Whole Blood Leukocyte vs. Separated Mononuclear Cell Blastogenesis in Calves: Time-Dependent Changes After Shipping

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ABSTRACT

The blastogenic response of peripheral blood mononuclear cells to mitogenic stimulation by concanavalin A was lower ($P<0.01$) after transporting 60 dairy calves 480 km than it was either one or two weeks later. The response was similar for phytohemagglutinin. There was a decrease ($P<0.05$) in the number of peripheral blood monocytes and neutrophils two weeks after shipping. The transportation of calves did not affect plasma IgG_1 or IgM levels.

The mitogenic stimulation of peripheral blood leukocytes by both phytohemagglutinin and concanavalin A in whole blood cultures was more variable than with the culture of peripheral blood mononuclear cells. Technique variation, which was defined as the coefficient of variation among quadruplicate cultures, was greater than 20% for whole blood assays and less than 10% for cultures of peripheral blood mononuclear cells. The variation among different calves tested at the same time and the variation within single calves tested at different times were also lower in peripheral blood mononuclear cell cultures than in whole blood assays. It is suggested that the variation among replicate cultures be reported in blastogenesis studies.

RÉSUMÉ

La réponse blastogénique des mononucléaires du sang périphérique à la stimulation mitogénique par la concanaviline A, se révéla moins forte ($P<0,01$), immédiatement après le transport de 60 veaux Holstein, sur une distance de 480 kilomètres, qu'au bout d'une ou deux semaines plus tard. La phytohémagglutinine donna des résultats similaires. Deux semaines après le transport, le nombre des monocytes et des neutrophiles du sang périphérique afficha une diminution ($P<0,05$) qui n'affecta toutefois pas la teneur du plasma en IgG_1 ou en IgM.

La stimulation mitogénique des leucocytes du sang périphérique, tant par la phytohémagglutinine que par la concanaviline A, se révéla plus variable dans les cultures de sang entier que dans celles de mononucléaires du sang périphérique. La variation de la technique, qui se définissait comme le coefficient de variation des cultures quadruples, dépassa 20%, pour les cultures de sang entier; elle demeura toutefois inférieure à 10%, pour les cultures de mononucléaires du sang périphérique. La variation observée d'un veau à l'autre, à l'occasion d'une seule et même épreuve, et celle qu'on détecta chez certains veaux, à la faveur d'épreuves effectuées à différentes périodes, se révélèrent aussi plus basses dans les cultures de mononucléaires du sang périphérique que dans celles de sang entier. Les auteurs suggèrent de reporter dans des études sur la blastogénèse, la variation enregistrée dans des cultures répétées.

INTRODUCTION

Shipping young calves often exacerbates the incidence of clinical disease and leads to increased mortality losses (2, 9). Death losses double when calves are shipped in the winter (25). It is possible that these adverse environmental stimuli are predisposing factors in the etiology of important infectious diseases in cattle, such as enzootic pneumonia, pneumonic pasteurellosis and neonatal diarrhea. Stress has been shown to affect the susceptibility of animals to disease and alter immune function in several animal species (12). However, the influence of environmental stressors on immune function in calves is relatively unknown.

Shipping stress alters antibody-mediated immune events. In one report, peritoneal and spleen cell

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suspensions from mice that were recently transported were tested for their capability to synthesize antibody against sheep erthyrocytes *in vitro* (6). A series of experiments showed that shipping stress reduced the number of plaque-forming cells from both types of cell suspensions when compared with the nonshipped controls. Hartmann *et al* (8) also showed that shipping altered the capability of calves to synthesize antibody *in vivo*. These results suggested that the stress response of animals to shipping could compromise antibody-mediated host defense immune mechanisms. This stress-induced immunosuppression might explain the increased susceptibility of calves to diseases that occur soon after shipment.

While several assays have been developed to measure various components of antibody-mediated immunity in cattle, a simple and reliable assay for cell-mediated immunity is not readily available. During the past decade, numerous laboratories have used mitogen-induced blastogenesis assays to monitor cell proliferation events. Collectively, these studies indicate that the stimulation of lymphoid cells with mitogens varies greatly from one laboratory to another. However, very few investigators have reported an estimate of the within-assay or technique variation. This source of variation probably differs considerably between laboratories and may partially explain the tremendous variation that is reported in the scientific literature.

The purpose of the studies reported herein was twofold: (a) to develop an assay that would optimize the blastogenic response of peripheral blood mononuclear cells (PBMC) to concanavalin A (Con A) and phytohemagglutinin (PHA) while minimizing within-assay variation. We also compared the biological variation among several calves tested at one time with the variation within single calves tested at several times; (b) the second objective was to monitor the effect of shipping and acclimating calves to a new environment on mitogen-induced blastogenesis of PBMC. In addition, we measured the number of blood leukocytes and plasma IgG_1 and IgM during this period.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Four groups of Holstein bull calves, each consisting of 15 animals, were purchased from a single commercial source. Calves ranged in age from one to 20 days ($\bar{X}$ = 10 days). They received a minimum of two feedings of colostrum within 24 hours of age and were then maintained on whole milk prior to transport. Immediately before shipment, calves were bled, injected with 3 mg sodium selenite and 6.6 mg vitamin E and the navels were treated with iodine. Animals were fed prior to transport but were without feed or water during the eight to ten hour trip. Calves were transported 480 km via truck to facilities at Washington State University. Upon arrival, calves were weighed and housed in individual cages (0.8 m x 1.6 m) with expanded metal floors and wire mesh sides. All cages were raised 23 cm above the floor and were cleaned twice daily. Calves were maintained in experimental laboratory facilities at 21°C under constant fluorescent light. A standard milk replacer was fed twice daily. Five days after arrival, all calves were offered alfalfa-barley pellets *ad libitum*. Calves were examined before each morning and evening feeding for clinical signs of disease, including gastroenteritis, respiratory distress or dehydration. They were treated as necessary with prescribed regimens of electrolyte solutions or antibiotics.

BLOOD SAMPLES

Approximately 15 mL of blood were collected by jugular venipuncture into vacutainers containing sodium heparin (10 units/mL). Blood samples were collected from all calves before shipment. Pretransport, whole blood samples were then carried on the truck during shipment of calves. After arrival, blood samples were placed in a laboratory maintained at 22°C. Calves were bled again eight hours after arrival. Therefore, blood collected at the farm had been stored for approximately 18 hours before processing for mitogen-induced blastogenesis. All other blood samples were either diluted for whole blood leukocyte cultures or processed for PBMC cultures within two hours after collection.

Total erythrocyte and leukocyte counts were performed on all blood samples with a Coulter Counter ZBI[1] equipped with a 100 μm aperture tube. Differential leukocyte counts were made from blood smears stained with Camco Wright's stain.[2]

PREPARATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC)

Heparinized whole blood was mixed with an equal volume of RPMI 1640 tissue culture medium,[3] pH 7.3, that was supplemented with penicillin (100 units/mL), fungizone (0.25 μg/mL), streptomycin (100 μg/mL), sodium bicarbonate (24 mM), and Hepes buffer (20 mM). Four and a half mL of the diluted blood was gently layered over 3.6 mL histopaque-1077,[4] a solution of ficoll and sodium diatrizoate that was adjusted to a density of 1.077 g/cm^3, in 16 x 125 mm glass tubes fitted with screw caps. All samples were centrifuged at 400 x g for 40 minutes at room temperature. Cells at the opaque interface were washed with 10 mL of 0.87% ammonium chloride to lyse contaminating ery-

[1]Coulter Electronics, Inc., Hialeah, FL 33010
[2]Scientific Products, McGaw Park, IL 60085
[3]Grand Island Biological Co., Grand Island, NY 14072
[4]Sigma Chemical Co., St. Louis, MO 63178

throcytes, and mononuclear leukocytes were sedimented by centrifugation at 150 x g for ten minutes. Cells were washed twice with RPMI 1640 medium before counting. Cell concentrations were determined and adjusted to 2 x 10^6 cells/mL by adding RPMI 1640 medium that contained 5% heat-inactivated fetal bovine serum.[5] In experiments that were designed to evaluate the effect of cell concentrations on mitogen-induced blastogensis, PBMC concentrations ranged from 0.5 x 10^6 to 4.0 x 10^6 cells/mL. Cell viability was determined by 0.1% trypan blue dye exclusion and was >97% in all cell suspensions collected in this manner. Differential leukocyte counts with Wright's stained smears revealed >98% mononuclear cells.

MITOGEN STIMULATION OF PBMC CULTURES

After cells were appropriately diluted, 100 μL aliquots of the suspension were dispensed into wells of sterile, 96-well flat-bottomed microtitration plates.[6] All samples were assayed in quadruplicate cultures. Phytohemagglutinin[7] and Con A[7] were reconstituted to the specified concentration with sterile RPMI 1640 medium and 100 μL of mitogen were added to the appropriate culture wells. Dose response curves were used to determine the concentration of mitogen and the incubation time which gave the optimum stimulation of bovine PBMC in this culture system. Cultures were incubated at 37°C for varying periods of time in a 5% $C0_2$ humidified air atmosphere in a SHEL-Lab incubator.[8] Eighteen hours prior to harvesting, 25 μL of RPMI 1640 that contained various amounts of methyl[^{3}H]thymidine[9] ([^{3}H]TdR, 6.7 Ci/ mmole) were added to each well. Cells were harvested for liquid scintillation counting using a semiautomatic cell harvester[10] which automatically rinsed each well several times with deionized water. The glass filter paper discs were dried and then transferred to 20 mL vials with 7 mL of scintillation fluid.[11] The vials were counted for 2 min in a scintillation spectrometer.[12]

WHOLE BLOOD CULTURES

Preliminary experiments similar to those described for PBMC cultures indicated that the following procedures gave optimal stimulation in whole blood cultures. Whole blood cell suspensions were prepared by adding one part heparinized whole blood to twenty-nine parts of RPMI 1640 that was supplemented as described for PBMC preparation. One hundred microliters of this suspension were pipetted into sterile microtiter plates and 100 μL of mitogen preparation were pipetted into quadruplicate cultures; PHA at 0.2 μg/culture and Con A at 0.1 μg/culture. Similar to the PBMC cultures, all plates were incubated for 96 hours. Eighteen hours prior to harvesting, 0.2 μCi [^{3}H]thymidine (6.7 Ci/mmole) in 25 μL of RPMI 1640 was added to each culture. Harvesting and counting were performed as described for PBMC cultures.

IgG_1 AND IgM LEVELS

Plasma was collected by centrifugation at 800 x g for 15 min and concentrations of IgG_1 and IgM were monitored using the single radial immunodiffusion technique of Mancini (15), as modified by Fahey and McKelvey (5). Antiserum was obtained from goats after multiple injections with either bovine IgG_1[13] or IgM[14] emulsified in Freund's complete or incomplete adjuvant as described in a companion paper now being prepared. Anti-IgG_1 was rendered monospecific by adsorption with soluble bovine IgG_2[15] and anti-IgM by adsorption with soluble bovine gamma globulin[16] and fetal bovine serum.[17] Adsorbed antisera were shown to be monospecific by exhibiting single precipitin bands with whole bovine serum when examined by immunoelectrophoresis, Ouchterlony double immunodiffusion and single radial immunodiffusion. In addition, the monospecific antisera did not cross react with other classes of bovine immunoglobulins. All samples were assayed in duplicate. The intraassay and interassay coefficients of variation for the IgG_1 assay were 8.3% and 7.0% respectively, and 8.3% and 4.2% for the IgM assay.

VIROLOGICAL METHODS

The techniques utilized for isolation and identification of viruses from calves have been recently reviewed (4). Briefly, samples in the forms of swabs (fecal and nasal) and/or tissues (lung, liver, kidney and spleen) obtained at necropsy were processed and inoculated onto secondary cultures of bovine embryonic testicular cells. Virus identification was conducted by specific virus neutralization using antisera obtained from the National Animal Disease Center, Ames, Iowa.

STATISTICAL METHODS

Results of the mitogen-stimula-

[5]Grand Island Biological Co., Grand Island, NY 14072
[6]Flow Laboratories, Inglewood, CA 90302
[7]Sigma Chemical Co., St. Louis, MO 63178
[8]Sheldon Manufacturing, Inc., Aloha, OR 97005
[9]New England Nuclear, Boston, MA 02118
[10]Brandel, Model M-12V, Biomedical Research and Development Laboratories, Inc., Rockville, MD 20852
[11]Omniflour, 4 g/L toluene, New England Nuclear, Boston, MA 02118
[12]Packard TriCarb Model 3330, Packard Instrument, Inc., Downer's Grove, IL 60515
[13]Miles Research Products Division, Elkhart, IN 46515
[14]Kindly provided by Dr. David Olson, Veterinary Science Department, University of Idaho, Moscow, ID 83843.
[15]Miles Research Products Division, Elkhart, IN 46515
[16]Bovine gamma-globulins, Fraction II, Calbiochem-Behring Corp., La Jolla, CA 92037.
[17]Grand Island Biological Co., Grand Island, NY 14072

tion assays were expressed as the mean value of counts per minute (cpm) of quadruplicate cultures or as Δcpm, the mean cpm of stimulated cultures minus the mean cpm of control cultures containing no mitogen (background cpm). For comparative purposes, results were sometimes expressed as a stimulation index (SI).

$$SI = \frac{\text{cpm of cultures stimulated with mitogen}}{\text{cpm of cultures without mitogen}}$$

Data were subjected to analysis of variance (26) procedures with group, time and the group by time interaction as sources of variation. Differences were tested with Duncan's new multiple range test (3).

RESULTS

DOSE RESPONSE OF PMBC CULTURES

The effects of varying concentrations of Con A and PHA, number of PBMC per culture, incubation time and amount of [^{3}H]thymidine were determined. Values reported are the means of three or four calves. Mononuclear cells were diluted to cell concentrations ranging from 0.5 x 10^5 to 4 x 10^5 per culture. Phytohemagglutinin was tested at concentrations varying from 1.0 to 6.0 μg per culture. In general, [^{3}H] thymidine incorporation increased as the number of cells in culture increased (Fig. 1). In the present study, 2.0 μg PHA per 2 x 10^5 cells was chosen because of high stimulation and cell economy. Concanavalin A was tested at concentrations of 0.1 to 2.5 μg per culture (Fig. 2). At a cell concentration of 2 x 10^5 per culture, the highest uptake of [^{3}H]thymidine was achieved with 1.0 μg of Con A per culture, and those conditions were employed throughout future studies. Results of experiments examining culture periods from 72 to 144 hours indicated that both the PHA and Con A responses were optimum at 96 hours (Figs. 3 and 4), an incubation period which was used in all subsequent experiments. Similar to

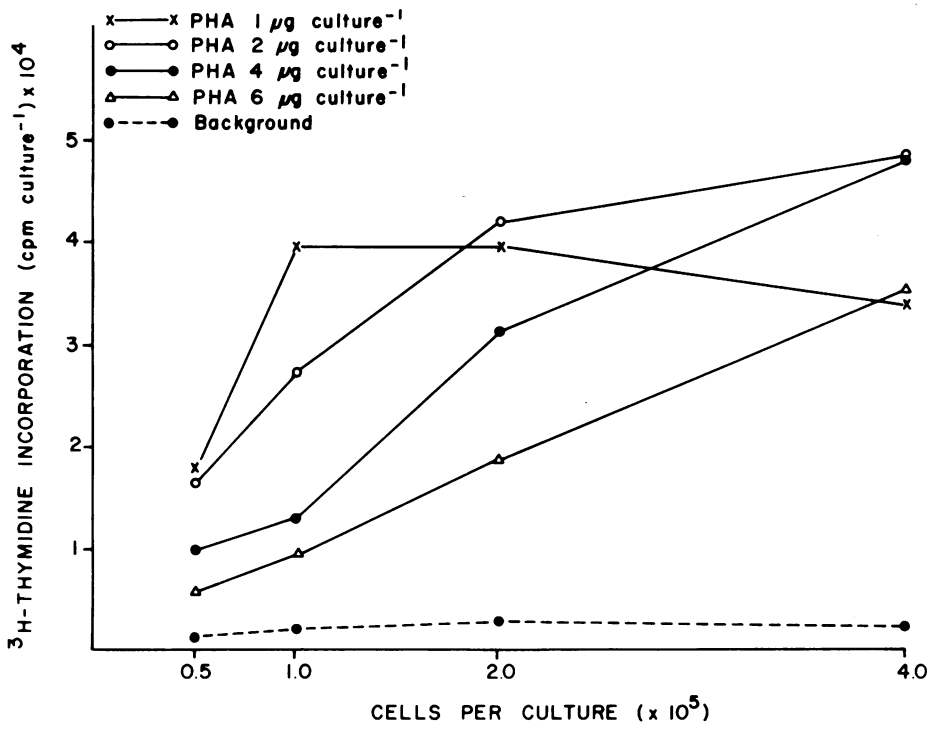

Fig. 1. Phytohemagglutinin (PHA) dose-response curves with varying numbers of bovine peripheral blood mononuclear cells (PBMC). All cultures were incubated for 96 hours and were pulsed with 0.2 μCi [^{3}H]thymidine. The averages for four calves were determined from the mean value of quadruplicate cultures from each animal for each cell concentration tested.

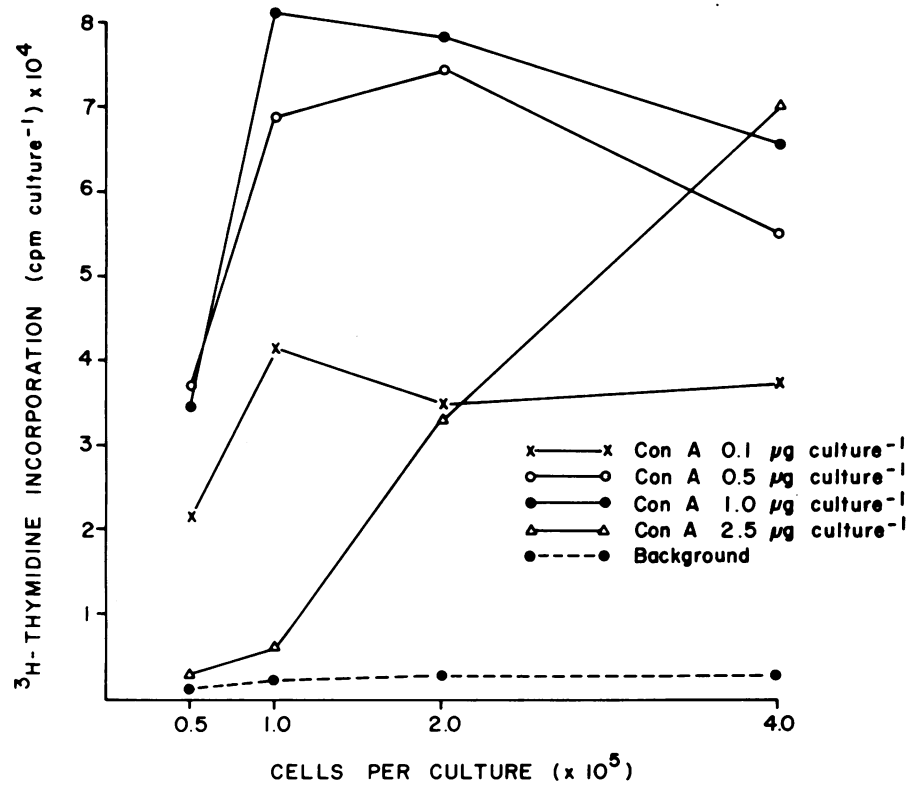

Fig. 2. Concanavalin A (Con A) dose-response curves with varying numbers of bovine PBMC. All cultures were incubated for 96 hours and were pulsed with 0.2 μCi [^{3}H]thymidine. The averages for four calves were determined from the mean value of quadruplicate cultures from each animal for each cell concentration.

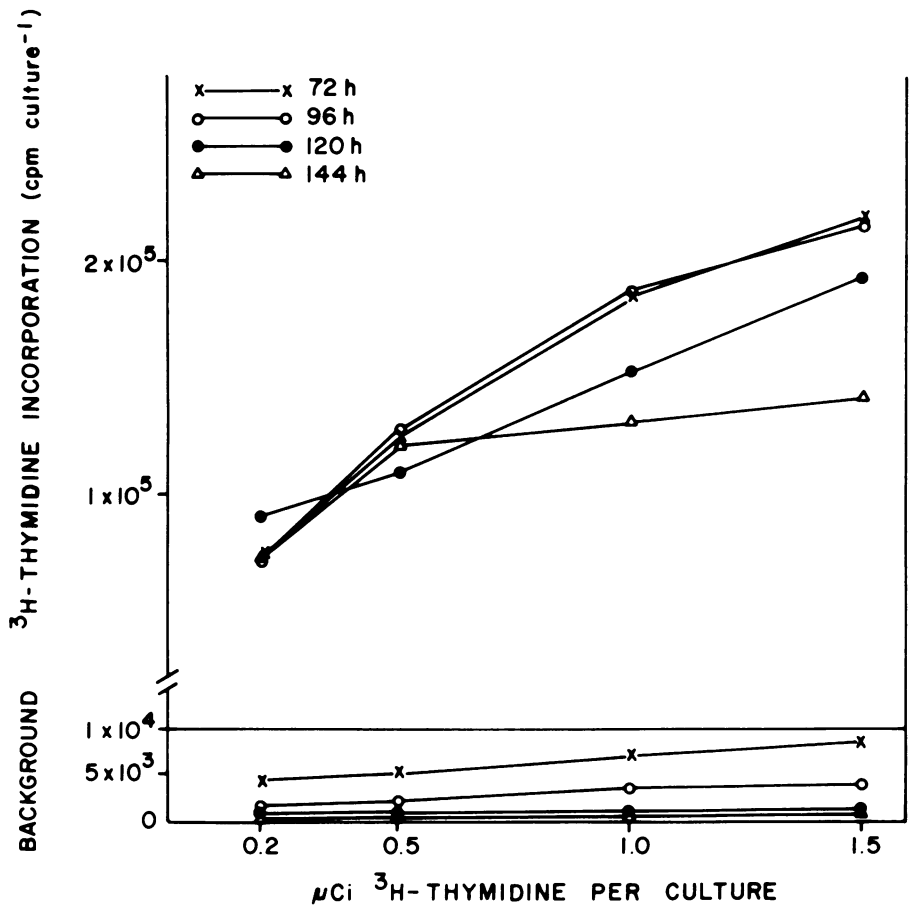

Fig. 3. Phytohemagglutinin (PHA) response curves with varying culture times and varying amounts of [^{3}H]thymidine. All cultures consisted of 2 x 10^5 PBMC stimulated with 2.0 μg PHA. The averages for three calves were determined from the mean value of quadruplicate cultures from each animal for each quantity tested.

the results of Paty and Hughes (19), we noted maximal stimulation of cell cultures to both PHA and Con A when the [^{3}H]thymidine was at 1.5 μCi (Figs. 3 and 4). However, for reasons of economy, we chose a standard dose of 1.0 μCi [^{3}H]thymidine per culture except in an initial experiment with 15 calves (Table I) where 0.2 μCi was used.

WHOLE BLOOD ASSAY

A 1/30 dilution for the whole blood assay gave the highest stimulation when 0.2 μCi [^{3}H]thymidine was used to pulse cultures. Different dilutions gave too many or too few mononuclear cells and yielded suboptimal stimulation. These optimal culture conditions for the whole blood assay were very similar to those that were found optimal for PBMC cultures when quantity of mitogen was calculated on the basis of the number of mononuclear cells per culture. Total leukocytes in whole blood averaged 10,000/μL, approximately 70% of which were mononuclear cells (Table IV). A 1/30 dilution of whole blood yielded approximately 2 x 10^4 mononuclear cells per 100 μL culture, which was one-tenth the number of PBMC in cell separated cultures. The quantity of mitogen used per whole blood culture, 0.1 μg Con A and 0.2 μg PHA, was also one-tenth the amount found to give optimal stimulation of PBMC cultures. Therefore, the mitogen: lymphocyte ratio was approximately constant for both assay systems.

WHOLE BLOOD COMPARED TO PBMC CULTURES

Three blood samples were collected from each of approximately 45 calves at weekly intervals. Blood from 15 calves was assayed for blastogenesis using whole blood cultures labelled with 0.2 μCi [^{3}H]thymidine. Mitogen-induced blastogenesis of leukocytes from another group of 15 calves was monitored with PBMC cultures that were also labelled with 0.2 μCi [^{3}H]thymidine. Finally, assays from the remaining 15 calves were conducted with PBMC cultures labelled with 1.0 μCi [^{3}H]thymidine.

Technique variation in whole

TABLE I. A Tabular Comparison of Variability in Mitogen-Induced Blastogenesis Assays Conducted on Whole Blood Cultures and Peripheral Blood Mononuclear Cells

	Whole Blood, 0.2 μCi[^{3}H]TdR[a]	PBMC, 0.2 μCi[^{3}H]TdR[b]	PBMC, 1.0 μCi[^{3}H]TdR[b]
Background	351±45	1722±432	4057±421
PHA-Δcpm[c]	3391±574	49,847±3269	238,939±11,550
PHA-SI[d]	15±3	89±15	82±8
Technique coefficient of variation[e]	27	8	10
Con A-Δcpm[c]	11,156±1192	77,307±3423	290,930±10,845
Con A-SI[d]	49±9	140±29	102±11
Technique Coefficient of Variation[e]	20	6	5

Values reported are means calculated from the raw quadruplicate cpm±SEM from at least 11 calves for each type of assay. Each calf was bled three times for a total of at least 33 quadruplicate cultures per type of assay

[a]Blood diluted 1/30; 96 h incubation; 0.2 μg PHA or 0.1 μg Con A per culture

[b]Cell concentration = 2 × 10^5 cells per culture; 96 h incubation; 2.0 μg PHA or 1.0 μg Con A per culture

[c]Δcpm = cpm of stimulated cultures - cpm of unstimulated cultures (background)

[d]Stimulation Index = cpm of stimulated cultures ÷ cpm of unstimulated cultures (background)

[e]This is the mean coefficient of variation of raw cpm among quadruplicate cultures of each calf and reflects technique variation rather than biological variation

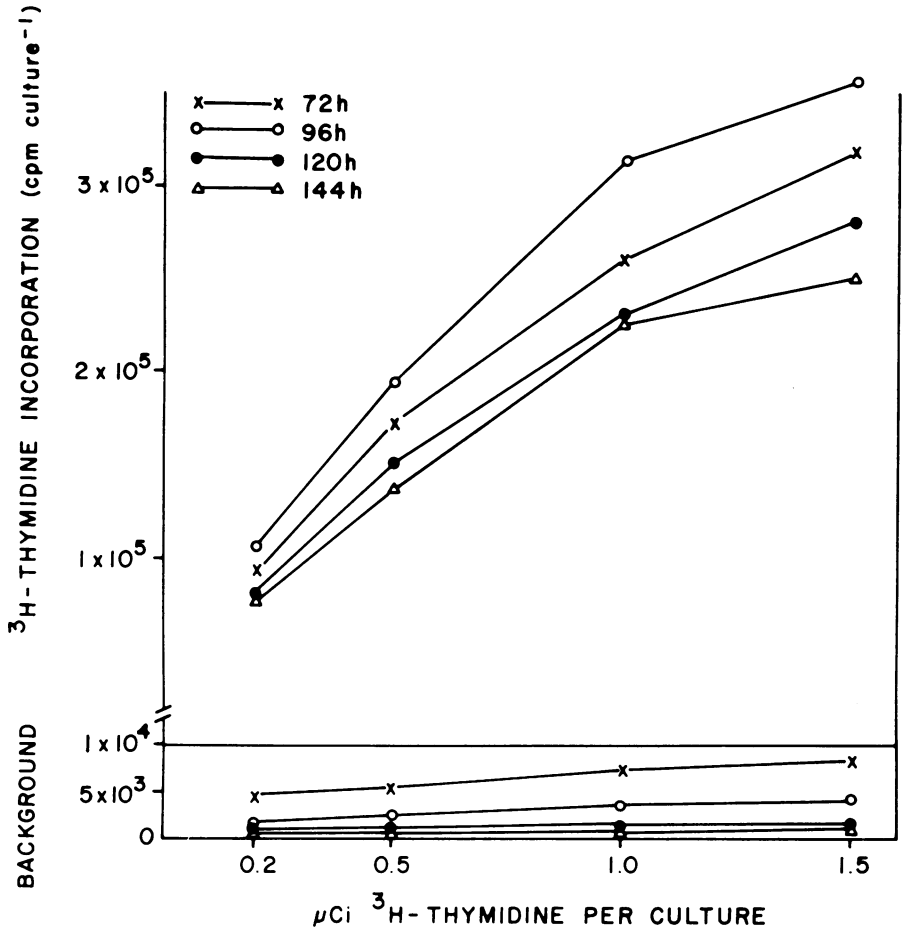

Fig. 4. Concanavalin A (Con A) response curves with varying culture periods and varying amounts of [³H]thymidine. All cultures consisted of 2 x 10⁵ PBMC stimulated with 1.0 μg Con A. The averages for three calves were determined from the mean value of quadruplicate cultures from each animal for each quantity tested.

blood cultures labelled with 0.2 μCi [³H]thymidine and in PBMC cultures labelled with 0.2 μCi or 1.0 μCi [³H]thymidine is shown in Table I. The variation is expressed as the coefficient of variation (CV) of raw cpm among quadruplicate cultures from each calf. The mean technique CV from both PHA- and Con A-stimulated cultures was three times higher in whole blood cultures than in either of the PBMC cultures. The higher technique variation with whole blood cultures was not due to differences in cell numbers between the groups of calves, because neither the number of leukocytes nor the percentage of lymphocytes was different ($P>0.05$) among the groups of calves. The ratio of mitogen to cells was also held constant for all groups of calves. Furthermore, when the data were expressed as Δcpm, both within— and among-calf variation at each bleeding were at least twofold less for PBMC than for whole blood cultures (Table II). The lower percent variation for PBMC was not due simply to higher counts, because the standard deviation in both culture systems was expressed as a percentage of the mean in each group (CV).

WITHIN VS. AMONG CALF VARIATION

Individual calf variation over time was determined from the three weekly bleedings of each calf. Results are shown in Table II as the standard deviation expressed as a percentage of the mean. Variation among calves was determined from the mitogenic response of at least 11 different calves that were bled at the same time. Variation among calves was consistently greater than the variation within calves for both the whole blood and PBMC cultures, although the differences were not large (Table II). These results are in agreement with the results of Renshaw *et al* (22). The least variation was measured with PBMC cultures with 1.0 μCi of [³H]TdR. The CV (Δcpm) among and within calves for PHA and Con A aver-

TABLE II. Coefficients of Variation in Mitogenesis Assays Within and Among Calves Tested With Either Whole Blood or PBMC Cultures[a]

Type of Assay	Mean Coefficients of Variation Among Calves[b]				Mean Coefficients of Variation Within Calves[c]
Whole Blood	Week 1	Week 2	Week 3	$\bar{X}$	Weeks 1, 2 and 3
0.2 μCi[³H]TdR					
PHA-Δcpm[d]	112	105	84	102	68
PHA-SI[d]	82	81	99	86	65
Con A-Δcpm[d]	69	64	73	68	51
Con A-SI[d]	112	78	102	98	50
No. of calves	15	13	11		
PBMC 0.2 μCi[³H]TdR					
PHA-Δcpm	44	45	35	42	36
PHA-SI	114	100	69	95	68
Con A-Δcpm	22	25	14	21	26
Con A-SI	95	127	60	95	63
No. of calves	15	14	13		
PBMC 1.0 μCi[³H]TdR					
PHA-Δcpm	34	29	30	31	26
PHA-SI	60	65	58	61	51
Con A-Δcpm	27	20	21	23	16
Con A-SI	60	84	50	65	49
No. of calves	15	15	15		

[a] All coefficients of variation are based on quadruplicate cultures of n calves
[b] Variation among different calves tested at the same time
[c] Variation among individual calves during three weekly bleedings
[d] See footnotes at bottom of Table I

aged, respectively: 31 and 26 and 23 and 16 (Table II).

SHIPPING STRESS AND BLASTOGENESIS

Four groups of 15 calves each were transported a distance of 480 km via truck. Blastogenesis of PBMC cultures was monitored in blood drawn before shipment, eight hours after shipment and one and two weeks later. Unfortunately, PBMC cells from pretransport blood samples did not respond to the mitogens Con A and PHA. Subsequent experiments showed that this effect was due to the length of time (18 hours) and colder temperatures to which the blood was exposed during transit. Therefore, because the cells had aged and did not incorporate [^{3}H]TdR, pretransport blastogenesis values were not valid.

Blastogenesis values after shipping are shown in Table III. The Δcpm of Con A-stimulated cultures was lower ($P<0.01$) upon arrival than at either one or two weeks after arrival. The SI of both Con A- and PHA-stimulated cultures was also depressed ($P<0.05$) soon after arrival when compared with one week later. The SI's then decreased to somewhat higher values than those determined upon arrival. Although there was a general increase in PHA-responsiveness (Δcpm) with time, this increase was not significant ($P>0.05$).

Regression analysis of blastogenic responses to Con A and PHA using exact calf age as the independent variable showed that these correlations were low and nonsignificant ($r^2 = 0.05$ to 0.08). This finding suggested that a factor other than age was the cause for the increase in blastogenic responses to Con A from week 1 to week 2 and the plateau from week 2 to week 3.

SHIPPING STRESS AND PLASMA IgG_1 AND IgM LEVELS

There was no significant change in plasma IgG_1 or IgM concentrations after shipping when compared to concentrations that were measured before shipping. Plasma IgG_1 concentrations in pretransport blood samples averaged 23.9 ± 2 mg/mL and blood drawn eight hours after transport averaged 21.3 ± 2 mg/mL. One and two weeks after shipping, IgG_1 levels were 21.4 ± 2 and 26.2 ± 2 mg/mL, respectively. The respective values for IgM were 1.02 ± 0.1 mg/mL in pretransport blood samples, 1.04 ± 0.1 mg/mL eight hours after transport, and 1.00 ± 0.1 mg/mL and 0.96 ± 0.1 mg/mL one and two weeks after shipping.

VIRAL ISOLATION

No virus isolations were made from swabs obtained from calves prior to shipment. After transport, infectious bovine rhinotracheitis (IBR) virus was isolated on four occasions: once from the nasal swab of one calf and three times from the lung tissue of calves succumbing to pneumonia. The IBR virus was isolated one to two weeks after the calves were shipped. Although the initial mitogenic responses of the calves that were infected with the IBR virus were not different from other calves, blastogenesis data from these three animals were excluded from the statistical analysis.

OTHER PHYSIOLOGICAL PARAMETERS

Body weights of the calves averaged 42.4 ± 0.7 kg immediately after transport. Two weeks later, the average body weight had increased to 46.1 ± 0.6 kg. Average erythrocyte counts ranged from $9.8 \pm 0.2 \times 10^6/\mu L$ before shipment to $9.4 \pm 0.2 \times 10^6/\mu L$ two weeks later (Table IV). Shipping did not affect total leukocyte counts, which remained constant over time with a mean of $10,150/\mu L$. Differential leukocyte counts (Table IV) revealed a decrease ($P<0.05$) in the number of neutrophils at one and two weeks after transport. The percent neutrophils at one and two weeks after shipping was also reduced ($P<0.05$) when compared with neutrophils at eight hours after shipping. There was also a significant reduction in both the percent and number of monocytes two weeks after shipping. The absolute number of lymphocytes remained unchanged throughout the entire study, even though the percent lymphocytes was elevated ($P<0.05$) one week after transport (Table IV).

DISCUSSION

This study was designed to measure time-dependent changes in immune events that occur in calves after shipping. Both plasma IgG_1 and IgM after shipping were similar to the pretransport concentrations. Shipping also did not alter the absolute number of lymphocytes. However, the blastogenic responses of PBMC to Con A was significantly lower in calves immediately after the 480 km shipment than at one or two weeks later. When the results were expressed as SI's, both Con A- and PHA-stimulated blastogenesis were also lower soon after shipping than they were one week later.

Unfortunately, storage of the

TABLE III. Time-Dependent Changes in Mitogen-Stimulated Blastogenesis

	Eight Hours After Shipping	One Week After Shipping	Two Weeks After Shipping
Background	9369±831[a] (60)	4013±845[b] (58)	4006±860[b] (56)
Δcpm-PHA	108,336±6455[a] (60)	111,129±6565[a] (58)	119,481±6682[a] (56)
SI-PHA	37.8±7.2[a] (60)	69.2± 7.3[b] (58)	53.1±7.46[ab] (56)
Δcpm-Con A	159,302±8466[a] (60)	209,596±8686[b] (57)	214,199±8763[b] (56)
SI-Con A	53.0±13.0[a] (60)	120.9±13.3[b] (57)	86.0±13.5[ab] (56)

$\bar{X}$±SEM; ( ) = Number of calves; means within the same row with different letter superscripts are different ($P<0.05$)

pretransport blood samples during transit prevented measurement of blastogenesis before the calves were shipped. Therefore, it is unknown whether these changes in blastogenesis were age-related or stress-related. Certainly, the limited number of IBR isolations, as well as lack of isolation of other important bovine viruses, suggested that an infection was not a causal factor in the critical depression of responsiveness to Con A. Furthermore, the blastogenic response to both mitogens was independent of age of the calves ($r^2 < 0.08$).

An age-related increase in the number of leukocytes also would not explain the blastogenesis results, as total leukocytes and lymphocyte numbers remained unchanged and the number of monocytes declined over the two week acclimation period. These results are in general agreement with those of Ruppanner *et al* (23), who also reported a reduction in the number of monocytes with little change in the number of circulating lymphocytes after shipping crossbred calves. However, these workers did report a reduction in the number of erythrocytes and leukocytes within two weeks after shipping cattle with little change in the number of neutrophils. It is therefore proposed that the reduced response of PBMC cultures to mitogens was caused primarily by the stress of shipping. Corroboration of this conclusion requires that other experiments be conducted that measures pretransport blastogenesis values or utilizes nonshipped calves.

Transportation had no effect on plasma IgG_1 or IgM levels. However, Hartmann *et al* (8) demonstrated with calves that the efficiency of antibody production to a specific antigen was compromised by shipping when the antigen was injected three days after transport. These combined results indicate that shipping may reduce the capability of calves to synthesize antigen-specific antibody, even though the existing total immunoglobulin pool is not changed. Mean concentrations of IgG_1 over the two week period ranged from 21 to 26 mg/mL. These levels are in close agreement with those of McGuire *et al* (16), who found in calves of mixed breeds that serum IgG_1 concentrations remained fairly constant at 24 mg/mL between eight and 30 days of age. Mean concentrations of IgM in the two week period ranged from 0.96 to 1.04 mg/mL, which are the same as McGuire's reported IgM concentrations of approximately 1.0 mg/mL.

Many investigators, using various modifications of the whole blood culture technique, have found the assay comparable or superior to enriched lymphocyte or mononuclear cell cultures (7, 10, 14, 18-22). Some laboratory groups claim greater reproducibility (20, 21) and comparable or greater blastogenic responses to mitogens (7, 14, 19, 21) with the whole blood assay, while others have reported greater sensitivity with a separated lymphocyte assay (27). Some workers have also reported that the whole blood assay gave a better correlation with delayed-type hypersensitivity (DTH) tests (21), yet others have found that the separated mononuclear cell assay was more representative of DTH (27). It is also commonly believed that the whole blood technique provides a better *in vitro* approximation of the *in vivo* situation because various serum factors and hormones are present during cell culture. Also, techniques that are used to enrich leukocytes can randomly select subpopulations of lymphocytes, giving a distorted view of *in vivo* events. In addition, the whole blood assay certainly requires less blood, time and technical preparation.

Results of this study with dairy calves clearly indicated that technique variation was less with the PBMC cultures than with whole

TABLE IV. Effect of Transportation and Acclimation on the Number of Peripheral Blood Erythrocytes and Leukocytes

Bleeding	Total Leukocytes per μL	Erythrocytes per μL ($X10^{-6}$)		Neutrophils	Bands	Basophils	Eosinophils	Monocytes	Lymphocytes
Before shipping									
			Percent	34.17±1.69^{ab}	0.15 0.09	0.10±0.05^{a}	0.17±0.05^{ab}	0.78±0.10^{a}	64.63±1.73^{bc}
	9943±481	9.77±0.20	Number	3522± 342^{ab}	16± 19	9± 6	17± 6^{ab}	79± 13^{a}	6299± 258
Eight hours after shipping									
			Percent	37.83±1.69^{a}	0.30±0.90	0.07±0.05^{b}	0.22±0.05^{a}	0.65±0.10^{ab}	61.10±1.73^{c}
	10,679±477	9.46±0.20	Number	4293± 339^{a}	37± 19	11± 6	21± 5^{a}	80± 14^{a}	6255± 256
One week after shipping									
			Percent	25.43±1.75^{c}	0.27±0.09	0.11±0.05^{a}	0.02±0.05^{b}	0.64±0.11^{ab}	73.34±1.79^{a}
	10,375±485	9.50±0.20	Number	3042± 351^{b}	54± 20	11± 6	3± 6^{b}	65± 15^{ab}	6998± 265
Two weeks after shipping									
			Percent	30.64±1.96^{bc}	0.22±0.10	0.24±0.06^{a}	0.13±0.06^{ab}	0.33±0.12^{b}	68.64±1.99^{ab}
	9986±494	9.37±0.21	Number	3142± 392^{b}	21± 22	21± 7	15± 6^{ab}	31± 16^{b}	6493± 296

$\bar{X}$±SEM; means within the same column with different letter superscripts are different ($P < 0.05$)

blood cultures under the conditions in our laboratory. Technique variation was reflected by CV's of replicate cultures and averaged 5-10% with PBMC cultures compared to 20-27% with whole blood cultures (Table I). While the results with other species are variable (10, 21), our results agree with the data of Paty and Hughes (19), who concluded that blastogenesis of separated human mononuclear cultures was less variable than with whole blood cultures (CV's of 20% and 30%, respectively). It is therefore likely that the presence of unknown serum factors increases the variability of lymphoid cells that are stimulated with mitogens.

These experiments also indicated that the total variability of the assay, that due to technique as well as biological variation within or among calves, was at least three times less with the PBMC assay than with the whole blood assay (Table II). This is in contrast to the results of Muscoplat *et al* (18), who found slightly less total variation with whole blood cultures (CV =31%) than with mononuclear cell cultures (CV = 38%) when the blood cells were stimulated with PHA. However, the total variation among calves in the present study (31% for PHA; 23% for Con A) compares favorably to the data reported by Muscoplat *et al* (17, 18) and others (24, 28).

Although technique variation is seldom reported with bovine blastogenesis assays, the work by Kaneene *et al* (11) indicated considerable within-assay variation, as evidenced by the range in stimulation within triplicate cultures stimulated by PHA. Since the degree of variability inherent in the technique is an important statement as to the sensitivity and validity of the assay, we feel that blastogenesis data would be more meaningful if such information was included in scientific reports. Regardless of whether a laboratory chooses a whole blood assay as the appropriate measure of the *in vivo* immune situation or an enriched mononuclear cell assay to assess the status of the leukocytes outside of the *in vivo* milieu, primary consideration must be given to the overall validity of the assay before biological conclusions can be reached.

For comparative purposes, it has been common to express blastogenic responses of leukocytes as a stimulation index (SI), which is the ratio of the cpm of stimulated to unstimulated (background) cultures. Yet, some investigators claim that this can be misleading because small changes in background counts can cause large changes in the SI while the actual stimulated counts may change very little or not at all (1, 13, 19). We have also found that the variation within or among calves is two to four times greater when results with PBMC's are summarized as SI's rather than Δcpm (Table II). Therefore, to minimize variation among calves, our results support the expression of blastogenic data as Δcpm.

In conclusion, the present data and those of Hartmann *et al* (8) indicate that transportation may compromise both antibody-and cell-mediated immune responses. Both specific antibody titers and lymphocyte mitogenesis are reduced one to three days after shipping but rise by seven to 14 days after transport. Shipping has little effect on systemic levels of IgG_1 or IgM.

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