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

PROGRESS REPORT NO. 17 TO THE U.S. ENERGY RESEARCH AND DEVELOPMENT ADMINISTRATION ON RESEARCH PERFORMED UNDER CONTRACT EY-76-S-02-0910 COVERING THE 15 MONTH PERIOD JULY, 1976- OCTOBER, 1977

Principal Investigator

D.K. Sorensen, Department of Large Animal Clinical Science

Co-Investigators

K.A. Pomeroy, Department of Large Animal Clinical Science
V.L. Larson, Department of Large Animal Clinical Science
A.F. Weber, Department of Veterinary Biology
J.B. Stevens, Department of Veterinary Pathobiology

College of Veterinary Medicine
University of Minnesota
St. Paul, Minnesota 55108

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DISCLAIMER

PROJECT ABSTRACT

The primary objective of the proposed research will be elucidation of the etiology and pathogenesis of bovine leukemia. We have consistently demonstrated C-type particles in mitogen stimulated lymphocyte cultures from leukemic cows and cows with a persistent lymphocytosis. These particles have been concentrated and partially purified by continuous flow, density gradient, ultracentrifugation. Newborn calves and late stage bovine fetuses have been inoculated with these concentrated cell free preparations. Our current study involves extensive monitoring of these inoculated animals to detect early pre-cancerous changes. The following parameters are being measured: 1) The serological titer against a bovine leukemia associated antigen. 2) The percentage of lymphocytes showing nuclear pockets. 3) The percentage of mitogen stimulated lymphocytes with C-type particles adherent to their surface. 4) The percentage of B-lymphocytes in the peripheral circulation. 5) The complete blood count. 6) The quantity of bovine leukemia virus (BLV) production as determined by the syncytia induction assay. Additional proposals include: a) Using the monitoring parameters to study animals with the juvenile and thymic forms of leukemia. b) The examination of adult lymphosarcoma cases to determine which tissues harbor BLV. c) Lymphocyte subpopulation work to further define which cell types are associated with BLV production and tumor formation.

GENERAL SUMMARY OF PROGRESS DURING THE 1976 - 1977 CONTRACT PERIOD

I. Progress on Monitoring Studies

1) All of the monitoring proposed in our 1976 - 1977 renewal request has been carried out.

2) Considering all of the data collected over the years on each of the parameters being studied, we believe that 8 of the 12 animals inoculated (303, 338, 650, 651, 655, 658, 660 and 661) are infected with BLV and that their infection is definitely progressing. Three of the inoculated animals (656, 657 and 659) are definitely infected with BLV but their infection has not progressed as rapidly as the others. One animal (652) is consistently in the normal range on all parameters and appears not to be infected at this time. In the accompanying renewal request we propose to eliminate this animal from the monitoring studies.

II. Progress on Proposals not Directly Related to Our Transmission Studies

1) A microculture assay for the detection of bovine leukemia virus (BLV) has been established and in most instances now replaces the cumbersome electron microscope assay previously used for detection and quantitation of BLV.

2) By separating peripheral blood lymphocytes into B-enriched and B-depleted subpopulations we established that the B subpopulation contains the target cells for BLV infection. In addition, we determined that the B subpopulation contains the nuclear pocket abnormality associated with BLV infection.

3) We developed a marker for Fc receptors on bovine lymphocytes and used it to determine the frequency of lymphocytes bearing Fc receptors in normal cattle, cattle with persistent lymphocytosis and cattle with confirmed lymphosarcoma.

DETAILED PROGRESS REPORT

This report summarizes the proposals made for the 1976-1977 contract period and describes our progress through June of 1977. 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 unnecessary repetition, the materials and animals used in our studies are described only in the accompanying renewal request.

For the contract year 1976-1977 our major proposal was to extensively monitor animals inoculated during prior contract periods. The proposed testing frequency for most parameters was monthly or bimonthly. We proposed to monitor and record changes in the following parameters: a) The serological titer against what we call the bovine leukemia associated antigen; b) The percentage of lymphocytes showing nuclear pockets; c) The percentage of lymphocytes in the peripheral blood coated with surface immunoglobulin; d) The complete blood count. In addition, every three months the percentage of cultured lymphocytes with C-type particles adherent to their surface will be determined.

The proposed monitoring has been carried out on 12 animals. Two of these animals (303 and 338) were inoculated shortly after birth. The remaining animals were all inoculated as fetuses in utero. Detailed results of our monitoring studies will be published when they are completed. For the purpose of this progress report, however, the February 1976 and most recent

Table I

Summary of February 1976 and Most Recent 1977 Samples

Animal	Age Months	% LNP's		%C-Type		Serology Titer		% SIg + Cells	
		76	77	76	77	76	77	76	77
303	64	3.3	2.1	18	24	32	128	44	36
338	40	1.8	1.1	8	7	32	128	22	42
650	48	1.5	2.1	1	5	32	128	14	15
651	48	3.6	4.6	24	31	32	32	37	21
652	48	1.1	1.0	0	0	2	2	17	15
653	Died at one month with aplastic anemia								
654	Died at one month with aplastic anemia								
655	46	4.1	4.3	34	49	32	32	49	46
656	38	3.8	1.2	7	3	8	8	10	17
657	30	0.5	1.2	3	2	2	32	11	17
658	37	2.6	6.8	5	16	32	8	3	31
659	36	2.1	2.6	4	0	2	8	17	17
660	36	2.0	2.0	28	24	128	128	24	43
661	35	2.2	1.3	1	3	2	8	22	38

1977 samples are summarized and compared. This allows for evaluation of the animals current status and changes during the past year. This data is presented in Table I.

ANALYSIS OF TABLE I

PERCENTAGE OF LYMPHOCYTIC NUCLEAR PROJECTIONS (% LNP'S)

Nuclear pockets have been shown to occur with increased frequency in peripheral blood lymphocytes of lymphocytotic and leukemic cattle as opposed to normal animals.[1,2] From our ongoing transmission experiments and a study of 50 Holstein bulls[3] we have established that the percentage of peripheral blood lymphocytes showing nuclear pockets is directly correlated with C-type viral infection. In fact, animals can be classified into three groups on the basis of lymphocytic nuclear pocket (LNP) evaluations. Animals showing 1.5% or greater LNP's are considered definitely infected and in all cases mitogen stimulated cultures of their lymphocytes have produced C-type particles. Animals with 0.5% to 1.5% are classed as suspicious for infection with mitogen stimulated cultures of their lymphocytes generally but not always producing low numbers of C-type particles. Animals with less than 0.5% LNP's are not infected and show no significant C-type particle production. Thus, with respect to Table I LNP values, our 12 inoculated animals can currently be classified as follows:

Definitely Infected: 303, 650, 651, 655, 658, 659 and 660

Suspect for Infection: 338, 652, 656, 657, 661

Negative for Infection: None

SUMMARY OF % LNP CHANGES DURING THE PAST YEAR

Most animals classified as definitely infected in February of 1976 retain this classification and show an increase in their LNP percentages indicating that infection and pathogenesis are definitely progressing in these animals (650, 651, 655, 658 and 659).

Two animals (303 and 660) remain in the definitely infected category but do not show an increase in LNP values.

The two animals classed as suspect for infection last year (652 and 657) remain suspects again this year.

Three animals previously classified as definitely infected (338, 656 and 661) have regressed in terms of LNP values and would currently be classified as suspects for infection by this parameter.

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. We believe that it is important to determine when these particles appear and how their quantity relates to changes in the other parameters we are monitoring. We therefore proposed to determine the percentage of PHA stimulated lymphocytes with C-type particles adhering to their surface (C-type cells). Standard electron microscopic techniques were used in this evaluation.

From previous field studies we have established that C-type particle production by cultured lymphocytes can be used to classify animals as definitely infected (5% or greater C-type cells), suspect for infection (1% - 4% C-type cells), and negative for infection (0% C-type cells). Thus

from Table I the animals comprising our transmission studies would currently be classified as follows:

Definitely Infected: 303, 338, 650, 651, 655, 658, and 660

Suspect for Infection: 656, 657 and 661

Negative for Infection: 652 and 659

SUMMARY OF % C-TYPE CHANGES DURING THE PAST YEAR

Most animals classified as definitely infected on the basis of C-type particle production in February of 1976 retained their definitely infected status in the most recent sampling (303, 338, 651, 658 and 660). The single exception is animal 656 which now falls into the suspect category.

Many of the definitely infected animals and some in the suspect category show an increase in viral production over that observed last year indicating that their infection is progressing (303, 650, 651, 655, 658 and 661).

In four animals (303, 651, 655 and 660) the % C-type cells is very high (24 to 49%) being in excess of the percentage usually observed in confirmed cases of bovine leukemia where we often see about 20% C-type cells.

Two animals (652 and 659) would currently be considered negative for infection as no BLV production can be demonstrated.

SEROLOGICAL TITER AGAINST BOVINE LEUKEMIA ASSOCIATED ANTIGENS

(Serology Titer)

Recently several methodologically different serological tests for the detection of bovine leukemia associated antigens or antibodies directed

against these antigens have been reported.[4,5,6] Recently we have significantly improved our own method for detecting and quantitating these antibodies. Using our test, uninfected animals consistently show no antibody titer while lymphocytotic and leukemic animals show titers up to 1:128.

With animals under study in our transmission experiments there has been a good correlation between changes in antibody titer and the other parameters we are simultaneously monitoring. From our preliminary data, it appears that animals with a titer of 1:8 or greater should be classified as definitely infected according to our nuclear pocket and C-type viral production criteria, while animals with a titer of 1:2 would be classified as suspicious for infection. If these observations are further confirmed during our monthly monitoring, it appears that the time consuming and relatively expensive nuclear pocket and viral production tests may be replaced by this fact inexpensive immunological test.

In accordance with the criteria detailed above, the animals in our transmission studies can currently be serologically classified as follows:

Definitely Infected: 303, 338, 650, 651, 655, 656, 657, 658, 659, 660 and 661

Suspect for Infection: 652

Negative for Infection: None

SUMMARY OF SEROLOGY TITER CHANGES DURING THE PAST YEAR

During the past year three animals (657, 659 and 661) have converted from serologically suspect for infection to definitely infected. This leaves only a single animal (652) not classed as definitely infected with respect to serological titer.

The serological titers of 6 animals (303, 338, 650, 657, 659 and 661) have increased over the last years and three animals (651, 655 and 660) have maintained a previously high titer suggesting that these animals have active infections with persistent BLV antigenic stimulation.

PERCENTAGE OF PERIPHERAL BLOOD LYMPHOCYTES COATED WITH SURFACE IMMUNOGLOBULIN (%SIg + 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.[7] 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 and in our monitoring studies we consider animals showing over 35% SIg+ cells or demonstrating a progressive increase in the percentage of SIg+ cells as suspects for BLV infection.

SUMMARY OF % SIg CHANGES DURING THE PAST YEAR

From Table I it can be seen that most of the animals under study currently show 35% SIg+ cells and/or have demonstrated an increase in the percent of SIg+ cells during the past year (303, 338, 650, 655, 656, 657, 658, 660 and 661). We consider this as additional evidence that these animals are infected with BLV and that their infection is progressing.

ABSOLUTE LYMPHOCYTE COUNT (Not shown on Table I)

It is widely accepted that persistent lymphocytosis represents an early pre-cancerous stage of bovine leukemia. By monitoring the complete blood count in all of our inoculated animals, we will be able to see when this sign develops in relation to changes in the other parameters we are simultaneously monitoring. This, we believe, will aid in demonstrating that reliable and better indications of bovine leukemia appear prior to the development of a persistent lymphocytosis.

SUMMARY OF ABSOLUTE LYMPHOCYTE COUNT CHANGES DURING THE PAST YEAR

On occasion, significantly elevated absolute lymphocyte values have been obtained on several of the animals under study. However, only animal 655 has demonstrated what we consider a persistent lymphocytosis.

PROGRESS ON PROPOSALS NOT DIRECTLY RELATED TO OUR TRANSMISSION STUDIES

In addition to the monitoring of all inoculated animals during the 1976-1977 contract period we also proposed the following:

1) To establish and modify for use in our laboratory a syncytial induction assay for the detection and quantiation of BLV.

2) To study lymphocyte subpopulations to determine which cell type is responsible for the production of C-type BLV particles.

Syncytial Induction Assay

During the past contract year considerable effort was expended working with and eventually modifying the BLV infectivity assay orginally described by Ferrer and Diglio.[8,9] The result has been the development of a reproducible microculture assay for the detection and titration of BLV. Our

microassay need not be detailed here because it is fully described in the accompanying preprint entitled "A Microculture Assay for Bovine Leukemia Virus." In addition, we have used the microculture syncytia assay for the detection of BLV in some of our lymphocyte subpopulation studies. An example of such use appears in the accompanying preprint entitled "Detection of Bovine Leukemia Virus in B-Lymphocytes by the Syncytia Induction Assay." It now appears likely that the microculture BLV assay will replace much of the quantitative work previously achieved only through use of the electron microscope.

Lymphocyte Subpopulation Studies

During the past contract year we have separated the peripheral blood lymphocytes of several BLV-infected cows with persistent lymphocytosis into B-enriched and B-depleted lymphocyte populations by fractionation through nylon-wool columns. The separated subpopulations were either cultured and examined for BLV by electron microscopy or examined for BLV by the syncytia induction microculture assay. BLV was present only in B-enriched populations and not in B-depleted populations.

Thus our studies established that B-lymphocytes are the target cells for BLV infection. Additionally, we established that the lymphocytic nuclear pockets associated with BLV infection are also contained only in the B-enriched lymphocyte subpopulation. A complete description of our methods and results can be found in the following reprint and preprints accompanying this report: a) "Evidence for the Replication of Bovine Leukemia Virus in the B-Lymphocytes." b) "Brief Communication: Evidence that B-Lymphocytes Carry

the Nuclear Pocket Abnormality Associated with Bovine Leukemia Virus Infection." c) "Detection of Bovine Leukemia Virus in B-Lymphocytes by the Syncytia Induction Assay."

Other lymphocyte subpopulation work involved the development of a marker for Fc receptors on bovine lymphocytes. This fluoresceinated, heat aggregated human immunoglobulin marker was then used to determine the frequency of lymphocytes bearing Fc receptors in normal cattle, BLV-infected cattle with persistent lymphocytosis and BLV-infected cattle with confirmed lymphosarcoma. The methods developed and results obtained during this study are described in the accompanying preprint entitled "Frequency of Lymphocytes Bearing Fc Receptors and Surface Membrane Immunoglobulins in Normal, Persistent Lymphocytotic and Leukemic Cows."

PUBLICATIONS DURING THE 1976 - 1977 CONTRACT PERIOD

1. Paul, P.S., Pomeroy, K.A., Johnson, D.W., Muscoplat, C.C., Handwerger, B.S., Soper, F.F., and Sorensen, D.K. Evidence for the Replication of Bovine Leukemia Virus in the B-Lymphocytes. Am. J. Vet. Res. 38: 873-876, 1977.

2. Pomeroy, K.A., Paul, P.S. Weber, A.F., Sorensen, D.K., and Johnson, D.W. Brief Communication: Evidence that B-Lymphocytes Carry the Nuclear Pocket Abnormality Associated with Bovine Leukemia Virus Infection. J. Nat. Cancer Inst. In Press.

3. Paul, P.S., Pomeroy, K.A., Castro, A.E., Johnson, D.W., Muscoplat, C.C., and Sorensen, D.K. Detection of Bovine Leukemia Virus in B-Lymphocytes by the Syncytia Induction Assay. J. Nat. Cancer Inst. In Press.

4. Kumar, P.S., Paul, P.S., Pomeroy, K.A., Johnson, D.W., Muscoplat, C.C., Van Der Maaten, M.J., Miller, J.M., and Sorensen, D.K. Frequency of Lymphocytes Bearing Fc Receptors and Surface Membrane Immunoglobulins in Normal, Persistent Lymphocytotic and Leukemic Cows. Accepted for publication in Am. J. Vet. Res.

5. Paul, P.S., Castro, A.E., Pomeroy, K.A., Johnson, D.W., Muscoplat, C.C., and Bronson, D.L. A Microculture Assay for Bovine Leukemia Virus. Submitted for publication in Archives of Virology.

PAPERS PRESENTED AT CONFERENCES DURING THE 1976 - 1977 CONTRACT PERIOD

1. Evidence That B-Lymphocytes Carry the Nuclear Pocket Abnormality Associated with Bovine Leukemia. Presented by K.A. Pomeroy at the 57th Annual Meeting of the Conference of Research Workers in Animal Disease, Pick-Congress, Chicago, Illinois, November 29-30, 1976.

2. Evidence for the B-Lymphocyte as a Progenitor for Bovine Leukemia Virus. Presented by P.S. Paul at the 57th Annual Meeting of the Conference of Research Workers in Animal Disease, Pick-Congress, Chicago, Illinois, November 29-30, 1976.

3. Current Status - Bovine Leukemia. Presented by D.K. Sorensen at the Public Health Conference for Veterinarians, Univeristy of Minnesota, St. Paul, Minnesota, April 27, 1977.

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4. Miller, J.M., and Olson, C , Precipitating Antibody to an Internal Antigen of the C-Type Virus Associated with Bovine Lymphosarcoma. J. Nat. Cancer Inst. 49: 1459-1462, 1972.

5. Ferrer, J.F., Avila, L., and Stock, N.D., Serological Detection of Type C Viruses Found in Bovine Cultures. Cancer Res. 32: 1864-1870, 1972.

6. Miller, J.M., and Van Der Maaten, M.J., A Complement-Fixation Test for Bovine Leukemia (C-Type) Virus. J. Nat. Cancer Inst. 53: 1699-1702, 1974.

7. Muscoplat, C.C., Johnson, D.W., Pomeroy, K.A., Olson, J.M., Larson, V.L., Stevens, J.B., and Sorensen, D.K., Lymphocyte Surface Immunoglobulin: Frequency in Normal and Lymphocytotic Cattle. Am. J. Vet. Res. 35: 593-595, 1974.

8. Ferrer, J.F., and Diglio, C.A., Development of an In Vitro Infectivity Assay for the C-Type Bovine Leukemia Virus. Cancer Res. 36: 1068-1073, 1976.

9. Diglio, C.A., and Ferrer, J.F., Induction of Syncytia by the Bovine C-Type Leukemia Virus. Cancer Res. 36: 1056-1067, 1976.