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

Progress Report for Period
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DISCLAIMER

ABSTRACT

The primary project objectives are to elucidate the cause(s) and early pathogenesis of the adult form of lymphosarcoma in cattle. This goal is to be accomplished through experimental transmission of the disease. For these studies large quantities of bovine leukemia virus (BLV) were propagated in short-term, mitogen stimulated, lymphocyte cultures. Cultures containing abundant BLV particles were pooled (33 liters total) and further processed by continuous flow, density gradient, ultracentrifugation. This highly concentrated, cell free, BLV preparation was then used as inoculum for 12 late stage bovine fetuses (inoculated in utero) and two newborn calves. Extensive monitoring studies have been carried out on these inoculated animals to detect precancerous changes and to obtain a detailed description of the events preceding the development of lymphosarcoma. These extensive records on lymphosarcoma associated blood parameters such as BLV production, nuclear pocket incidence, B-cell percentage, anti-BLV serology titer, and complete blood count have established that all of the inoculated animals became persistently BLV infected. However, after more than five years of incubation, no cases of lymphosarcoma developed. Consequently, during the past seven months, five of these well characterized animals have been subjected to frequent BLV re-exposure in order to study BLV-host interactions in previously infected adults and to potentially accelerate tumor formation in these animals.

PROGRESS REPORT 1979-1980

EXPERIMENTAL PLAN

Group Assignments -- The 11 animals currently on study were divided into three experimental groups (A, B and C). The five animals in group A received BLV infected, antigenically foreign lymphocytes. The three animals in group B received non-BLV infected, foreign lymphocytes. The three animals in group C received no inoculations and therefore served as controls. The actual group assignments are presented in Table 2.

Source of Lymphocytes for Inoculation -- Animals in group A (303, 652, 655, 656 and 658) received antigenically foreign lymphocytes from the best BLV producers identified during the May, 1979 blood monitoring study (see Table 2). Thus, animals 303, 652, 656 and 658 received lymphocytes from Jersey 655 and Jersey 655 received lymphocytes from Holstein 658. With the exception of animal 652, the lymphocyte recipient was of a different breed than the donor to maximize the antigenic foreignicity of the inoculated lymphocytes. Animals in group B (338, 657 and 658) received lymphocytes from a non-BLV infected, clinically normal, adult Jersey. This animal, maintained in the University of Minnesota dairy herd, has repeatedly been serologically negative for BLV infection. It was rechecked frequently during the study to assure that it remained negative.

Preparation of Lymphocytes from Donor Cattle -- Peripheral blood was obtained via jugular venipuncture and transferred to heparinized tube. Mononuclear cells were separated from red cells and granulocytes by centrifugation over Ficoll-Hypaque. Purified lymphocytes were then washed three times in Hanks' balanced salt solution and resuspended in saline for inoculation.

Processing and inoculation were performed on the same day to maximize lymphocyte viability. For the fifth, sixth and seventh inoculation of group A and B animals, the procedure was changed in that processed lymphocytes were cultured in the presence of 1% phytohemagglutinin M (PHA-M) for four hours prior to injection.

Inoculation Procedure -- Animals in groups A and B each received 16 x 10^6 lymphocytes per inoculation. This dose was evenly divided among 16 injection sites with 0.1 cc (1 x 10^6 lymphocytes) administered intradermally at each site. All injections were given in the withers region, eight on each side of the vertebral column.

Inoculation and Monitoring Schedule -- The inoculation and monitoring schedule for October, 1979 - May, 1980 is shown in Table 1. Animals in groups A and B were inoculated seven times during this period and all animals, including group C, evaluated twice. This evaluation included complete blood sampling to determine changes in the monitored parameters, and clinical examination to detect lymph node enlargements.

RATIONALE FOR 1979-1980 EXPERIMENTAL PLAN

The rationale for the 1979-1980 experimental plan is detailed in the accompanying renewal request and need not be duplicated here.

RESULTS THROUGH MAY, 1980

All lymphocyte processing, intradermal injections and blood analyses proposed for the October, 1979 - June, 1980 period have been successfully carried out. Complete blood analyses including BLV production, nuclear pocket incidence, anti-BLV serology titer, B-cell percentage and absolute lymphocyte count have been performed twice since the October, 1979 initiation of intradermal lymphocyte inoculation. The values obtained in December of 1979

and May of 1980, along with pre-experiment comparison values from May of 1979, are summarized in Table 2.

Table 1. Inoculation and Blood Monitoring Schedule for October, 1979 - May, 1980

Month	Procedure
October	Inoculate Animals in Groups A and B
November	Inoculate Animals in Groups A and B
December	Inoculate Animals in Groups A and B
December	Blood Analysis and Clinical Examination of All Animals
January	Inoculate Animals in Groups A and B
February	Inoculate Groups A and B with PHA-M Stimulated Lymphocytes
March	Inoculate Groups A and B with PHA-M Stimulated Lymphocytes
April	Inoculate Groups A and B with PHA-M Stimulated Lymphocytes
May	Blood Analysis and Clinical Examination of All Animals

Analysis of Table 2

Bovine Leukemia Virus (BLV) Production

In previous progress reports we have presented evidence indicating that persistent lymphocytosis and lymphosarcoma are manifestations of infection with a C-type RNA 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, 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 2 it can be seen that: a) Group A animals showed a substantial decrease in BLV production. b) Group B animals remained essentially unchanged with respect to BLV production. c) Animals in group C remained unchanged with the exception of 660 which showed sharply decreased viral production. d) All animals previously considered inactive ($< 5\%$ BLV production) remained so regardless of group assignment.

Table 2. Summary of Blood Values in Groups A, B and C Animals Before and After Animals in Group A and B Received Foreign Lymphocytes

	Animal			BLV Production[b]			LNP Incidence[c]			RIA-BLV Glycoprotein Titer[d]			B-Cell Percentage			Absolute Lymphocyte Count x 10^3		
				Before	After		Before	After		Before	After		Before	After		Before	After	
	Number	Sex	Breed[a]	5/79	12/79	5/80	5/79	12/79	5/80	5/79	12/79	5/80	5/79	12/79	5/80	5/79	12/79	5/80
Group A	303	F	H	38	25	21	2.0	1.5	3.4	3600	2560	3360	40	25	29	3.3	3.9	3.4
Received	652	M	J	1	1	1	0.4	0.6	1.2	1250	1280	2600	-	3	6	2.6	3.7	2.9
BLV Infected	655	F	J	67	41	17	2.1	4.0	2.9	6500	5120	4850	52	42	66	7.9	10.5	9.8
Lymphocytes	656	F	H	14	15	6	0.9	2.2	2.5	5400	3540	2905	24	6	23	4.1	3.6	3.0
	658	M	H	41	27	9	1.8	1.8	1.9	2560	1600	1550	34	12	19	4.4	4.0	4.0
Group B	338	M	H	8	7	6	0.5	0.6	1.6	9500	8250	6050	24	13	10	3.1	3.3	3.2
Received	657	M	H	2	0	1	0.2	0.5	0.9	1065	1750	1765	10	4	18	4.1	4.0	3.3
Non-BLV	659	F	H	0	2	2	1.1	0.1	1.1	3250	3000	2340	12	5	5	4.7	4.2	4.0
Infected Lymphocytes																		
Group C	650	M	J	7	10	9	0.4	0.7	1.7	8500	6125	9000	25	6	38	4.7	3.6	2.9
Received	660	F	H	39	25	15	1.8	1.3	2.2	6850	3800	4750	48	27	54	4.8	3.4	5.6
No Inoculum	661	M	H	6	8	7	0.6	2.1	3.3	1350	1550	1475	22	8	16	3.5	3.9	3.6

[a] H = Holstein, J = Jersey

[b] Percentage of cultured lymphocytes with BLV particles adherent to their surface.

[c] Percentage of buffy coat lymphocytes containing lymphocytic nuclear pockets.

[d] Radioimmunoprecipitin assay using ^{125}I-labeled BLV glycoprotein antigen.

Lymphocytic Nuclear Pocket (LNP) Incidence

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 lymphocytes 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.[4] Because of the relationships between LNP's, BLV production and bovine leukosis, we have been monitoring this parameter in all inoculated animals.

From Table 2 it can be seen that with the exception of animal 659, all animals showed increased LNP incidence. Those animals showing a substantial ($\triangleright$ 1%) increase were evenly divided between the three experimental groups with two animals (303 and 656) in group A, one (338) in group B and two (650 and 661) in group C.

Radioimmunoprecipitin Assay (RIA) Titers Against BLV-Glycoprotein Antigen

In conjunction with the ongoing transmission study we developed a serologic test for detecting and semi-quantitating antibodies directed against BLV associated antigens. This complement dependent immunofluorescence test is used as a measure of immune response against BLV infection and to determine if fluctuations in serotiter can be correlated with changes in the other parameters being monitored. Recently, however, a radioimmunoassay using ^{125}I-labeled BLV-glycoprotein became available. This highly sensitive, quantitative, serologic test is kindly being provided by Dr. Mary Jo Schmerr,

National Animal Disease Center, Ames, Iowa. Because of its much greater sensitivity, this RIA assay replaces our immunofluorescence test for quantitating serotiter changes in the experimental animals.

From Table 2 it can be seen that serotiter against BLV glycoprotein antigen has remained extremely constant in all animals during the past year. In fact, only a single animal has shown as much as a two-fold variation in serotiter during this time. This group A animal (652) developed a moderate (2.08 fold) serotiter increase after receiving intradermal inoculations with BLV infected lymphocytes. However, a slight decrease in serotiter was observed in the other group A animals showing active BLV production.

B-Cell Percentage

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.[5] In normal cows, approximately 28% of peripheral blood lymphocytes were identified as B-cells, whereas approximately 63% of the peripheral blood lymphocytes from cows with persistent lymphocytosis demonstrated surface immunoglobulins. Thus, our results suggested 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[6,7] and that the lymphocytic nuclear pockets associated with BLV infection are also present only in the B-lymphocyte subpopulation.[8] 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 2 it can be seen that most animals, irrespective of group assignment, showed some decrease in B-cell percentage during the past year. Only animals 655 from group A and 650 from group C showed a substantial ($>$ 10%) increase in this parameter. Two animals, 655 and 660 would currently be classified as preleukemic on the basis of their B-cell percentage.

Absolute Lymphocyte Count

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.

From Table 2 it can be seen that absolute lymphocyte counts have remained relatively stable with only animal 655 showing a substantial increase. Lymphocytosis has persisted for over 55 months in this animal with no clinical signs of lymphosarcoma.

Clinical Observations

Clinical signs of lymphosarcoma have not been observed in any of the experimental, BLV inoculated, animals as of May, 1980.

Conclusions from the Blood Value Monitoring Data Presented in Table 2

The lack of substantial blood value increases in group A animals during the past seven months suggests that repeated inoculations with BLV infected lymphocytes has had little effect. However, our monitoring detects only systemic changes reflected in the blood and does not reveal local changes in

the microenvironment. Given the extremely long latent period associated with naturally occurring bovine lymphosarcoma, such systemic changes cannot be expected to occur rapidly. Consequently, we will not try to draw conclusions at this time but will instead continue with the inoculations and monitoring as proposed in the accompanying renewal request.

ADDITIONAL PROGRESS DURING THE 1979-1980 CONTRACT YEAR

Recently four papers resulting from contract supported bovine leukosis studies have been published. The first 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 second entitled, "Enzootic Bovine Leukosis: Incidence of Blood Lymphocyte Nuclear Pockets in United States and Foreign Dairy Bulls," concludes that nuclear pockets incidence is significantly greater in the United States than in countries having active bovine leukosis surveillance programs. The third 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 fourth 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. Reprints of each article accompany this report.

PERCENTAGE OF PRINCIPAL INVESTIGATORS TIME

The principal investigator expects to devote approximately 15% of his time on a 12 month basis to carry out this project.

REFERENCES

1. Weber A, Andrews J, Dickinson B, et al: Occurrence of nuclear pockets in lymphocytes of normal, persistent lymphocytotic and leukemic adult cattle. J Natl Cancer Inst 43: 1307-1315, 1969.

2. Weber A, Bendixen HJ, Hammer RF, et al: Correlative studies of the frequency of blood lymphocytic nuclear pockets and persistent lymphocytosis in cattle. Am J Vet Res 35: 537-541, 1974.

3. Weber A, Fahning M, Hammer RF, et al: Relation between the presence of nuclear pockets in bovine peripheral blood lymphocytes and C-type virus particle incidence in cultures of these cells. J Natl Cancer Inst 51: 81-88, 1973.

4. Weber AF, Pomeroy KA, Dias E: Long-term stability of blood lymphocyte nuclear pocket prevalence in clinically normal adult dairy bulls. Am J Vet Res 41: 170-173, 1980.

5. Muscoplat CC, Johnson DW, Pomeroy KA, et al: Lymphocyte surface immunoglobulin: Frequency in normal and lymphocytotic cattle. Am J Vet Res 35: 593-595, 1974.

6. Paul PS, Pomeroy KA, Johnson DW, et al: Evidence for the replication of bovine leukemia virus in the B-lymphocytes. Am J Vet Res 38: 873-876, 1977.

7. Paul PS, Pomeroy KA, Castro AE, et al: Detection of bovine leukemia virus in B-lymphocytes by the syncytia inducation assay. J Natl Cancer Inst 59: 1269-1272, 1977.

8. Pomeroy KA, Paul PS, Weber AF, et al: Evidence that B-lymphocytes carry the nuclear pocket abnormality associated with bovine leukemia virus infection. J Natl Cancer Inst 59: 281-283, 1977.