BOVINE LYMPHOCYTIC LEUKEMIA: STUDIES OF
ETIOLOGY, PATHOGENESIS, AND MODE OF TRANSMISSION. 345

MASTER

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PROJECT ABSTRACT

The primary objective of our current DOE supported research is to elucidate the cause(s) and early pathogenesis of the adult form of lymphosarcoma in cattle. Consequently, a major portion of our research is centered around experimental transmission of this disease. In our transmission attempts we have been guided by the previously successful leukemia transmission studies in other animal species. These studies demonstrated that increasing the number of viral particles in the inoculum increased the incidence of leukemia and decreased the latent period. A similar enhancing effect was provided by inoculating animals at progressively earlier ages, especially when they were inoculated in utero as fetuses.

Bovine leukemia is believed to be caused by an oncogenic RNA virus designated bovine leukemia virus (BLV). In our laboratory, using electron microscopy, we have consistently demonstrated the presence of BLV particles in lymphocyte cultures from leukemic cattle and cattle with a persistent lymphocytosis. These particles were partially purified and highly concentrated by continuous flow, density gradient, ultracentrifugation to provide a potent inoculum as discussed above. The concentrated, cell free, BLV preparations were then used to inoculate 12 late stage bovine fetuses (in utero) and two newborn calves.

Our current study involves extensive monitoring of these inoculated animals to detect precancerous changes and obtain a detailed description of the events preceding the development of lymphosarcoma. Currently the following parameters are being evaluated: a) the serological titer against a bovine leukemia associated antigen; 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 peripheral blood lymphocytes coated with surface immunoglobulin; e) the complete blood count.

From our ongoing monitoring study we will have a complete record of all changes in the various leukemia associated parameters listed above. We will then be able to detail when, in what sequence, and to what extent each parameter changes in the course of lymphosarcoma development. Thus, if a single inoculated animal develops leukemia, its record will contribute significantly to our understanding of the early events in the pathogenesis of this disease.

Fourteen animals were successfully inoculated during the study. Eleven remain alive, and comprise the current monitoring program. All 11 of these animals are definitely infected with BLV, and in nine the infection has substantially progressed with respect to the parameters being monitored.

In addition to transmission and monitoring studies we have examined various lymphocyte subpopulations in an attempt to determine which cell type(s) are involved in the pathogenesis of bovine lymphosarcoma. These studies have conclusively established that B-lymphocytes are the target cells for BLV infection and that they carry the morphologic nuclear abnormality associated with this disease. Recently, subpopulation studies have led to the development of a marker for Fc receptors on bovine lymphocytes and to the development of a reproducible microculture assay for detecting and quantitating BLV.

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I. Current Fate of the 14 Inoculated Animals

Three animals (651, 653 and 654) have died. Animal 651 which, based on our monitoring studies had a steadily progressing BLV infection, died at 52 months of age. Gross and microscopic necropsy findings established that death was due to extensive necrosis and hemorrhage of the jejunum. No evidence of lymphosarcoma, bone marrow alterations or other pathologic changes could be found. The cause of the jejunal destruction could not be determined, however, the high level of BLV infection may have played a role, because recently a multitude of lymphosarcoma unrelated pathologies have been associated with feline leukemia virus infection in the cat.

The two other deaths occurred approximately one month after the birth of apparently healthy calves. Calf 653, inoculated 21 days prior to birth, died 36 days after birth. Calf 654, inoculated 30 days before birth, died 33 days after birth. The clinical, hematological, macroscopic and histopathological findings in these cases were essentially identical, and conclusively established that the calves died from severe bone marrow hypoplasia. We concluded that these unique cases were associated with BLV inoculation because:

- a) The remarkable case similarities strongly suggested a common etiology, and both calves had active BLV infections;
- b) Marrow hypoplasia is extremely infrequent in calves, yet, in this in utero transmission study, two out of six experimental animals died from this rare condition;
- c) Previously known inducers of marrow hypoplasia could not explain these cases;
- d) An etiologic association between

an oncornavirus and marrow hypoplasia has previously been demonstrated in the feline species. Complete details concerning these unique marrow hypoplasia cases and their association with BLV inoculation are contained in the accompanying preprint entitled, "In Utero Bovine Leukemia Virus Inoculation Associated with the Development of Fatal Bone Marrow Hypoplasia in Calves."

The 11 animals remaining on study are infected with BLV as determined by two different methods: a) The demonstration of BLV specific syncytia in the syncytia induction assay; b) The electron microscopic demonstration of C-type particles in short term PHA stimulated lymphocyte cultures. In nine of these animals (303, 338, 650, 655, 656, 657, 658, 660 and 661) changes in the monitored parameters have been substantial and currently indicate strong BLV infections. Recent monitoring of the other two animals (652 and 659) revealed only a low level of infection showing no signs of progressing.

II. Progress on Monitoring Studies

The monitoring proposed in past renewal requests has been carried out and we now have 130 values recorded for our youngest animal (661, 60 months old) and over 200 for our oldest (303, 88 months old). These extensive records on leukemia associated parameters such as viral production, nuclear pockets, B-cell percentages, serological titers and CBC's provide us with an excellent blood history for each animal and serve as a detailed baseline for detecting future changes.

III. Specific Changes in Monitored Parameters During the Past Three Years

For the purpose of this report values from 1977 and 1978 progress reports are tabulated along with those obtained during May, 1979 (Table I). This allows for observation of the trend for each animal as well as its current status.

Analysis of Table I

Percentage of Lymphocytic Nuclear Projections (% LNP'S)

We have shown that nuclear pockets occur with increased frequency in peripheral blood lymphocytes from lymphocytotic and leukemic cattle as opposed to normal animals.^{1,2} In a quantitative study of LNP'S in buffy coat cells from domestic Holstein-Friesian bulls, we observed that elevated LNP incidence (2% and above) was significantly correlated with the ability of an animal's lymphocytes to produce C-type, BLV particles in culture.³ In addition, budding BLV particles were observed on the cell surface of some lymphocytes containing nuclear pockets, indicating that LNP containing cells produce this virus.

Because of the relationships between LNP'S, BLV production, and bovine leukosis mentioned above, we have been monitoring this parameter in our inoculated animals. It was anticipated that a direct relationship between LNP frequency and BLV production would be observed. However, as shown in Table I, fluctuations in LNP incidence have not correlated well with BLV production during the past three years, and currently many animals (303, 655, 656, 657 and 658) show decreased levels of LNP'S while maintaining, or substantially increasing, their BLV production.

Table I

Comparison of 1977, 1978 and 1979 Blood Values for 14 Bovine
Leukemia Virus Inoculated Animals

Animal Number	Age Months	% LNP's ^a			% C-Type ^b			Serology Titer ^c			% B-Cells		
		77	78	79	77	78	79	77	78	79	77	78	79
303 ^d	88	2.1	3.0	2.0	24	33	38	128	128	128	36	42	42
338 ^d	64	1.1	0.7	0.5	7	18	8	128	32	8	42	21	33
650	73	2.1	1.4	0.4	5	25	7	128	128	32	15	32	28
651	Died at 52 months with extensive necrosis of the jejunum												
652	72	1.0	0.4	0.4	0	4	1	2	8	8	15	16	24
653	Died at one month from bone marrow hypoplasia (BLV related death)												
654	Died at one month from bone marrow hypoplasia (BLV related death)												
655	70	4.3	4.8	2.1	49	34	67	32	128	32	46	45	73
656	62	1.2	3.3	0.9	3	3	14	8	8	32	17	12	30
657	63	1.2	0.6	0.2	2	2	24	32	8	32	17	12	17
658	61	6.8	2.6	1.8	16	42	41	8	32	32	31	35	34
659	60	2.6	0.7	1.1	0	1	0	8	32	8	17	3	16
660	60	2.0	2.7	1.8	24	49	39	128	32	32	43	45	43
661	60	1.3	1.4	0.6	3	14	6	8	32	32	38	33	24

^a Percentage of lymphocytic nuclear pockets.

^b Percentage of cultured lymphocytes with C-type particles adherent to their surface.

^c Serological titer against bovine leukemia virus associated antigens.

^d 303 and 338 inoculated as newborn calves, all other animals inoculated as fetuses in utero.

Percentage of Cultured Lymphocytes with C-Type Particles Adherent to Their Surface (% C-Type)

In previous progress reports we have presented our evidence indicating that persistent lymphocytosis and the ensuing leukemic condition are the manifestations of infection with a C-type virus designated BLV. Consequently, throughout this study we have been quantitating the production of this virus. These values, obtained by electron microscopic examination of cultured lymphocytes as detailed in the accompanying preprint, serve as a standard measure of each animal's degree of infection. Cultures in which 5% or more of the lymphocytes show C-type particles adherent to their surface, are considered highly indicative of an active BLV infection.

From Table I it can be seen that: a) Nine of the 11 animals on study (all except 652 and 659) have active BLV infections. b) In four animals (303, 655, 656 and 657), the BLV infection level has increased substantially during the past year. c) In four animals (338, 650, 660 and 661), infection level has recently decreased. d) In one animal (655), the level of infection has reached an extreme (67%) previously not observed even in highly leukemic field cases.

Serological Titer Against Bovine Leukemia Associated Antigens

(Serology Titer)

In conjunction with the ongoing transmission study we have developed a serologic test for detecting and semi-quantitating antibodies directed against BLV associated antigens. This complement dependent immunofluorescence test is fully described in the accompanying preprint entitled, "In Utero Bovine Leukemia Virus Inoculation Associated with the Development of Fatal Bone Marrow Hypoplasia in Calves." In a preliminary study we found that

normal animals have no anti-BLV titer in this assay but infected animals, those whose mitogen stimulated lymphocytes produce BLV in culture, consistently show a titer of 1:8 or greater. Consequently, we now routinely determine the anti-BLV serotiter of all animals on study, both as a measure of infection, and to determine if fluctuations in titer can be correlated with changes in other parameters being monitored.

From Table I it can be seen that serologically all animals currently show strong evidence of BLV infection, each having a titer of 1:8 or greater. During the past year, however, only two animals (656 and 657) showed a serotiter increase. In five of the remaining animals (303, 652, 658, 660 and 661) serotiter remained unchanged, and four animals (338, 650, 655 and 659) showed a titer decrease. This year fluctuations in anti-BLV serotiter correlated well with increases and decreases in BLV production (338, 650, 656, 657 and 659). However, as in the past, the correlation was not absolute (655).

Percentage of Peripheral Blood Lymphocytes Coated with Surface Immunoglobulin (% B-Cells)

We have been studying the frequency of surface immunoglobulin, a B-lymphocyte marker, on peripheral blood lymphocytes obtained from normal cows and cows with a persistent lymphocytosis.⁴ In normal cows, approximately 28% of peripheral blood lymphocytes were identified as B-cells, whereas approximately 63% of peripheral blood lymphocytes from cows with persistent lymphocytosis demonstrated surface immunoglobulins. Thus, our results suggest that preleukemic lymphocytosis is due to an increase in

B-lymphocytes. Recently, we have further implicated B-lymphocytes in the pathogenesis of bovine lymphosarcoma by establishing that they are the target cells for BLV infection^{5,6} and that the lymphocytic nuclear pockets associated with BLV infection are also present only in the B-lymphocyte subpopulation.⁷

In our monitoring study we consider a persistently elevated B-cell percentage to be a reliable indicator of progression toward clinical lymphosarcoma and animals consistently showing B-cell percentages of 40% or more are regarded as being in a preleukemic state. From Table I, it can be seen that three animals (303, 655 and 660) can currently be classified as preleukemic on this basis. The others all remain in the normal range.

Absolute Lymphocyte Count (Not Shown on Table I)

It is widely accepted that persistent lymphocytosis represents an early precancerous stage in some, but not all, cases of bovine lymphosarcoma. By monitoring the complete blood count in our inoculated animals, we will be able to see if and when this sign develops in relation to changes in other parameters simultaneously being monitored. We believe that this study will demonstrate that reliable and superior indicators of impending lymphosarcoma appear prior to, and often instead of, the development of a persistent lymphocytosis.

During this study significantly elevated absolute lymphocyte values have occasionally been obtained on several of the animals under study. However, only a single animal (655) has remained lymphocytotic. In this case the lymphocytosis has persisted for over 44 months without progressing into clinical lymphosarcoma.

IV. Conclusions from the Monitoring Studies

The purpose of the monitoring studies is to compile extensive blood parameter records from birth until lymphosarcoma development, detailing all changes observed along the way, and then in retrospect to determine which parameter(s) best predicted ensuing lymphosarcoma development. It is also of great interest to see how the various parameters change in relation to each other throughout this time. Since no cases of lymphosarcoma have yet developed, the monitoring studies must continue in order to be successful and reach meaningful conclusions. However, because the latent period has extended beyond our expectation, we now propose that certain modifications be made. These changes, aimed at accelerating lymphosarcoma development, are discussed in the accompanying renewal request.

V. Additional Progress During the 1978 - 1979 Contract Year

Recently five papers resulting from contract supported bovine leukosis studies have been submitted for publication. The first entitled, "In Utero Bovine Leukemia Virus Inoculation Associated with the Development of Fatal Bone Marrow Hypoplasia in Calves," details the bone marrow hypoplasia deaths occurring early in this transmission study. The second entitled, "Long Term Stability of Blood Lymphocyte Nuclear Pocket Incidence in Clinically Normal Adult Dairy Bulls," deals with the stability over time of LNP incidence in individual animals. The third entitled, "Enzootic Bovine Leukosis: Incidence of Blood Lymphocyte Nuclear Pockets in United States and Foreign Dairy Bulls," concludes that nuclear pocket incidence is significantly

greater in the United States than in countries having active bovine leukosis surveillance programs. The fourth entitled, "Clinical Manifestations of Bovine Leukosis," reviews the clinical manifestations, diagnostic features of the different clinical forms and the relevant epidemiologic features of the disease. The fifth entitled, "Prevalence and Economics of Bovine Leukosis in the United States," concerns the prevalence of bovine leukosis in the United States and documents the economic significance of this disease. Preprints of each article accompany this report.

Preprints Removed

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