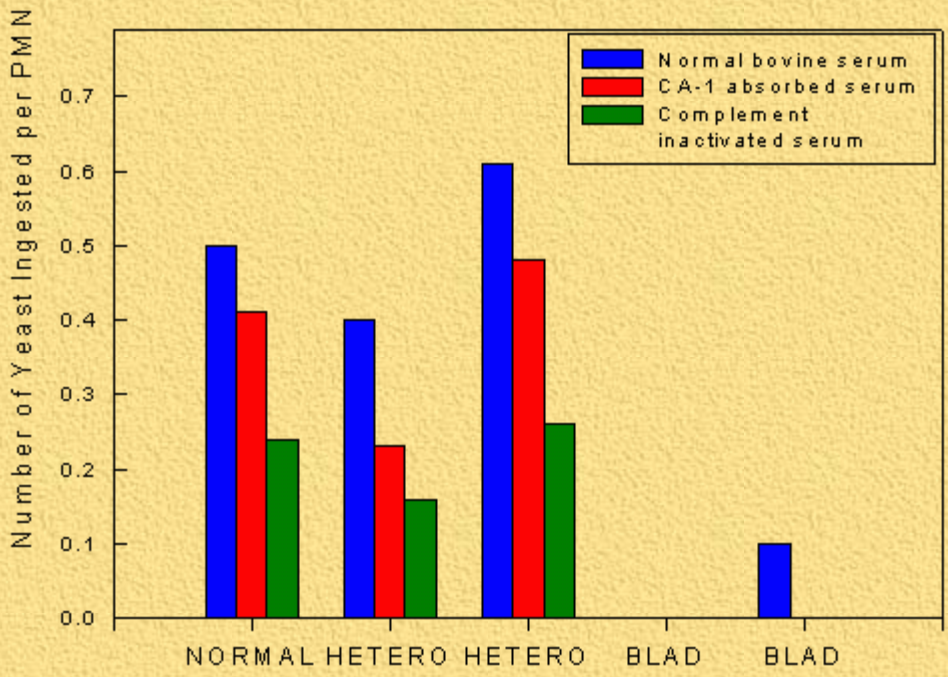
Surface Markers on Bovine Neutrophils

Ab Name	Binding Site	Normal	Heterozygous	Homozygous
GD 3.1	γδ T-cell	-	-	-
GD 197	γδ T-cell	-	-	-
GD 3.5	γδ T-cell	-	-	-
GD 3.8	γδ T-cell	-	-	-
GD 6-10	CD 45	+	+	+
GD 4-22	Bovine L-selectin	+	+	+
CC 17	Lymphocytes CD 5	-	-	-
CC 30	CD 4	-	-	-
CC 58	CD 8	-	-	-
CC 42	CD 2	-	-	-
CC 21	B-cells	-	-	-
Hermes 3	CD 44	+	+	+
GD 121	Unknown	+	+	-
R 15.7	CD 18	+	+	-
MHM 23	CD 18	+	+	-
IB4	CD 18	+	+	-
MM12A	CD 11b	+	+	-

Surface Markers on Bovine Lymphocytes

Ab Name	Binding Site	Normal	Heterozygous	Homozygous
GD 3.1	γδ T-cell	+	+	+
GD 197	γδ T-cell	+	+	+
GD 3.5	γδ T-cell	+	+	+
GD 3.8	γδ T-cell	+	+	+
GD 6-10	CD 45	+	+	+
GD 4-22	Bovine L-selectin	+	+	+
CC 17	Lymphocytes CD 5	+	+	+
CC 30	CD 4	+	+	+
CC 58	CD 8	+	+	+
CC 42	CD 2	+	+	+
CC 21	B-cells	+	+	+
Hermes	CD 44	+	+	+
GD 121	Unknown	+	+	-
R 15.7	CD 18	+	+	-
MHM 23	CD 18	+	+	-
IB4	CD 18	+	+	-
MM12A	CD 11b	+	+	-

Phagocytosis Assay



All samples were run in duplicate. A 500µl aliquot of PMN at 5.4 x 10^6PMNs/ml was added to tissue culture treated coverslips inside the wells of a 6 well tissue culture plate. The PMNs were allowed to adhere for 30 min to 1 hr at 37^{0}C. The RPMI was removed and 500µl of FITC labeled yeast in RPMI + 10% normal bovine serum, complement inactivated serum, or CA-1 absorbed serum prewarmed to 37^{0}C was added. Serum was complement inactivated at 56^{0}C for 30 min. CA-1 absorbed serum was produced by mixing 10 parts of serum with 1 part of packed formalin killed CA-1 yeast cells, 10 min on ice. Yeast cells were pelleted out and the supernatant removed and absorbed two more times. PMNs and yeast were allowed to interact for 1 hr at 37^{0}C, stationary. Cold 1% paraformaldehyde was added to each well to stop the interaction. The FITC on yeast cells not ingested was quenched using 2mg/ml trypan blue in 0.02M citrate buffer containing 0.15M NaCl, pH 4.4. Trypan blue does not penetrate the PMNs so that ingested yeast can be differentiated from attached yeast by using fluorescent microscopy.

Neutrophil Differential Count

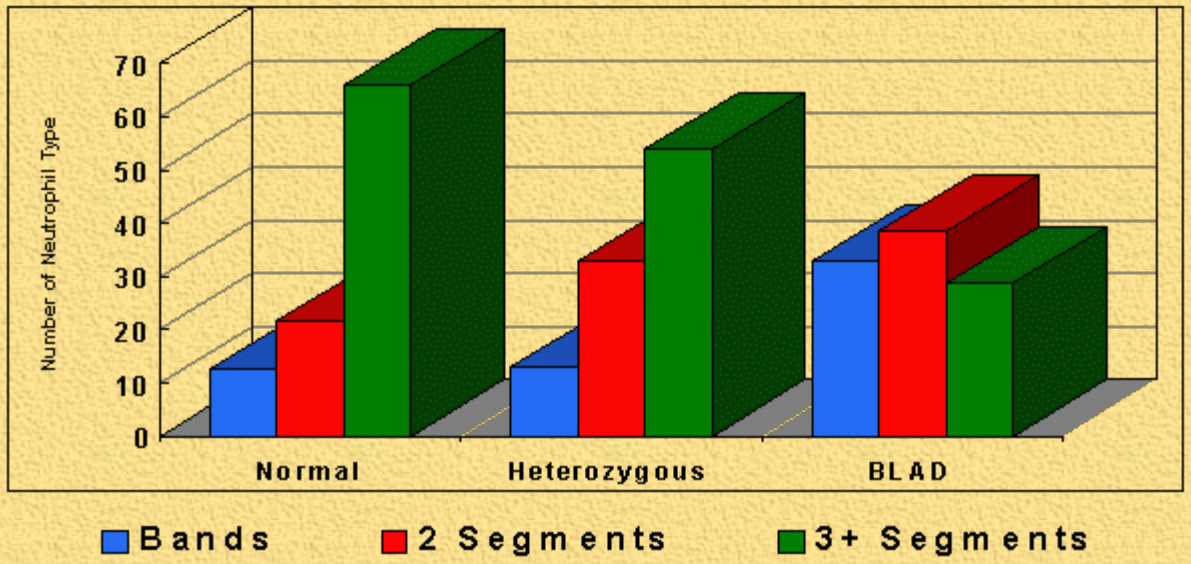


FIGURE 4) Duplicate whole blood smears were prepared from normal, heterozygous and homozygous BLAD calf specimens. Dried smears were stained with Wright's stain, and differential counts were performed on the neutrophil population. Neutrophils were scored as bands if no nuclear segments were visible; 2 segments if the nucleus had two segments; and 3+ segments if the nucleus had at least three segments.

Oxidant Production Measured by DHR in a Flow Cytometric Analysis

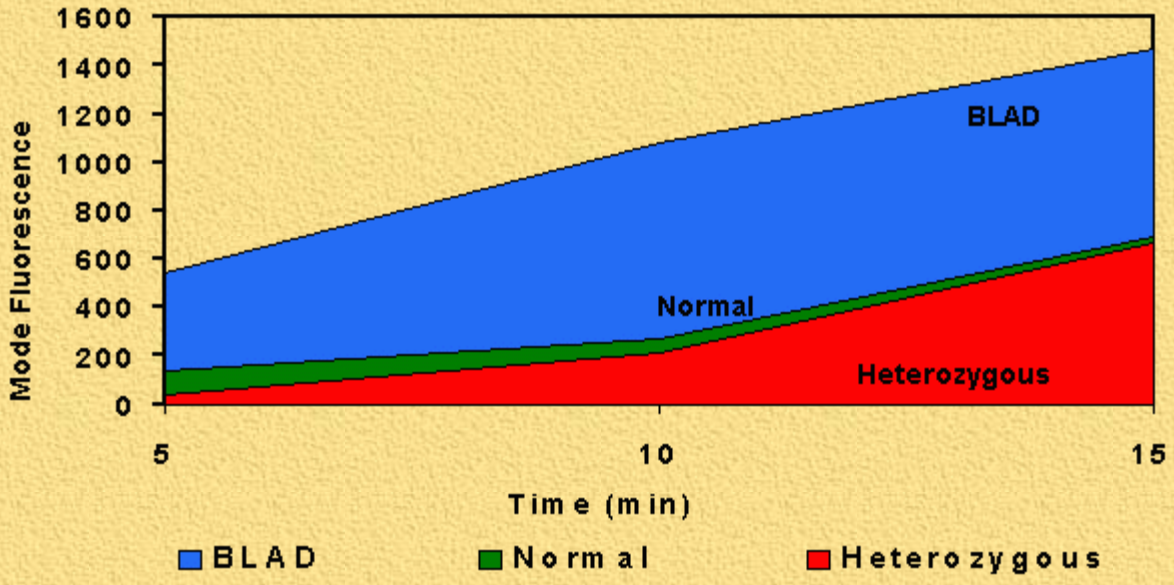


FIGURE 5) Bovine neutrophils from normal, heterozygous and homozygous BLAD calves were loaded with dihydrorhodamine 123 (DHR) at 370 C for 5 minutes. These neutrophils were activated with PMA at 370 C, and samples were taken at various time intervals and placed on ice. The DHR fluorescence was measured with a flow cytometer, and the mode fluorescence of each sample is plotted verses time.

G00040

Enhanced NADPH Oxidase Activity in Neutrophils Isolated from Cattle with Bovine Leukocyte Adhesion Deficiency

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Abstract:
Neutrophils isolated from cattle with bovine leukocyte adhesion deficiency (BLAD) provide a unique opportunity to study the role of beta 2 integrins in different aspects of neutrophil function. Flow cytometric analysis of leukocytes isolated from normal calves, heterozygous calves, and homozygous BLAD calves revealed comparable surface molecules on all calves regardless of genotype or duration between collection and leukocyte isolation, with the exception of the beta 2 integrin molecules which were absent from homozygous BLAD leukocytes, as expected. Consistent with the absence of beta 2 integrins, BLAD neutrophils were unable to phagocytose opsonized *C. albicans*, while cells from normal and heterozygous calves phagocytosed and killed *C. albicans* normally. Reactive oxygen metabolites were measured on all three genotypes of calves using various stimuli and two different measurement protocols. Surprisingly, homozygous BLAD neutrophils stimulated with PMA or E. coli showed enhanced NADPH oxidase activity in a cytochrome c assay compared to normal and heterozygous calves. Dihydrorhodamine 123 fluorescence was also increased in the homozygous BLAD neutrophils when measured using a fluorescent microtiter plate reader or by flow cytometry after stimulation with PMA or E. coli. These findings suggest that the beta 2 integrins may play a regulatory role in the respiratory burst. NIH 5T32AIO7465-02.

Introduction:

The genetic absence of beta-2 integrins have been shown to be the causative agent in human leukocyte adhesion deficiency (LAD) and bovine leukocyte adhesion deficiency (BLAD). These surface molecules are known to be the major receptors in leukocyte tight adhesion thus their absence results in increased neutrophil numbers in the peripheral blood and decreased neutrophils in the tissues. The bovine model for LAD has provided many researchers the opportunity to study the role of the beta-2 integrins in neutrophils, and a number of papers have been published which describe certain cell functions in normal bovine neutrophils compared to BLAD neutrophils. The purpose of our study was to characterize the BLAD neutrophil by measuring the phagocytic function and NADPH oxidase activity. Surface molecule antigens were marked to assess the effects of shipment on normal, heterozygous and homozygous BLAD bovine blood. In this report, we show the phagocytic and killing potential, the oxidant production and the peripheral blood maturation of BLAD neutrophils as compared to normal or heterozygous bovine neutrophils.

Materials and Methods:
Duplicate whole blood smears were prepared from normal, heterozygous and homozygous BLAD calf specimens. Dried smears were stained with Wright's stain, and differential counts were performed on the neutrophil population. Neutrophils were scored as bands if no nuclear segments were visible; 2 segments if the nucleus had two segments; and 3+ segments if the nucleus had at least three segments.

Bovine neutrophils from normal, heterozygous and homozygous BLAD calves were placed in a microtiter plate with cytochrome c and activated with PMA. The rate of superoxide production is presented as the change in Cytochrome C absorbance at 550nm over time.

Bovine neutrophils from normal, heterozygous and homozygous BLAD calves were loaded with dihydrorhodamine 123 (DHR) at 37 degrees C for 5 minutes. These neutrophils were activated with PMA at 37 degrees C, and samples were taken at various time intervals and placed on ice. The DHR fluorescence was measured with a flow cytometer, and the mode fluorescence of each sample is plotted verses time.

Bovine neutrophils from normal, heterozygous and homozygous BLAD calves were loaded with dihydrorhodamine 123 (DHR) at 37 degrees C for 5 minutes. These neutrophils were placed in a microtiter plate and activated with PMA. The fluorescence was measured on a Bio-Tec FL500 microtiter plate fluorometer using two different filters at two minute time intervals for 28 minutes. The fluorescence is plotted verses time.

Bovine leukocytes were stained with monoclonal antibodies against various surface molecules. Fluorescence was measured by flow cytometry on a Becton Dickinson FACScan and/or a Becton Dickinson FACSCalibur. Neutrophils and lymphocytes were separately gated and the fluorescence compared between normal, heterozygous and homozygous BLAD calf blood.

Homozygous BLAD, heterozygous, and normal bovine neutrophils were allowed to interact with yeast cells in RPMI + 10% normal bovine serum for one hour. Samples were serially diluted and aliquots were spread on Sabouraud's Dextrose Agar plates at 370 C. Colony forming units (CFU's) were enumerated after 48 hours of growth. The percent killed was determined by calculating the number of CFU's per sample after one hour of interaction, divided by the amount of yeast originally used and subtracting that percent from 100%.

Neutrophils from homozygous BLAD, heterozygous, and normal bovine calves were adhered to tissue culture treated coverslips and allowed to interact with FITC-labeled yeast cells at a neutrophil to yeast ratio of 1:2. FITC fluorescence yeast attached to the cell surface was quenched using trypan blue. Fluorescent microscopy was used to differentiate engulfed versus attached yeast cells.

Results:

1) Overnight shipment had no effect on the expression of leukocyte surface molecules of homozygous BLAD, heterozygous or normal bovine blood as compared to leukocytes isolated from fresh normal bovine blood. (Figure #1 & #2)

2) BLAD neutrophils have decreased phagocytic and killing functions as compared to normal and heterozygous bovine neutrophils. (Figure #3)

3) The peripheral blood neutrophils of homozygous BLAD calves consist of an increased percentage of cells with immature morphology. (Figure #4)

4) BLAD neutrophils show an enhanced NADPH oxidase activity when activated with PMA and measured with a cytochrome c assay or DHR fluorescence. (Figure #5)

Discussion and Conclusions:

Neutrophils from animals with BLAD have an increased capacity to generate microbicidal oxidants. This host defense response may represent an adaptive mechanism used by the cell to compensate for their inability to adhere to and phagocytose foreign pathogens.

References:

Comments:
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Address questions and comments about this abstract to Karen Sipes (uvsmqks@gemini.oscs.montana.edu).

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Questions and Answers

Q: Very interesting study. This appears to be in contrast to results published by Kehrli's group, and what we have found with mouse and human LADs. Do you have an explanation for the difference? We know that IgG-mediated phagocytosis is decreases in BLAD, as well as human LAD. Have you looked at IgG-opsonized particles with respect to the ability to generate RB in the BLADs?

A: Most measurements of the oxidative burst reported for bovine LAD are stimulated by phagocytosis. Since there is a decrease in the phagocytic ability of the BLAD neutrophils, a decrease in the oxidative burst is expected. Other studies using PMA to stimulate the oxidative burst in BLAD neutrophils used luminol chemiluminescence for measurement. These reports state variable results ranging from decreased ROS production to nonconclusive results. We chose dihydrorhodamine 123 fluorescence as our measurement device because it is extremely sensitive to internal ROS production. I feel that if neutrophil isolation protocols and ROS measurement protocols were standardized for all the LAD species available, a more relevant comparison could be made.

Q:Do any of the MoAbs you used to stain the bovine neutrophils recognize the activated form of the beta-2 integrins better than the constitutively expressed form?

A: No, not to my knowledge. Our beta-2 integrin MoAbs were only used to confirm the presence or absence of the surface molecule.

Q:You indicated that L-selectin was expressed on neutrophils and lymphocytes from BLAD animals. Did you notice any difference in MFI of staining? We have seen that L-selectin is present on neutrophils of BLAD calves at birth but that it's MFI declines with age. I wonder if this does not represent in vivo activation of circulating neutrophils that are unable to leave the blood stream? Might this relate to your observed increase in NADPH-oxidase activity (i.e., the neutrophils in BLAD animals have been primed in vivo by chronic exposure to inflammatory cytokines, etc?

A: We stained L-selectin on the isolated neutrophils to assure that they had not been activated during the 24 hour shipping of the whole blood or during the isolation procedure. We also did a measurement of L-selectin shedding during our activation to arrive at a proper dose of PMA for our protocols, but we did not quantitate the L-selectin. We agree that the circulating BLAD neutrophils may be primed due to the chronic infections endured by these calves. The L-selection measurement could be a good indicator of this activation.

Q: We did not quantitate the hypersegmented neutrophils differently from the 3+ group. The left shift we reported was from three separate weekly shipments on the same calves. The differential counts were performed on three whole blood slides prepared from each calf each week. It may be interesting to see if we are loosing the hypersegmented neutrophil population during the shipping process. Please note that there was a Arelative@ decline in the number of neutrophils with 3+ segments. Due to the large increase in the absolute numbers of total neutrophils, the absolute numbers of 3+ segmented is still increased.

A: I was surprised by the decline in number of neutrophils with 3+ nuclear segments. Did you quantitate the percentage of hypersegmented neutrophils? It has been reported in dogs, humans and cattle with LAD that there is an increase in this subpopulation of neutrophils and likely represents neutrophils that were dying in the circulation since their circulating half-life was dramatically increased. A left shift has not always been a feature of BLAD calves reported in the literature.