological data accumulated during the past year on adult cattle in the

study herds is comparable to that reported in previous years. High incidences of persistent lymphocytosis continues to be a characteristic of this herd type. Familial and temporal-spatial relationships reported in past years are still noted.

CONTROL HERD STUDIES:

Control herds by our definition are herds of cattle which have had no cases of leukemia, do not contain cows with lymphocytosis and have had no new animal introduction for at least five years. Presently, 15 herds of this type are under observation and have served as source herds for experimental animals used in our transmission studies.

PROGRESS IN AREAS NOT SPECIFICALLY PROPOSED:

In addition to our research on the chronic adult form of bovine leukemia we have extensively studied two cases of the acute calf form of this disease. Our findings will be presented in a paper we are currently preparing for publication.

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