Application of a leukocyte migration inhibition assay for detecting cellular responses in cattle exposed to *Brucella abortus*

by

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I. INTRODUCTION

The study of brucellosis in cattle, leading to the eventual eradication of the disease, is of importance from both public health and economic aspects (1). Since brucellosis is not generally transmitted from person to person, the prevention of human infection depends upon the control and elimination of the disease in animals (2).

Brucella are small aerobic gram-negative rods or coccobacilli. These facultative intracellular parasites are devoid of capsules, flagella, spores, and exotoxins. When brucellae are introduced into skin, polymorphonuclear leukocytes (PMNS) are mobilized within hours and phagocytosis occurs (3).

The PMN is produced in the bone marrow from a common ancestral stem cell (4). The primary function of the PMN is ingestion and digestion of microorganisms in tissues of the host animal (5). For the PMN to ingest the microorganism it must first migrate to the infection site. A variety of soluble factors and other components, including lymphokines, released at the site of infection attract PMNS in a process called chemotaxis (6). Lymphokines are produced and released by lymphocytes. A lymphokine of importance in the in vitro leukocyte migration inhibition assay is the leukocyte migration inhibition factor (7).

Leukocyte migration inhibition assays and lymphocyte blastogenesis are considered in vitro correlates of cell-mediated immune responsiveness. Leukocyte migration inhibition has been used as an indicator of cell-mediated immune responses in humans (8, 9, 10), cattle (11, 12, 13, 14, 15), chickens (16), and swine (17). There are few reports of the use of

leukocyte migration assays to evaluate cell-mediated responses of cattle exposed to Brucella. Lee (13) and Azadegan et al. (14) examined the responses of calves vaccinated with B. abortus strain 19. Dorsey and Deyoe examined responses of heifers experimentally infected with B. abortus strain 2308 (15). Lymphocyte blastogenesis has been used to evaluate the cell-mediated immune responses of cattle exposed to B. abortus (18, 19, 20). Kaneene et al. used lymphocyte blastogenesis to differentiate the responses of cattle vaccinated with B. abortus strain 19 from the responses of cattle infected with field strain B. abortus (20). No reports are available comparing the leukocyte migration responses of adult vaccinated cattle to the responses of experimentally infected cattle. In some geographic areas adult vaccination of cattle with B. abortus strain 19 is used as a means of controlling the spread of brucellosis, thereby causing difficulty in differentiating the responses of vaccinated cattle from the responses of infected cattle (21).

The objectives of this study were to:

1. Examine some variables affecting an agarose leukocyte migration inhibition assay.
2. Examine the applicability of the assay for detecting and differentiating cellular responses of cattle following exposure to virulent B. abortus strain 2308 or attenuated B. abortus strain 19.

II. LITERATURE REVIEW

A. Brucella

Brucella are small aerobic gram-negative rods or coccobacilli. These facultative intracellular parasites devoid of capsules, flagella, spores, and exotoxins infect a wide variety of animal species, including humans. Brucellosis is transmissible by ingestion, inhalation, and contact (1). The primary routes of infection are gastrointestinal (ingestion) and penetration of skin or mucous membranes, including conjunctiva (1).

When cattle are exposed to virulent strains of B. abortus, any of three consequences are possible: 1. Infection may fail to result from that exposure; 2. Infection may occur without resulting in clinical disease; 3. Infection may result in clinical disease (22). The most readily recognized symptom of clinical disease in cattle is abortion. This is pertinent only when the infected animal is pregnant, and over 20% of pregnant cattle infected with B. abortus fail to abort (23).

The ability of B. abortus to multiply in the host tissues apparently determines the appearance of clinical symptoms. The growth of virulent strains of B. abortus is stimulated by erythritol, a carbohydrate substance produced by the uterus of cattle (24). The greatest concentration of erythritol occurs in the placenta and foetal fluids; this is associated with localization of infection in these tissues (25).

The study of brucellosis in cattle, leading to the eventual eradication of the disease, is of importance from both public health and economic aspects (1). Since brucellosis is not generally transmitted from person to

person, the prevention of human infection depends upon the control and elimination of the disease in animals, such as cattle. The economic losses caused by bovine brucellosis in the U.S.A. have been estimated to be in the millions of dollars annually (2).

B. Cellular Response in Host

When Brucella are introduced into skin, polymorphonuclear leukocytes (PMNS) are mobilized within hours and phagocytosis occurs (3). There are four phases of infection: 1. An initial phase characterized by the early growth and multiplication of Brucella; 2. A bacteriocidal phase when activated PMNS and macrophages ingest and kill the Brucella; 3. A plateau phase characterized by the presence of relatively constant low numbers of bacteria; 4. The final phase occurs when either all the Brucella are killed or the animal dies (26). Chronic infection occurs when the plateau phase is extended and may be associated with the relative resistance of B. abortus to PMN and macrophage killing (27). Granulomatous lesions containing lymphocytes, epitheloid cells, multinucleated giant cells, and plasma cells may be formed (28).

1. Polymorphonuclear leukocytes (PMNS)

The PMN originates in the bone marrow from a common stem cell. The cell divisions in PMN maturation include: myeloblast, promyelocyte, metamyelocyte, band cell, and finally the mature PMN (4). The PMN is an end cell of myeloid differentiation and does not divide.

The primary function of the PMN is ingestion and digestion of microorganisms in tissues of the host animal (5). For the PMN to kill these microorganisms it must first migrate to the site of infection. Molecular

agents released at the site of infection from microbial, cellular or humoral origins attract PMNS in a process called chemotaxis (6). Opsinization of the particle with immunoglobulins and complement components may be required before the PMN can bind to and ingest the microorganism (6).

The antimicrobial systems of the PMN can be divided into two broad groups: the oxygen-dependent systems and the oxygen-independent systems. The oxygen-dependent systems include: myeloperoxidase (29), hydrogen peroxide (30), superoxide ion (31), hydroxyl radical (32), and singlet oxygen (33). The oxygen-independent systems include: lowered cellular pH (34, 35), lysozyme (30), lactoferrin (36), and granular cationic proteins (37). The hexose monophosphate shunt enzymes are believed to be involved in the oxygen-dependent systems (38). It has been suggested that this energy pathway is not stimulated by the ingestion of B. abortus; therefore, the organism can survive because degranulation does not occur (39).

2. Lymphocytes

Lymphocytes develop from pluripotent hemopoietic stem cells found in bone marrow (40). The lymphoid cell may develop into either a T-lymphocyte (maturation is thymus dependent) or a B-lymphocyte (maturation is thymus independent and dependent on the bursa of Fabricius in birds or possibly its equivalent in other animals) (41). The T-lymphocyte is primarily associated with cell-mediated immunity because it is capable of reacting to antigen either directly or indirectly through the recruitment and activation of various inflammatory cells (42). The B-lymphocyte is primarily associated with humoral immunity because it is capable of differentiating into a plasma cell capable of producing immunoglobulin (43).

In vitro the two lymphocyte types are differentiated by the ability of T-lymphocytes to form rosettes with sheep red blood cells and by the presence of surface immunoglobulin on B-lymphocytes (44, 45). Peripheral blood lymphocytes from normal cattle consist of approximately 63% T-lymphocytes, 11% B-lymphocytes, and 26% null cells (46); null cells lack the characteristics of either mature T- or B-lymphocytes.

A complex cooperation occurs between the inflammatory cells involved in the immune response. Many of these processes seem to be governed by specifically reactive T-lymphocytes which are capable of enhancing or suppressing the activities of B-lymphocytes, macrophages, PMNS, and non-committed T-lymphocytes (7).

The term "lymphokine" was used by Dumonde et al. in 1969 to include all biologically active non-antibody mediators generated after lymphocyte activation (47). More recently, Bendtzen restricted the term to antigen-nonspecific biologically active proteins produced by lymphoid cells (7). These mediators are classified according to their biological functions *in vitro* and their hypothesized roles *in vivo* (7, 47).

Some lymphokines affect T- and B-lymphocytes; these include mitogenic factor (48), antibody inhibitory material (49), Type II interferon (50), and a histamine-induced suppressor factor (51). Other lymphokines affect the behavior of other cells participating in the immune response, including macrophages (52), eosinophiles (53), platelets (54), and PMNS (55, 56, 57).

A lymphokine of primary importance in the leukocyte migration inhibition assay is the leukocyte migration inhibitory factor (LIF) (7). The LIF appears to be produced by an interaction of T- and B-lymphocytes or by a

subpopulation of lymphocytes because neither T-lymphocytes nor B-lymphocytes alone or with monocytes produce the lymphokine. However when T- and B-lymphocytes are mixed LIF is produced in abundance (58). LIF selectively inhibits the migration of PMNS; macrophage migration is not inhibited by LIF (55). LIF inhibition of PMNS is not species specific because guinea pig PMNS are susceptible to the action of human LIF (59).

LIF produced in cattle has not been extensively physiochemically analyzed. Therefore, for the purposes of this discussion the properties of human LIF will be described with the assumption that bovine LIF is similar. LIF has a molecular weight of 50,000 to 70,000 (60, 61). LIF is considered a protein because of its buoyant density in isopycnic centrifugation studies, its susceptibility to chymotrypsin, and its resistance to neuraminidase (60). LIF is stable when exposed to 50° C for 1 hour, but is destroyed when exposed to 80° C for 30 minutes; LIF is stable when treated for 30 minutes to a pH between 4 and 10, but partially destroyed at pH 3 or 11 (62). LIF appears to be an esterase and a protease (51, 62, 63). LIF obtained from sensitized lymphocytes stimulated with an antigen or with Concanavalin A (a non-specific mitogen) have similar physiochemical and immunochemical properties (55, 60, 64).

Exposure of PMNS to LIF for 1 hour at 37° C appears to be sufficient to inhibit the motility of cells for the next 24 hours (7). A receptor mechanism of LIF-PMN interaction is indicated because only PMNS, not mononuclear cells or erythrocytes, are capable of absorbing the migration inhibitory activity from LIF-rich supernatants (7).

C. Leukocyte Migration Inhibition Assays

Leukocyte migration inhibition assays are considered *in vitro* correlates of cell-mediated immune responses. Holst in 1922 observed that the migration of human blood leukocytes out of capillary tubes was sometimes inhibited by tuberculin (65). Later, Rich and Lewis demonstrated tuberculin-induced migration inhibition of spleen and buffy coat explants from guinea pigs exhibiting delayed-type hypersensitivity to tuberculin (66). Leukocyte migration inhibition has been used as an indicator of cell-mediated immune responses in humans (8, 9, 10), cattle (11, 12, 13, 14, 15), chickens (16), and swine (17).

To study the migration of leukocytes, usually either capillary tubes or agarose plates are used (8, 67, 68, 69). In the capillary tube technique, peritoneal exudate cells or peripheral blood leukocytes are placed in glass capillary tubes. The tubes are placed horizontally in chambers containing cell culture media; during incubation the cells migrate out of the tubes and spread on the bottom of the chambers. In the agarose plate technique, petri dishes containing a mixture of agarose and cell culture media are used. Suspensions of peripheral blood leukocytes are placed in wells cut in agarose; during incubation the cells migrate out of the wells in the space between the agarose and the supporting dish (12).

There are few reports of the use of leukocyte migration assays to study brucellosis in cattle. Lee (13) and Azadegan et al. (14) examined the responses of calves vaccinated with *B. abortus* strain 19. Dorsey and Deyoe examined experimental infection in heifers (15). There are no reports available comparing the leukocyte migration responses of adult vaccinated

cattle to the responses of experimentally infected cattle. Because in some geographic areas adult vaccination of cattle with B. abortus strain 19 is used as a means of controlling the spread of brucellosis, there is a problem of differentiating the responses of vaccinated cattle from the responses of cattle infected with B. abortus (21).

III. MATERIALS AND METHODS

A. Blood Sample Collection

Approximately 90 ml of peripheral blood were aseptically collected by jugular venapuncture and placed in 2 sterile silicone-treated 15 x 120 mm screw-cap tubes. The anticoagulant was a 50% concentrate of acid-citrate-dextrose[1] (ACD) at a 1:10 v/v ratio with the blood. The tubes were rotated to prevent the blood from clotting and kept at 22-25° C. Coagulated samples were discarded.

B. Harvesting Cells from Blood

1. Polymorphonuclear leukocytes (PMNS)

The PMN-rich suspension was prepared by the method described by Roth and Kaeberle (70). Approximately 80 ml of whole blood containing ACD were centrifuged for 20 minutes at 1000 x g. The plasma layer, buffy coat, and a few ml of red blood cells were aseptically removed by aspiration. The erythrocytes were lysed by aseptically diluting them 2:1 with sterile phosphate buffered (0.01 M) triple distilled water (pH 7.4, 22-25° C) in an Erlenmeyer flask. The flask was rotated for 45 seconds and one volume of sterile phosphate buffered (0.03 M) triple distilled water containing 2.7% NaCl (pH 7.4, 22-25° C) was added to restore isotonicity. This preparation was transferred to 2 sterile silicone-treated 15 x 120 mm screw-cap tubes and centrifuged at 200 x g for 10 minutes.

The pelleted PMNS were resuspended and washed twice in approximately 10 ml phosphate buffered (0.01 M) saline (PBS, pH 7.4, 22-25° C). The

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[1] 50% concentrate ACD:

Citric Acid	80 gm
Sodium Citrate	20 gm
Dextrose	25 gm
Triple distilled water	500 ml

cells were centrifuged at 200 x g for 10 minutes between each wash. After the second wash, the pelleted PMNS were resuspended in 0.8 ml bicarbonate buffered (0.0268 M) Medium 199 without Ca^{++} and Mg^{++} (M199)[2]. The cell viability, as determined by trypan blue staining, was a minimum of 95%. The cell suspension, as determined by Wright's stain, was 96-98% PMNS, 2-4% erythrocytes, and less than 1% miscellaneous cells.

2. Lymphocytes

The lymphocyte-rich suspension was prepared by a modification of the method described by Boyum (71). Fifteen ml of whole blood containing ACD were diluted 1:3 in PBS (pH 7.4). Twenty ml of the diluted blood were carefully layered onto 8 ml Histopaque-1077[3] (22-25° C) in each of 2 sterile 15 x 120 mm silicone-treated screw-cap tubes.

The tubes were centrifuged at 400 x g for 45 minutes. The center milk-like phase, containing the lymphocytes, was removed using a sterile 5 ml glass pipette with a pro-pipetter. The lymphocytes were transferred to a sterile silicone-treated tube and washed with bicarbonate buffered (0.0268 M) Hanks balanced salt solution without Ca^{++} and Mg^{++} (HBSS)[4]. The ratio of HBSS to lymphocyte suspension was 3:1. After centrifugation at 200 x g for 10 minutes, the supernatant was aseptically removed and the pelleted lymphocytes resuspended in 1 ml of M199 (22-25° C). The cell viability, as determined by trypan blue staining, was a minimum of 97%. The cell suspension, as determined by Wright's stain, was 80-85% lymphocytes,

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[2] Gibco, Grand Island, NY (Lot Nos. 20N6210 and 18P7701).

[3] Sigma, St. Louis, MO (Lot Nos. 11F-6099 and 41F-6109).

[4] Gibco, Grand Island, NY (Lot Nos. 20K1011 and C899305).

10-15% monocytes/macrophages, and 1-5% miscellaneous cells including eosinophiles and basophiles.

C. Antigen Production

A Brucella whole cell antigen was prepared as described by Thoen et al. (72). Lyophilized B. abortus strain 19 was reconstituted with sterile diluent.[5] Tryptose broth in 5 ml quantities was inoculated with 0.2 ml of the B. abortus solution and incubated for 72 hours at 37° C. Roux flasks containing tryptose agar were inoculated with 2 ml aliquots of the 72 hour broth cultures. After incubation at 37° C for 4 days, the cells were harvested by washing the agar surface with sterile PBS (pH 7.4). The PBS containing the B. abortus cells was autoclaved for 30 minutes at 121° C with 17 p.s.i. and centrifuged for 45 minutes at 800 x g. After aseptic removal of the supernatant, the cells were resuspended and washed once with approximately 20 ml sterile PBS (pH 7.4). The cells were resuspended in 3 ml sterile PBS (pH 7.4); this concentrated antigen stock was stored at 22-25° C.

D. Concanavalin A Preparation

Concanavalin A (Con A) from Conavalia ensformis (Jack beans)[6] was the mitogen used. Aliquots of stock solution containing 1 mg/ml in M199 were stored at -70° C. The stock was diluted and used the same day.

E. Preparation of Agarose Plates

Agarose plates were made daily. Each 60 x 15 mm plastic petri dish[7]

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[5] National Veterinary Services Laboratory, Ames, IA.

[6] Sigma, St. Louis, MO (Lot No. 79C-7620).

[7] Falcon, Oxnard, CA.

contained a 5 ml mixture of agarose indubiose A45[8], 50% M199, and 10% heat-inactivated serum. The agarose was dissolved in triple distilled water using a 100° C water bath and autoclaved for 5 minutes at 121° C and 17 p.s.i. All components were equilibrated at 50° C before combining. The plates were incubated at 4° C for 1 hour; six 2.5 mm equidistant wells were cut in each plate using a sterile stainless steel punch and plexiglass template. The agarose plugs were aseptically removed using a sterile Pasteur pipette connected to an aspirator. The plates were incubated at 37° C in a humidified atmosphere containing 5% CO_2 until used later that day. Immediately prior to use the wells were aseptically aspirated to remove any condensation.

F. Leukocyte Migration Inhibition Assay Procedures

The cells from each animal were divided into six test suspensions:

1. PMNS (4×10^6 cells)
2. PMNS with Brucella antigen
3. PMNS with Con A
4. PMNS (4×10^6) plus lymphocytes (2×10^6 cells)
5. PMNS plus lymphocytes with Brucella antigen
6. PMNS plus lymphocytes with Con A

The v/v ratio of cells to antigen/mitogen was 2:1; the suspensions that did not contain antigen or mitogen contained M199. The total volume of each suspension was 500 µl (200 µl PMNS, 200 µl lymphocytes, 100 µl antigen/mitogen). The suspensions were placed on a horizontal shaker for 1 minute and incubated at 37° C in a humidified atmosphere containing 5%

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[8] Gallard-Schlesinger Chemical Mfg., Carle Place, NY (Pharmindustrie agarose Lot No. 3160).

CO^2 for 1 hour. Each suspension (Nos. 1-6) was mixed on a vortex; then 7 µl of each suspension were placed in each of 2 wells cut in agarose plates. The plates were incubated at 37° C in a humidified atmosphere containing 5% CO_2 for 12 hours.

G. Evaluation of Data

After incubation, the plates were flooded with reagent grade methanol for 30 minutes (22-25° C). The methanol was poured off and 45% reagent grade formaldehyde added. After 30 minutes the formaldehyde and agarose were carefully removed. The cells fixed to the petri dish were stained for 15 minutes with 2% crystal violet.

Two measurements of the diameter of cell migration were made at 90° angles to each other. Measurements (mm) were averaged and the area of migration was calculated ($\pi(1/2 \text{ diameter})^2$).

Percent migration (%M) was calculated as follows:

$$\%M = \left[\frac{\text{area of migration of cells with mitogen minus area of well}}{\text{area of cells without mitogen minus area of well}} \right] \times 100$$

Percent migration inhibition (%MI) was calculated as 100-%M. Results were statistically compared by analysis of variance.

IV. EXAMINATION OF SOME VARIABLES IN A LEUKOCYTE MIGRATION INHIBITION ASSAY

A. Serums Used to Supplement Cell Culture Media

1. Serums from different animal species

Heat-inactivated horse[9], fetal calf[10], and newborn calf[11] serums were evaluated by comparing the areas of migration of PMN-rich suspensions collected from 3 cows not exposed to B. abortus. Agarose plates containing 0.8% agarose, 50% M199, and 10% of each respective serum were prepared. Cell suspensions from each of 3 cows were placed in duplicate wells cut in agarose plates. Three agarose plates containing each serum were used for each PMN sample. After incubation for 12 hours at 37° C in a humidified atmosphere containing 5% CO_2 the diameter of migration was measured and the area of migration calculated. The largest areas of migration were observed in plates containing newborn calf serum (mean = 78 mm^2). The mean area of migration observed in plates containing horse serum was 50 mm^2. The lowest areas of migration were observed in plates containing fetal calf serum (mean = 27 mm^2). Newborn calf serum was selected as the media supplement.

2. Comparison of different lots of newborn calf sera

Six lots[12] of heat-inactivated newborn calf sera were evaluated using PMN-rich suspensions prepared from 3 cows not exposed to B. abortus. Agar-

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[9] Gibco, Grand Island, NY (Lot No. 23K2002).

[10] Gibco, Grand Island, NY (Lot No. 19P1131).

[11] Gibco, Grand Island, NY (Lot No. 26P9301).

[12] Gibco, Grand Island, NY (Lot Nos. 20K9305, 24P9301, 25P9301, 26P9301, 27K1202 and 30N2902).

ose plates containing 0.8% agarose, 50% M199, and 10% of each respective lot of serum were prepared. Cell suspensions from each of 3 cows were placed in duplicate wells cut in agarose plates. Three agarose plates containing each serum lot were used for each PMN sample. The plates were incubated for 12 hours at 37° C in a humidified atmosphere containing 5% CO_2. The cells were fixed to the plates and the area of migration calculated. Results are shown in Table 1.

Table 1. Area of migration (mean mm^2) of PMNS obtained from 3 cows (Nos. 1, 2 and 3) not exposed to B. abortus. Agarose plates contained 0.8% agarose, 50% M199, and 10% of each respective lot (Nos. 20K9305, 24P9301, 25P9301, 26P9301, 27K1202 and 30N2902) of newborn calf serum obtained from Gibco, Grand Island, NY

Serum Lot number	Cow No. 1	Cow No. 2	Cow No. 3
20K9305	59.0	146.8	78.5
24P9301	15.9	28.3	50.3
25P9301	33.2	78.5	70.9
26P9301	73.4	140.4	98.0
27K1202	<5	<5	<5
30N2902	75.9	161.3	89.4

PMN suspensions incubated in agarose plates containing Lot 27K1202 did not migrate out of the wells. The greatest areas of migration (mean) of PMNS obtained from 2 of 3 cows (Nos. 1 and 2) were observed using plates containing Lot 30N2902; therefore, Lot 30N2902 was chosen to supplement cell culture media.

B. Concentration of Agarose

Three concentrations of agarose were evaluated using PMN-rich suspensions obtained from 3 cows not exposed to B. abortus. Plates containing 0.8%, 0.7%, or 0.6% agarose, 50% M199, and 10% newborn calf serum[13] were

[13] Gibco, Grand Island, NY (Lot No. 30N2902).

prepared. Duplicate wells in each plate were filled with each PMN suspension. Three plates containing each agarose concentration were used for each sample. The plates were incubated for 12 hours at 37° C in a humidified atmosphere containing 5% CO_2.

The plates containing 0.6% agarose were considered soft and unsuitable for use in the assay. In plates containing 0.7% agarose the areas of migration were 10-15 mm^2 greater than in plates containing 0.8% agarose; therefore the concentration of agarose in the plates used for the duration of the study was 0.7%.

C. Titration of Brucella Antigen

Seven concentrations of a Brucella abortus whole cell antigen were evaluated. The antigen concentrations were equivalent to McFarland nephelometer tubes 3, 2, 1, and dilutions of tube 1 (1:4, 1:8, 1:16 and 1:32). Lymphocyte and PMN suspensions from 2 cows (Nos. 26 and 36) from which B. abortus strain 2308 had been isolated, 2 cows (Nos. 1280 and 3746) vaccinated with B. abortus strain 19, and 1 cow (No. 3396) not exposed to B. abortus (control) were used. The cell suspensions contained 4 x 10^6 PMNS and 2 x 10^6 lymphocytes in 400 µl. One hundred µl of antigen were added to cell suspensions and incubated at 37° C in 5% CO_2 for 1 hour. Control cell suspensions (no antigen) contained 100 µl of M199. Each cell suspension was placed in duplicate wells cut in agarose plates. The assay was performed in triplicate. The plates were incubated for 12 hours at 37° C in a humidified atmosphere containing 5% CO_2. The cells were fixed to the petri dish, stained, and the percent migration inhibition calculated. Results are shown in Table 2.

Table 2. Percent migration inhibition (mean) using PMNS plus lymphocytes obtained from 2 cows (Nos. 26 and 36) from which B. abortus strain 2308 was isolated, 2 cows (Nos. 1280 and 3746) vaccinated with B. abortus strain 19, and 1 cow (No. 3396) not exposed to B. abortus. PMNS plus lymphocytes were exposed to whole cell Brucella antigen concentrations equivalent to McFarland nephelometer tubes 3, 2, 1 and dilutions of tube 1 (1:4, 1:8, 1:16 and 1:32)

Cow number	Concentration of Brucella antigen						
	Mc3	Mc2	Mc1	Mc1 1:4	Mc1 1:8	Mc1 1:16	Mc1 1:32
26	89.2	86.8	96.7	83.3	81.4	83.3	0.0
36	79.9	75.0	84.0	64.0	72.4	66.9	0.0
1280	87.7	86.6	78.2	81.2	87.8	85.3	72.0
3746	94.0	96.0	96.0	84.0	87.8	79.8	0.0
3396	14.0	13.8	13.8	7.0	7.0	3.4	0.0

When cell suspensions were exposed to Brucella antigen at a 1:32 dilution of a McFarland nephelometer tube 1, the migration of PMNS plus lymphocytes (PMNS/L) obtained from both cows (Nos. 26 and 36) from which B. abortus was isolated and 1 of 2 cows (No. 3746) vaccinated with B. abortus strain 19 was not inhibited. At a 1:8 or a 1:16 dilution of a McFarland nephelometer tube 1 the migration of PMNS/L from cows (Nos. 26, 36, 1280 and 3746) exposed to B. abortus was inhibited; the migration of PMNS/L from the cow (No. 3396) not exposed to B. abortus was not inhibited. The antigen concentration equivalent to a 1:8 dilution of a McFarland Nephelometer tube 1 (O.D. = 0.055 at 525 nm) was chosen for use in the assay. Each time the antigen was used 0.5 ml of the suspension was placed on a blood agar plate and incubated for 48 hours at 37° C to evaluate for contamination.

D. Titration of Concanavalin A

The PMNS and lymphocytes from 1 cow (No. 54) from which B. abortus strain 2308 was isolated, 1 cow (No. 63) vaccinated with B. abortus strain

19, and 2 cows (Nos. 1329, 3396) not exposed to B. abortus (controls) were used to titrate Concanavalin A (Con A). Five concentrations of Con A (20 µg/ml, 10 µg/ml, 5 µg/ml, 3 µg/ml, and 2.5 µg/ml) were evaluated. One hundred µl of each Con A suspension were added to 400 µl containing 4×10^6 PMNS plus 2×10^6 lymphocytes and incubated for 1 hour at 37° C with 5% CO_2. Cell suspensions containing 100 µl of M199 instead of Con A were migration controls. Each suspension was placed in duplicate wells cut in plates containing 0.7% agarose, 50% M199, and 10% newborn calf serum. The assay was performed in triplicate. The plates were incubated for 12 hours at 37° C in a humidified atmosphere containing 5% CO_2. Percent migration inhibition was calculated. Results are shown in Table 3.

Table 3. Percent migration inhibition (mean) using PMNS plus lymphocytes obtained from 1 cow (No. 54) from which B. abortus strain 2308 was isolated, 1 cow (No. 63) vaccinated with B. abortus strain 19, and 2 cows (Nos. 1329 and 3396) not exposed to B. abortus. PMNS plus lymphocytes were exposed to 20 µg/ml, 10 µg/ml, 5 µg/ml, 3 µg/ml or 2.5 µg/ml Concanavalin A.

Cow number	Concentration of Concanavalin A				
	20 µg/ml	10 µg/ml	5 µg/ml	3 µg/ml	2.5 µg/ml
54	64.0	62.5	57.4	57.4	0.0
63	81.0	81.0	66.1	54.0	0.0
1329	91.2	91.2	71.9	75.0	0.0
3396	84.4	84.4	78.0	54.5	0.0

A Con A concentration of 2.5 µg/ml did not inhibit the migration of PMNS plus lymphocytes. The concentration of 5 µg/ml inhibited the migration of PMNS plus lymphocytes obtained from all 4 cows and was selected for use in the assay.

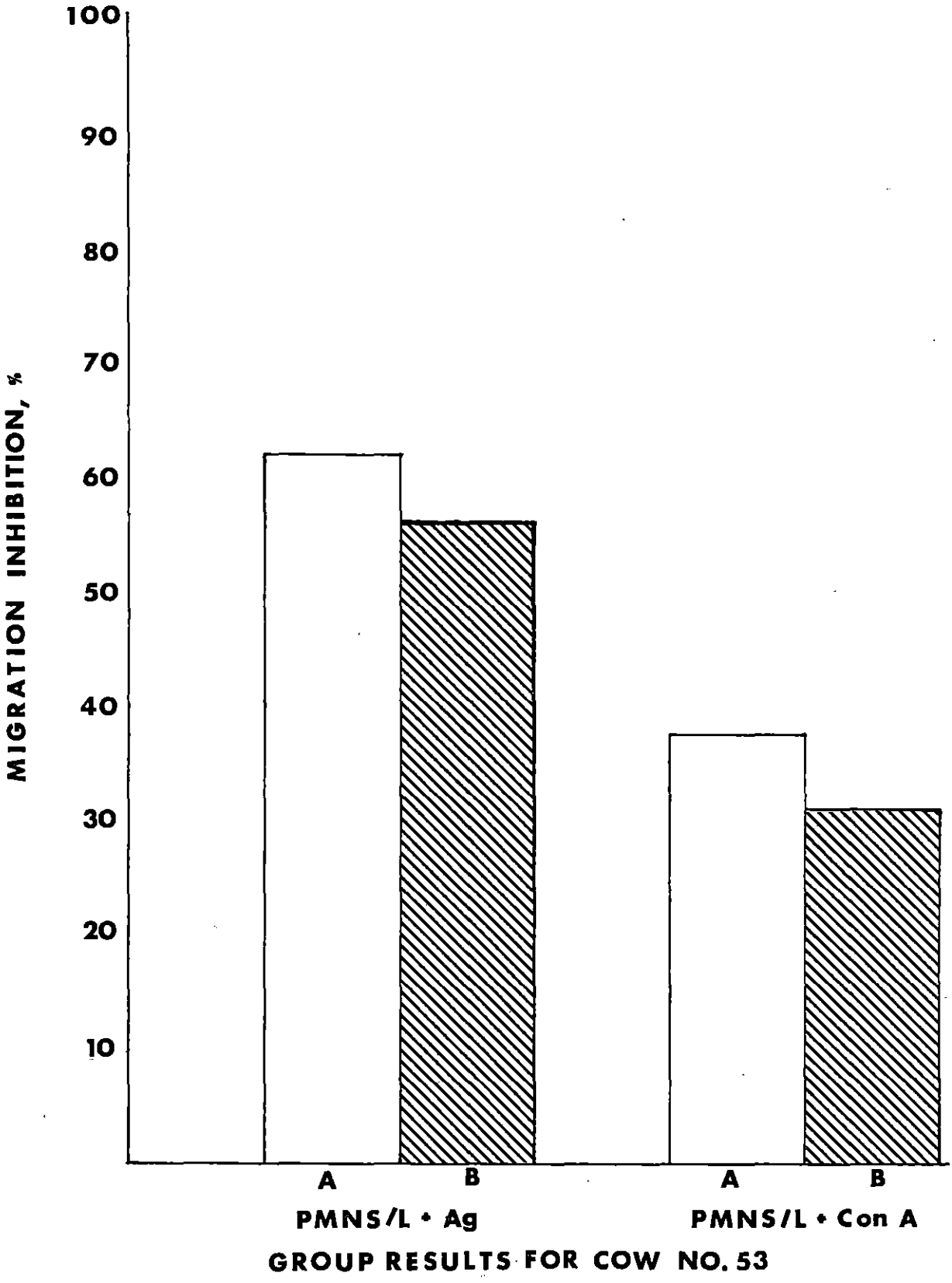
E. Reproducibility of the Assay

To examine the variability of results obtained in a leukocyte migration inhibition assay 200 ml of peripheral blood were asceptically collected from each of 3 cows; B. abortus strain 2308 was isolated from 2 cows (Nos. 53, 67), 1 cow (No. 3396) was not exposed to B. abortus. Fifty ml of blood were placed in each of 4 sterile silicone-treated 15 x 120 mm screw-cap tubes containing ACD. The 4 tubes of blood from each cow were randomly placed into two groups, A and B (2 tubes in each group). These groups were processed separately. The groups were identified as 53A, 53B, 67A, 67B, 3396A, and 3396B.

The PMNS and lymphocytes were harvested and the cell suspensions exposed to a killed whole cell Brucella antigen or to Con A as previously described. Cell suspensions from each group were placed in duplicate wells cut in agarose plates containing 0.7% agarose, 50% M199, and 10% newborn calf serum. After incubation for 12 hours at 37^{o} C in a humidified atmosphere containing 5% CO_2, the percent migration inhibition was calculated. Results are shown in Graphs 1-3.

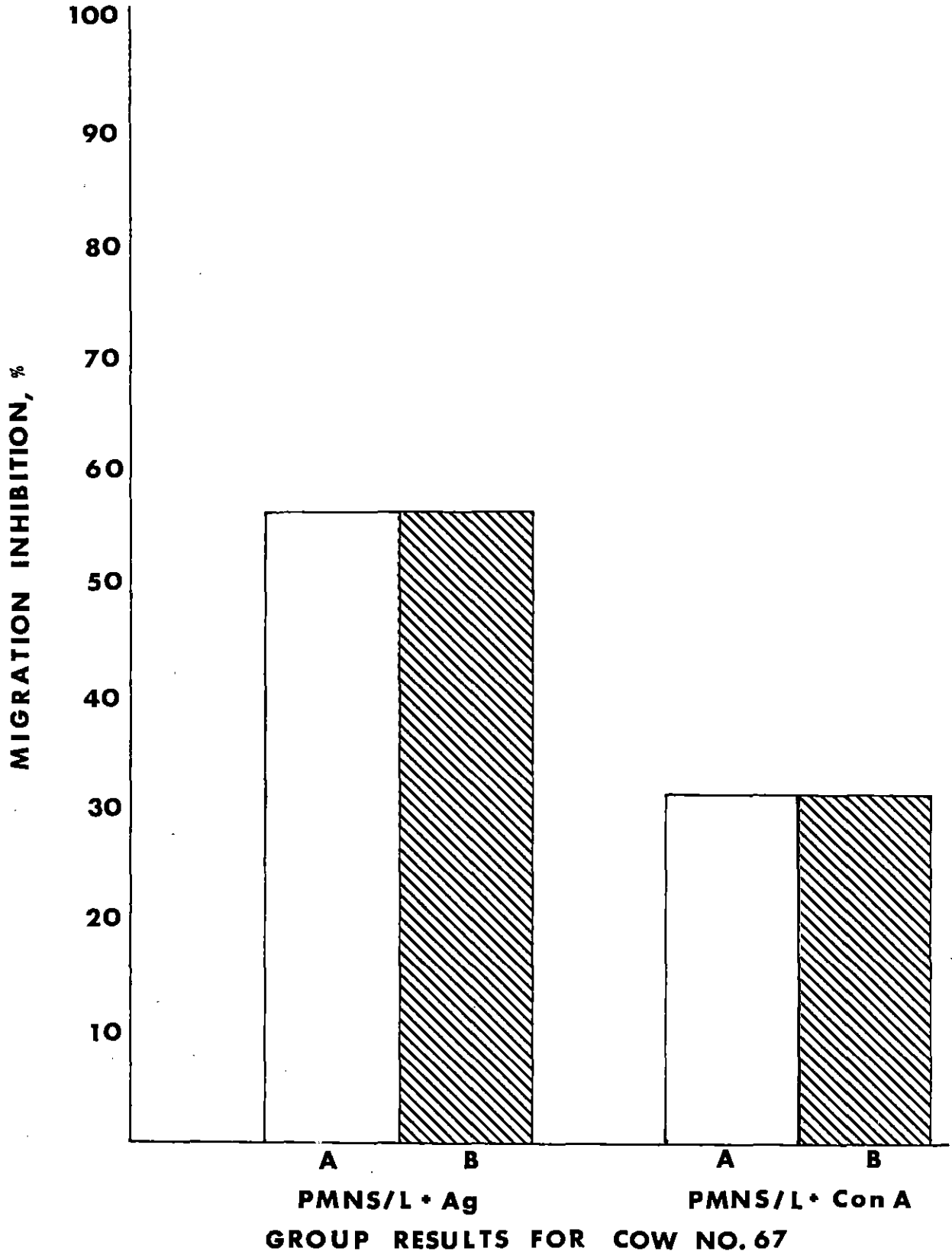
The percents migration inhibition (%MI) of PMNS plus lymphocytes (PMNS/L) from groups 53A and 53B following incubation with a Brucella antigen were 61.5 and 55.6 respectively. The %MI of PMNS/L from groups 53A and 53B following incubation with Con A were 37.1 and 30.6 respectively. The %MI was 55.6 for PMNS/L from each of groups 67A and 67B following incubation with the antigen. The %MI was 30.6 for PMNS/L from each of groups 67A and 67B following incubation with Con A. Although no differences were detected in the calculated %MI for PMNS/L from groups 67A and 67B, the areas

Graph 1. Percent migration inhibition (mean) using PMNS/L obtained from groups 53A and 53B (B. abortus strain 2308 isolated) and incubated with Brucella antigen (PMNS/L + Ag) or Con A (PMNS/L + Con A)



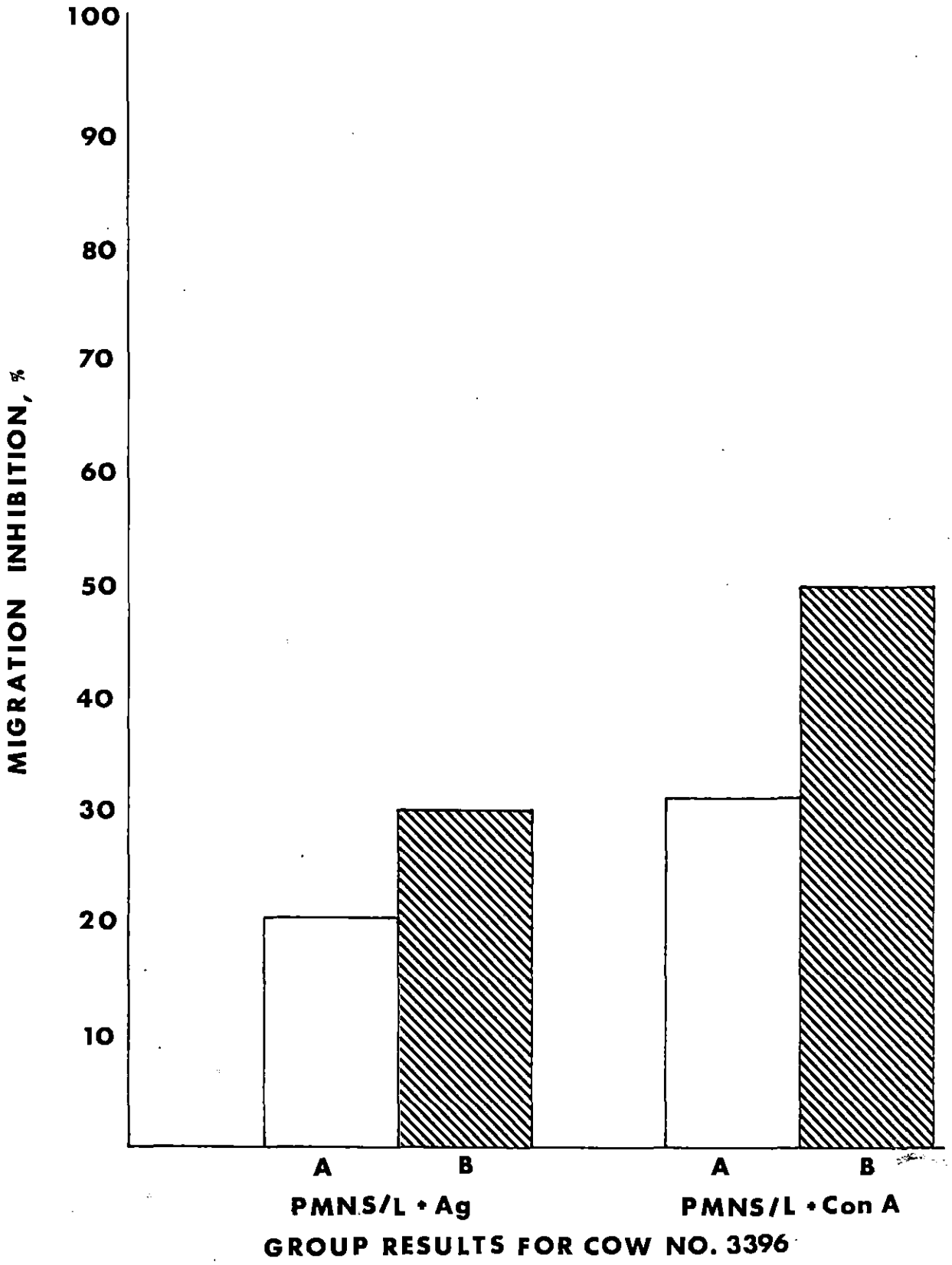
GROUP RESULTS FOR COW NO. 53

Graph 2. Percent migration inhibition (mean) using PMNS/L obtained from groups 67A and 67B (*B. abortus* strain 2308 isolated) and incubated with *Brucella* antigen (PMNS/L + Ag) or Con A (PMNS/L + Con A)



GROUP RESULTS FOR COW NO. 67

Graph 3. Percent migration inhibition (mean) using PMNS/L obtained from groups 3396A and 3396B (not exposed to B. abortus) and incubated with Brucella antigen (PMNS/L + Ag) or Con A (PMNS/L + Con A)



GROUP RESULTS FOR COW NO. 3396

of migration were different. The area of migration of PMNS/L from group 67A not exposed to antigen or Con A was 86.6 mm^2; the area of PMNS/L from group 67B not exposed to antigen or Con A was 113.1 mm^2. The %MI for PMNS/L from groups 3396A and 3396B following incubation with the antigen were 19.8 and 29.6 respectively. The %MI for PMNS/L from groups 3396A and 3396B following incubation with Con A were 30.6 and 49.0 respectively.

These findings provide evidence that suitable reproducibility was obtained with the assay using Brucella antigen or Con A.

V. LEUKOCYTE MIGRATION INHIBITION ASSAY OF CELLS FROM COWS FROM WHICH *BRUCELLA ABORTUS* STRAIN 2308 WAS ISOLATED

Blood was collected from each of 6 Charolais-Hereford cows (Nos. 26, 40, 53, 54, 61 and 62) at 111, 114, 116, 119 and 121 days postexposure to 2.98×10^7 colony forming units of *B. abortus* strain 2308 via the conjunctival route (0.45 ml in each eye). *B. abortus* was isolated from the vaginal discharge following parturition.[14] Two cows (Nos. 1329 and 3396) not exposed to *B. abortus* were included as controls.

The pattern of migration of PMNS and PMNS plus lymphocytes from cow No. 53 (*B. abortus* was isolated) is shown in Figure 1. The average area of migration of PMNS from cows (Nos. 26, 40, 53, 54, 61 and 62) exposed to *B. abortus* strain 2308 was greater in cultures containing PMNS alone as compared to cultures containing PMNS plus lymphocytes (average area of migration of PMNS was 98.6 mm^2; average area of migration of PMNS plus lymphocytes was 80.7 mm^2). The migration of PMNS alone or the migration of PMNS plus lymphocytes (PMNS/L) was greater in cultures not containing *Brucella* antigen or Con A as compared to the migration of PMNS and PMNS/L in cultures containing *Brucella* antigen or Con A. The %MI (group mean) was greater using cultures of PMNS/L incubated with *Brucella* antigen (62 ± 16) as compared to the %MI (group mean) using PMNS/L incubated with Con A (40 ± 24).

The average area of migration of PMNS from cows (Nos. 1329 and 3396) not exposed to *B. abortus* was greater in cultures containing PMNS alone as

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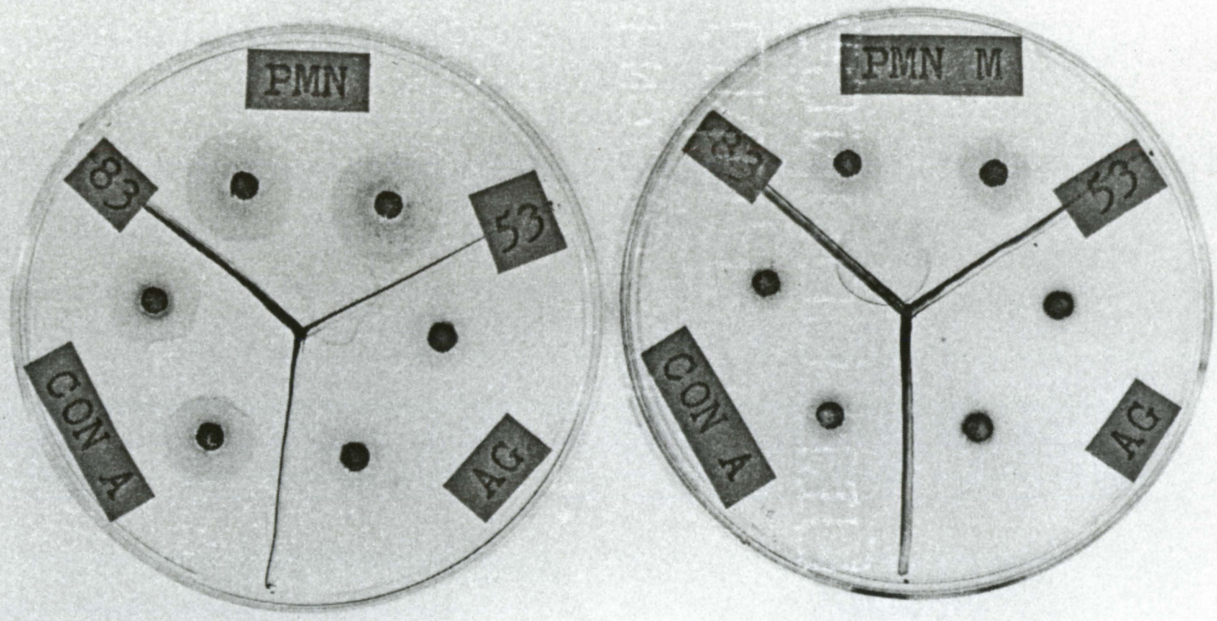
[14] Dr. Donald Pietz, U.S. Department of Agriculture, National Veterinary Services Laboratory, Ames, IA.

Figure 1. Pattern of migration, under agarose, of PMNS (A) and PMNS/L (B) obtained from cow No. 53 from which B. abortus strain 2308 was isolated. The migration of PMNS (PMN) or PMNS/L (PMN M) not incubated with Brucella antigen or Concanavalin A and the migration of these cells following incubation with Brucella antigen (Ag) or Concanavalin A (Con A) are shown



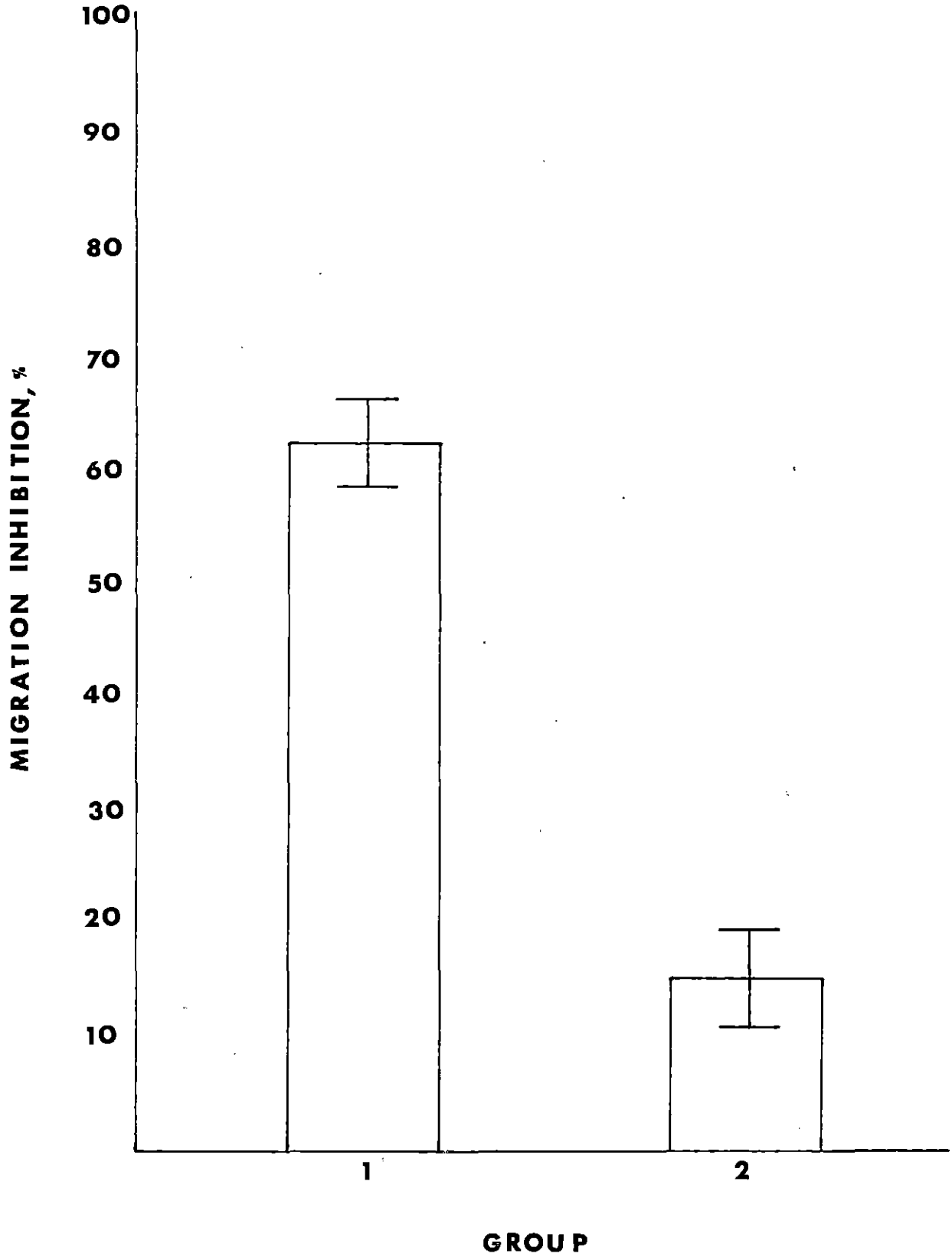
A B

compared to cultures containing PMNS/L (average area of migration of PMNS was 133 mm^2; average area of migration of PMNS/L was 108 mm^2). The migration of PMNS alone or the migration of PMNS/L was greater in cultures not containing Brucella antigen or Con A as compared to the migration of PMNS and PMNS/L in cultures containing Brucella antigen or Con A. The %MI (group mean) was greater using cultures of PMNS/L incubated with Con A (33 ± 16) as compared to the %MI (group mean) using PMNS/L incubated with Brucella antigen (15 ± 12).

The %MI using PMNS/L (group mean ± standard error) incubated with Brucella antigen or with Con A for two groups (6 cows from which B. abortus was isolated and 2 cows not exposed to B. abortus) are shown in Graphs 4 and 5 respectively. A significantly greater %MI (mean) of PMNS/L was observed for cows (Nos. 26, 40, 53, 54, 61, and 62) from which B. abortus was isolated as compared to control cows (Nos. 1329 and 3396) when PMNS/L were incubated with Brucella antigen ($p<0.002$). When PMNS/L were incubated with Con A, no significant differences were observed between the two groups ($p>0.05$).

The %MI using PMNS/L obtained from cows Nos. 26, 53, and 62 (B. abortus was isolated) and incubated with Brucella antigen or with Con A are shown in Graphs 6, 7, and 8 respectively. The %MI using PMNS/L obtained from cow No. 26 and incubated with Brucella antigen were 70.7, 57.2, 75.0 and 73.6 at 114, 116, 119 and 121 days postexposure. The PMNS/L incubated with Con A had the following %MI: 30.6, 14.8, 23.4 and 26.5 for the respective sample dates. The %MI using PMNS/L obtained from cow No. 53 and incubated with Brucella antigen were 65.1, 51.0, 44.8, 40.1 and 52.3 at 111, 114, 116, 119

Graph 4. Percent migration inhibition (group mean ± standard error) using PMNS/L obtained from cows (Nos. 26, 40, 53, 54, 61 and 62) from which B. abortus strain 2308 was isolated (group 1) and from cows (Nos. 1329 and 3396) not exposed to B. abortus (group 2). The PMNS/L were incubated with Brucella antigen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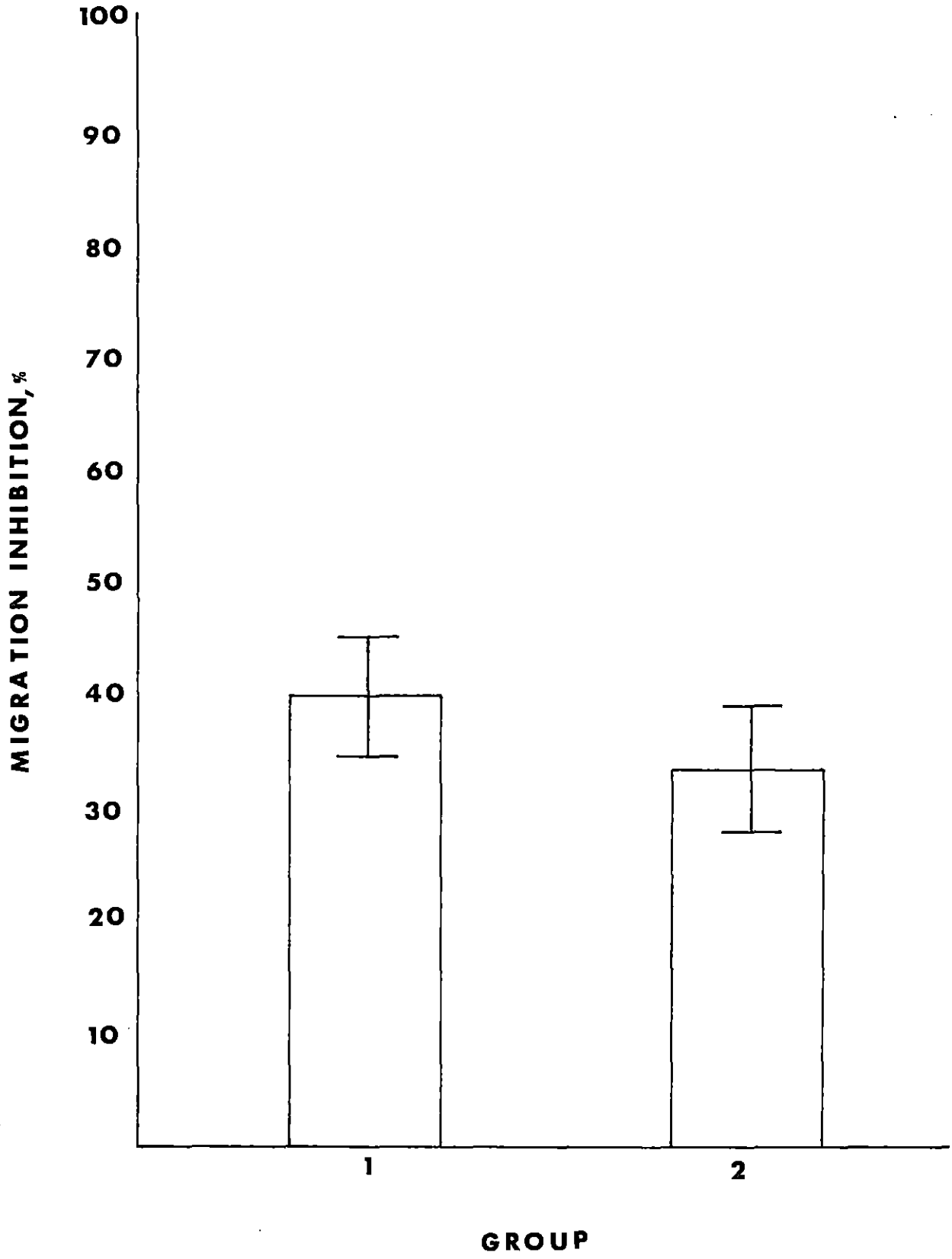
MIGRATION INHIBITION, %
1
2
GROUP

Graph 5. Percent migration inhibition (group mean ± standard error) using PMNS/L obtained from cows (Nos. 26, 40, 53, 54, 61 and 62) from which B. abortus strain 2308 was isolated (group 1) and from cows (Nos. 1329 and 3396) not exposed to B. abortus (group 2). The PMNS/L were incubated with Con A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MIGRATION INHIBITION, %
1
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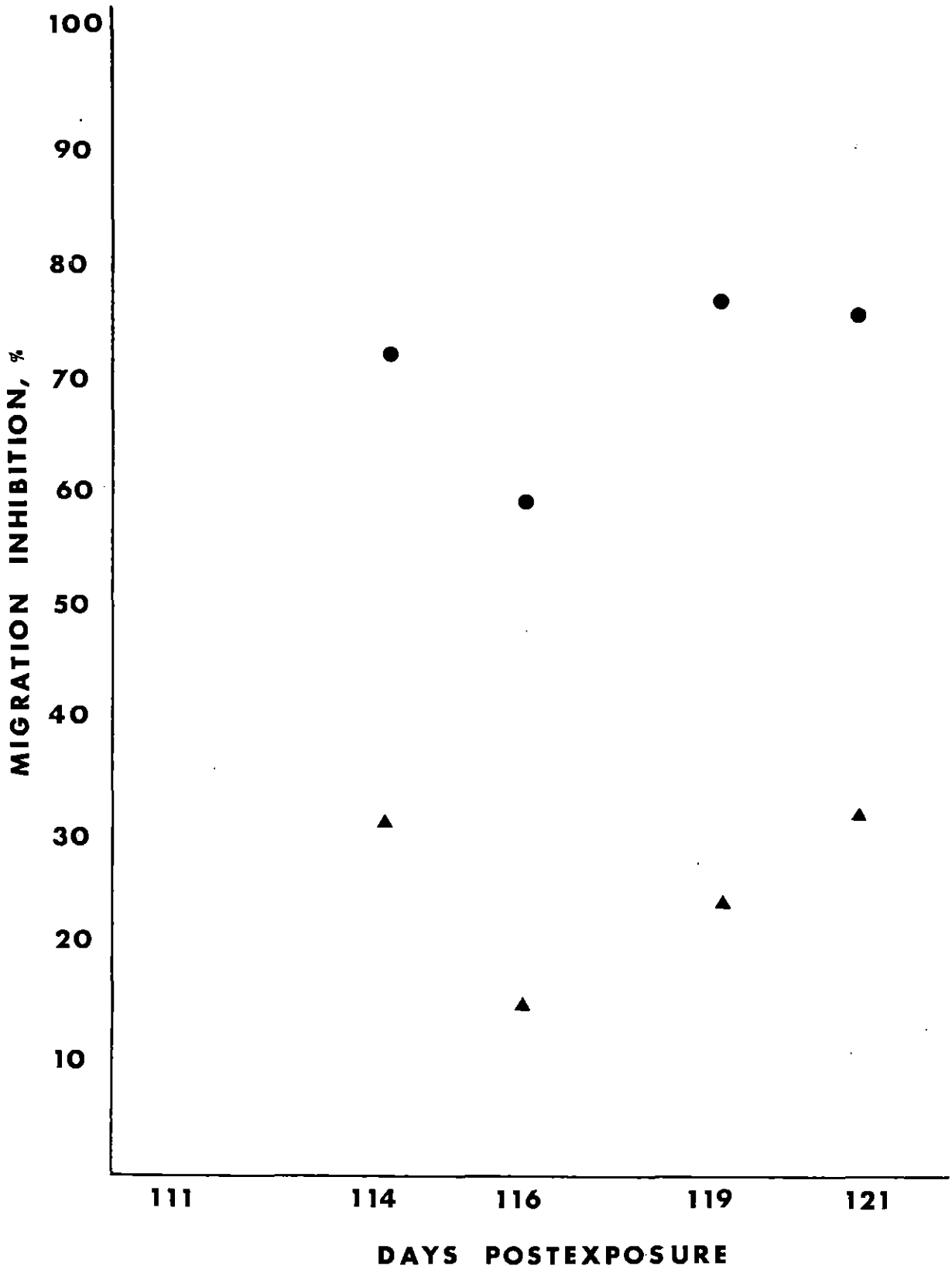
GROUP

Graph 6. Percent migration inhibition using PMNS/L obtained from cow No. 26 at 114, 116, 119 and 121 day postexposure to B. abortus strain 2308. The PMNS/L were incubated with Brucella antigen (●) or Con A (▲)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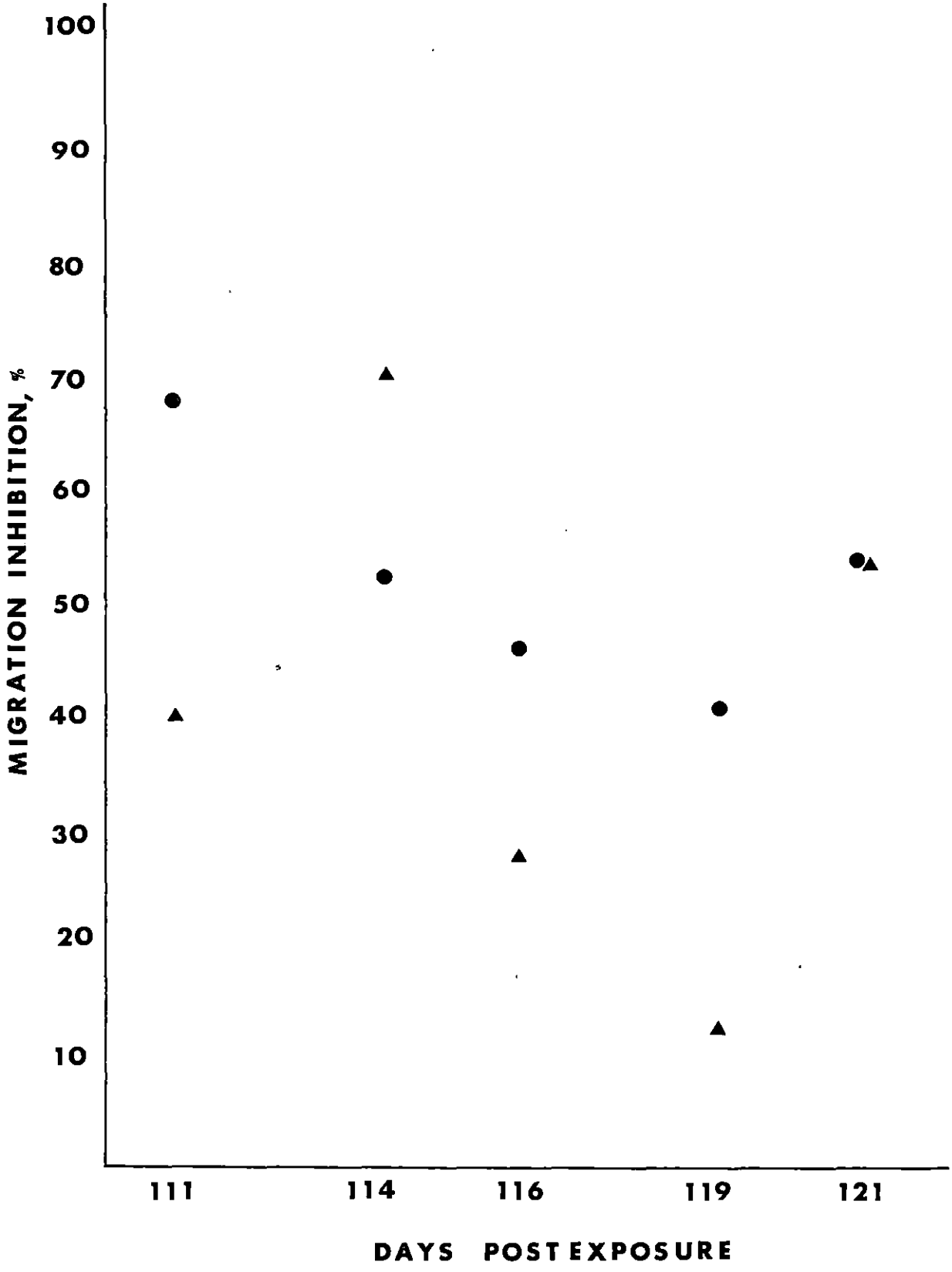
MIGRATION INHIBITION, %
111
114
116
119
121
DAYS POSTEXPOSURE

Graph 7. Percent migration inhibition using PMNS/L obtained from cow No. 53 at 111, 114, 116, 119 and 121 days postexposure to B. abortus strain 2308. The PMNS/L were incubated with Brucella antigen (●) or Con A (▲)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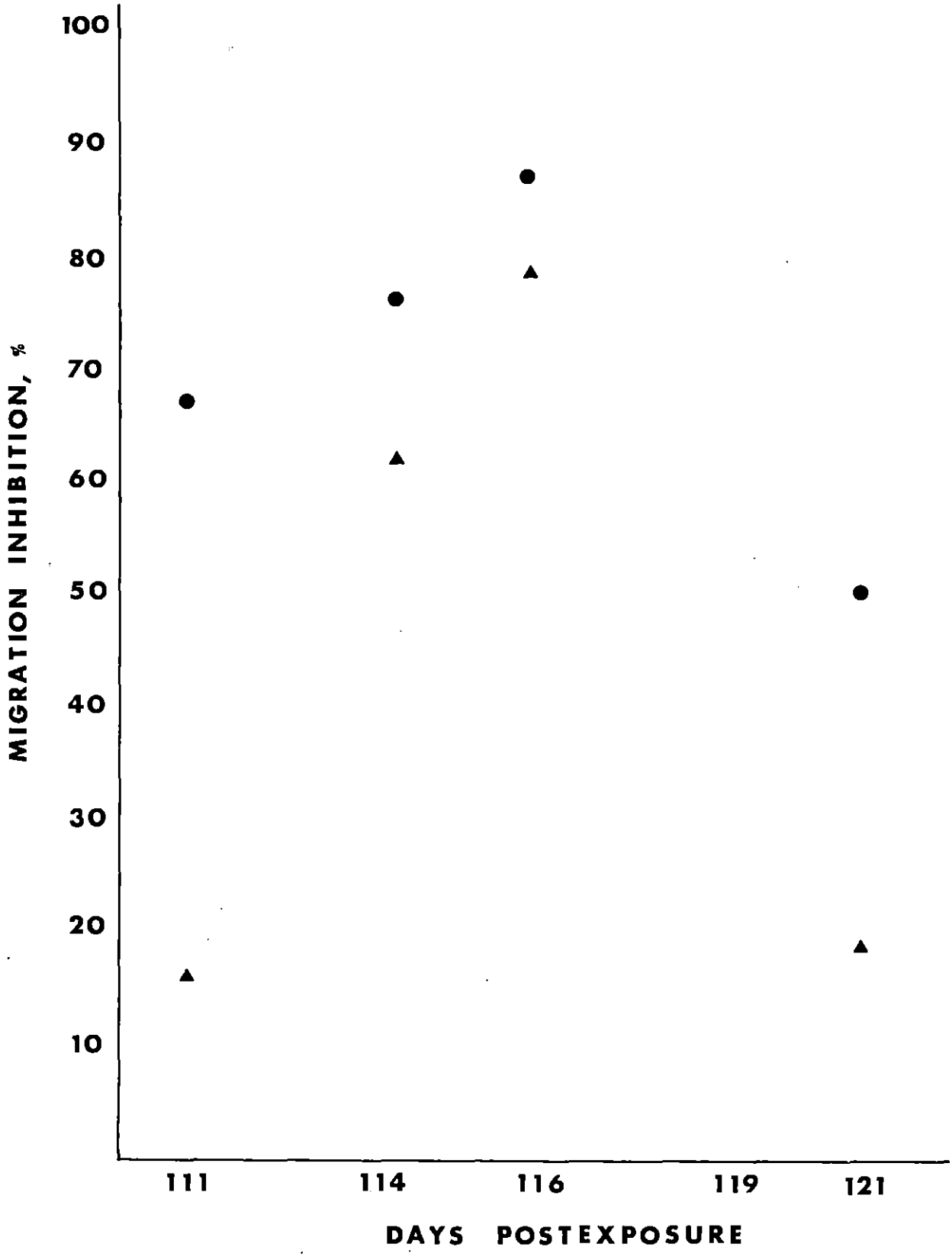
MIGRATION INHIBITION, %
111
114
116
119
121
DAYS POST EXPOSURE

Graph 8. Percent migration inhibition using PMNS/L obtained from cow No. 62 at 111, 114, 116 and 121 days postexposure to B. abortus strain 2308. The PMNS/L were incubated with Brucella antigen (●) or Con A (▲)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MIGRATION INHIBITION, %
111
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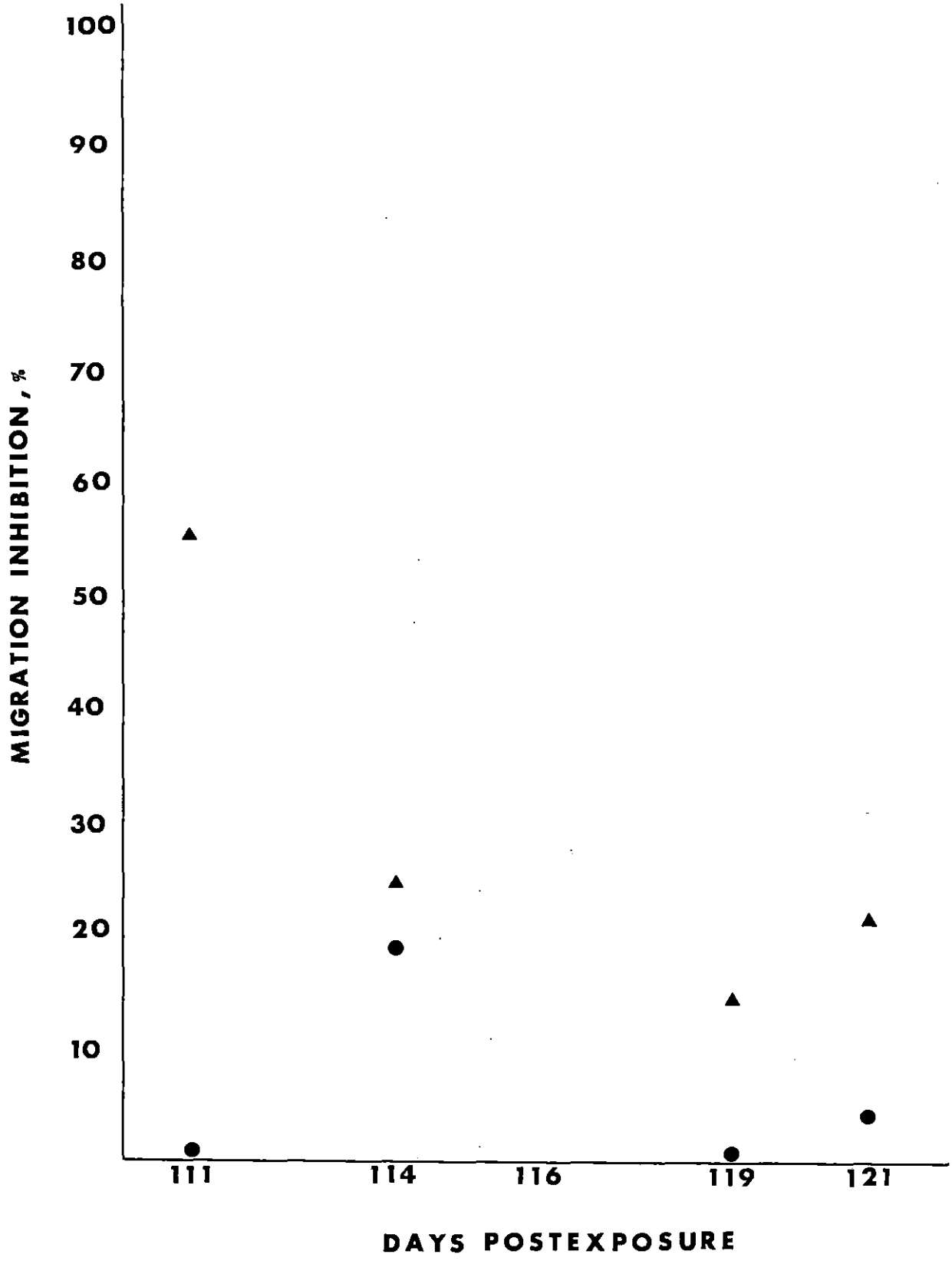
119
121
DAYS POSTEXPOSURE

and 121 days postexposure. The respective %MI obtained when PMNS/L were incubated with Con A were 40.3, 69.2, 26.5, 12.5 and 52.3. The %MI using PMNS/L obtained from cow No. 62 and incubated with Brucella antigen were 66.0, 75.0, 85.5 and 40.0 at 111, 114, 116 and 121 days postexposure. The respective %MI obtained when PMNS/L were incubated with Con A were 16.0, 60.9, 77.3 and 18.4. The %MI using PMNS/L obtained from cow No. 40 and incubated with Brucella antigen were 52.7, 58.7 and 88.1 at 116, 119 and 121 days postexposure. The respective %MI obtained when PMNS/L were incubated with Con A were 80.9, 26.5 and 76.7. The %MI using PMNS/L obtained from cow No. 54 and incubated with Brucella antigen were 43.8 and 47.1 at 119 and 121 days postexposure. The respective %MI obtained when PMNS/L were incubated with Con A were 68.4 and 17.4. The %MI using PMNS/L obtained from cow No.61 and incubated with Brucella antigen was 88.0 at 119 days postexposure. The respective %MI obtained when PMNS/L were incubated with Con A was 21.7.

The %MI using PMNS/L obtained from cow No. 3396 (not exposed to B. abortus) and incubated with Brucella antigen or Con A are shown in Graph 9. The %MI using PMNS/L obtained from cow No. 3396 and incubated with Brucella antigen were 0.0, 18.4, 0.0 and 4.4 corresponding to 111, 114, 119 and 121 days postexposure. The respective %MI obtained when PMNS/L were incubated with Con A were 54.1, 24.1, 14.2 and 21.0. The %MI using PMNS/L obtained from cow No. 1329 and incubated with Brucella antigen were 30.6, 18.1, 28.7 and 18.4 corresponding to 114, 116, 119 and 121 days postexposure. The respective %MI obtained when PMNS/L were incubated with Con A were 28.4, 48.5, 21.0 and 51.4.

Graph 9. Percent migration inhibition using PMNS/L obtained from cow No. 3396 (not exposed to B. abortus) on dates corresponding to 111, 114, 119 and 121 days postexposure to B. abortus strain 2308. The PMNS/L were incubated with Brucella antigen (●) or Con A (▲)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MIGRATION INHIBITION, %
111
114
116
119
121
DAYS POSTEXPOSURE

VI. LEUKOCYTE MIGRATION INHIBITION ASSAY OF CELLS FROM COWS VACCINATED WITH BRUCELLA ABORTUS STRAIN 19

Blood was collected from each of 2 Charolais-Hereford cows (Nos. 1280 and 3746) at 116, 118, 121 and 123 days postvaccination. The cows were each vaccinated by subcutaneous injection of 1.07×10^9 colony forming units of B. abortus strain 19. Two cows not exposed to B. abortus described in Part V were included as controls.

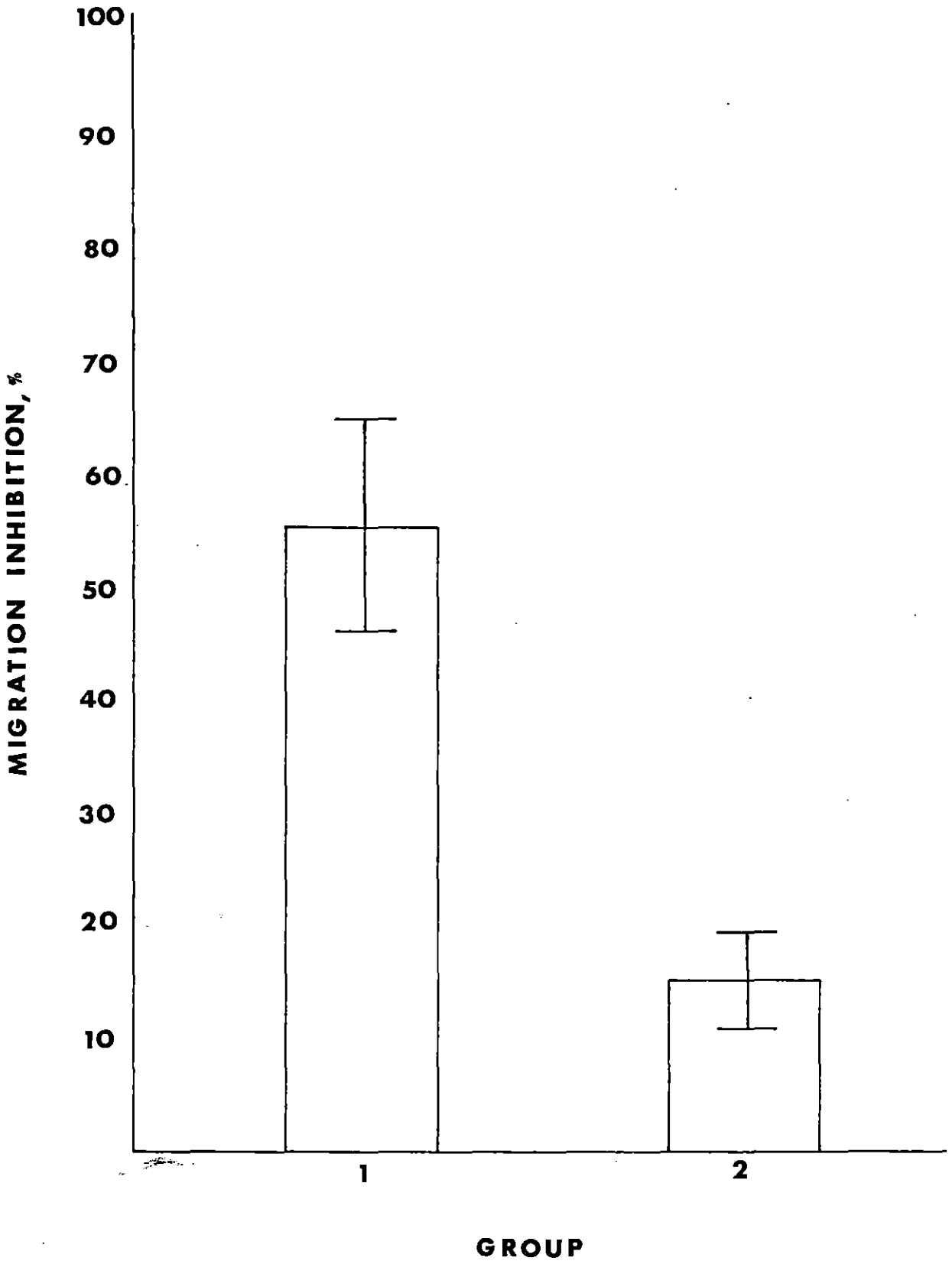
The average area of migration of PMNS obtained from cows (Nos. 1280 and 3746) vaccinated with B. abortus strain 19 was greater in cultures containing PMNS alone as compared to cultures containing PMNS plus lymphocytes (average area of migration of PMNS was 126.2 mm^2; average area of migration of PMNS/L was 115.0 mm^2). The migration of PMNS alone or the migration of PMNS/L was greater in cultures not containing Brucella antigen or Con A as compared to the migration of PMNS or PMNS/L in cultures containing Brucella antigen or Con A. The %MI (group mean) was greater using cultures of PMNS/L incubated with Brucella antigen (55 ± 23) as compared to the %MI (group mean) using PMNS/L incubated with Con A (32 ± 24).

The average area of migration of PMNS from cows (Nos. 1329 and 3396) not exposed to B. abortus was greater in cultures containing PMNS alone as compared to cultures containing PMNS/L (average area of migration of PMNS was 133 mm^2; average area of migration of PMNS/L was 108 mm^2). The %MI (group mean) was greater using cultures of PMNS/L incubated with Con A (33 ± 16) as compared to the %MI (group mean) using PMNS/L incubated with Brucella antigen (15 ± 12).

The %MI using PMNS/L (group mean ± standard error) incubated with Brucella antigen or with Con A for two groups (2 cows vaccinated with B. abortus strain 19 and 2 cows not exposed to B. abortus) are shown in Graphs 10 and 11. The %MI (group mean) was greater using PMNS/L obtained from cows (Nos. 1280 and 3746) vaccinated with B. abortus strain 19 as compared to PMNS/L obtained from cows (Nos. 1329 and 3396) when PMNS/L were incubated with Brucella antigen ($p<0.04$). When PMNS/L were incubated with Con A, no significant differences were observed between the two groups ($p>0.05$).

The %MI using PMNS/L obtained from cows Nos. 1280 and 3746 (vaccinated with B. abortus) and incubated with Brucella antigen or Con A are shown in Graphs 12 and 13. The %MI using PMNS/L obtained from cow No. 1280 and incubated with Brucella antigen were 28.8, 37.3 and 36.7 at 118, 121 and 123 days postvaccination. The PMNS/L incubated with Con A had the following %MI: 71.8, 16.0 and 33.1 for the respective sample dates. The %MI using PMNS/L obtained from cow No. 3746 and incubated with Brucella antigen were 75.0, 78.9 and 70.8 at 116, 118 and 121 days postvaccination. The respective %MI using PMNS/L incubated with Con A were 10.0, 46.7 and 15.4. The %MI using PMNS/L obtained from cow No. 3396 (not exposed to B. abortus) and incubated with Brucella antigen or Con A are shown in Graph 14. The %MI using PMNS/L obtained from cow No. 3396 and incubated with Brucella antigen were 18.4, 0.0 and 4.4 corresponding to 116, 121 and 123 days postvaccination. The respective %MI using PMNS/L incubated with Con A were 24.1, 14.2 and 21.0. The %MI using PMNS/L obtained from cow No. 1329 and incubated with Brucella antigen were 30.6, 18.1, 28.7 and 18.4 corresponding to 116, 118, 121 and 123 days postvaccination. The respective %MI

Graph 10. Percent migration inhibition (group mean ± standard error) using PMNS/L obtained from cows (Nos. 1280 and 3746) vaccinated with B. abortus strain 19 (group 1) and cows (Nos. 1329 and 3396) not exposed to B. abortus (group 2). The PMNS/L were incubated with Brucella antigen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MIGRATION INHIBITION, %
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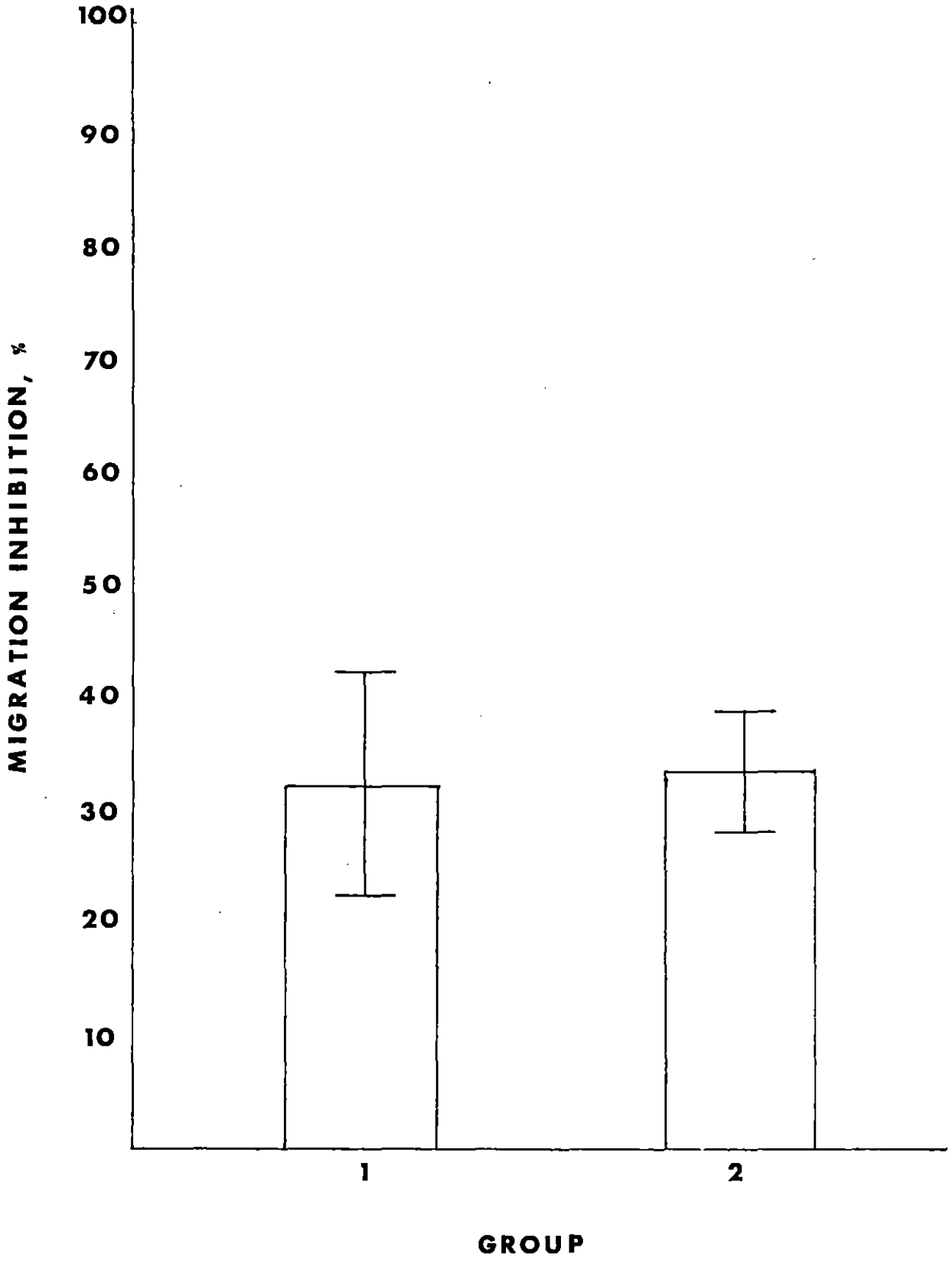
GROUP

Graph 11. Percent migration inhibition (group mean ± standard error) using PMNS/L obtained from cows (Nos. 1280 and 3746) vaccinated with B. abortus strain 19 (group 1) and cows (Nos. 1329 and 3396) not exposed to B. abortus (group 2). The PMNS/L were incubated with Con A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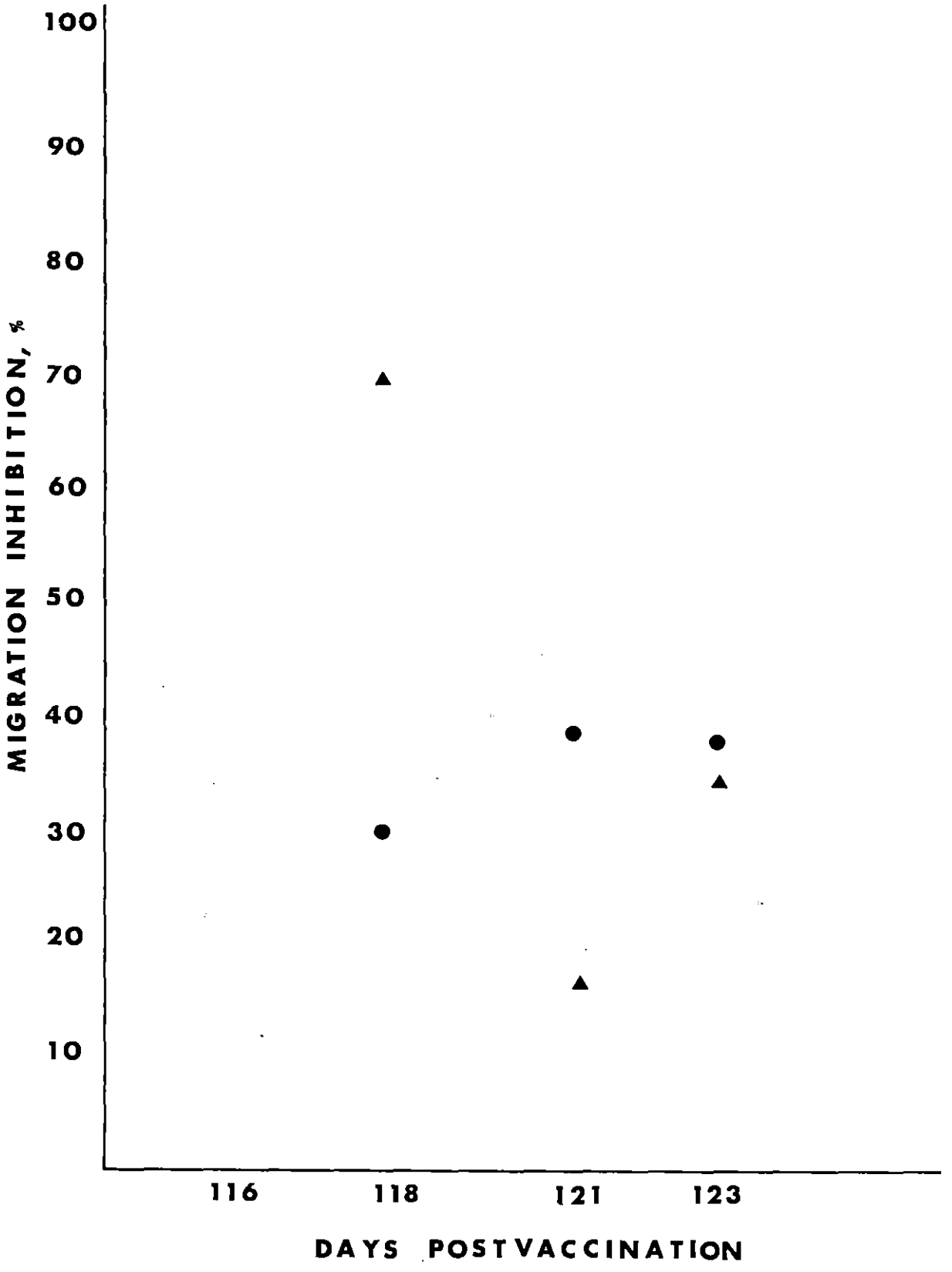
MIGRATION INHIBITION, %
1
2
GROUP

Graph 12. Percent migration inhibition using PMNS/L obtained from cow No. 1280 at 118, 121 and 123 days postvaccination with B. abortus strain 19. The PMNS/L were incubated with Brucella antigen (●) or Con A (▲)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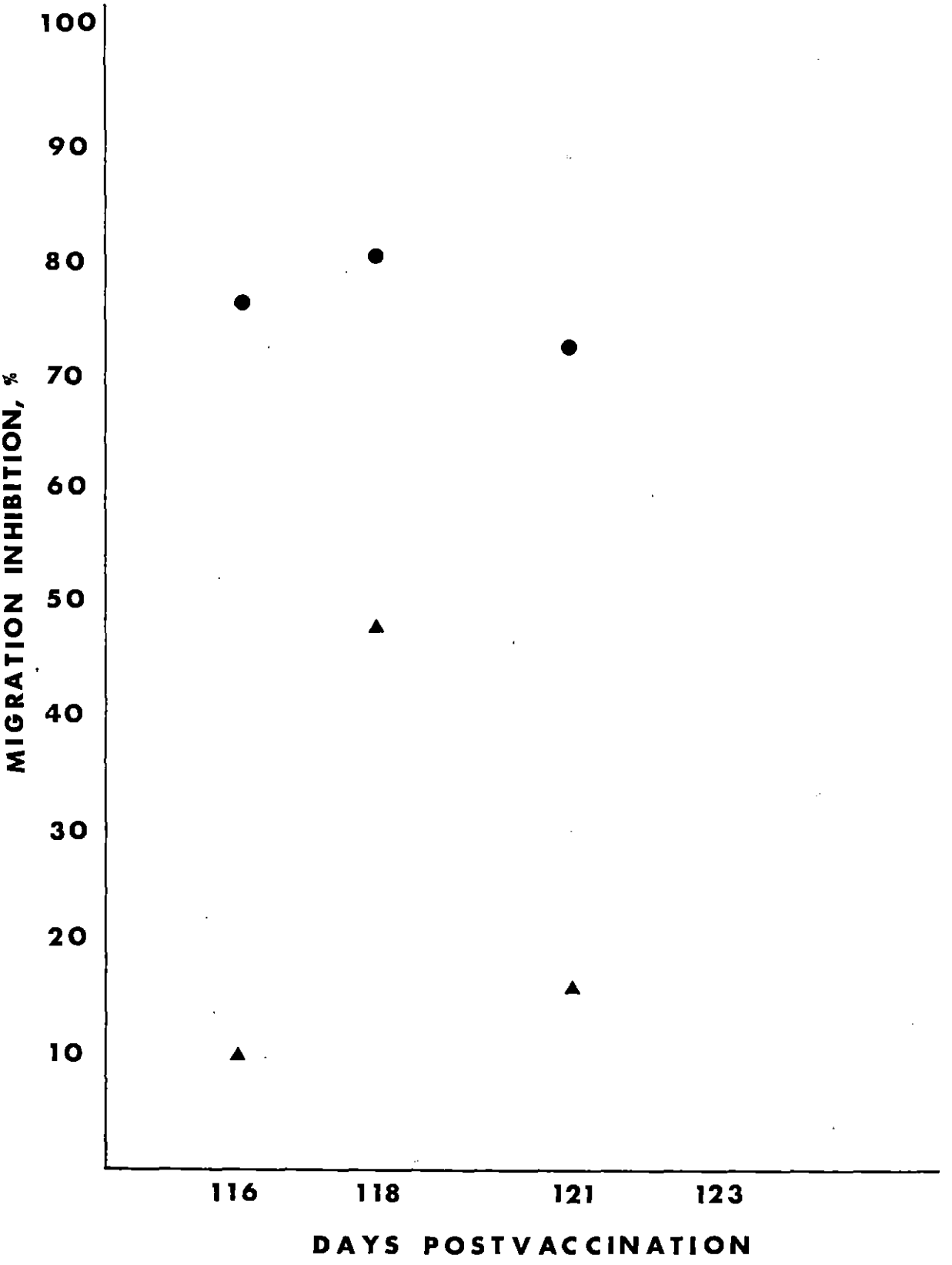
20
10
MIGRATION INHIBITION, %
116
118
121
123
DAYS POSTVACCINATION

Graph 13. Percent migration inhibition using PMNS/L obtained from cow No. 3746 at 116, 118 and 121 days postvaccination with B. abortus strain 19. The PMNS/L were incubated with Brucella antigen (●) or Con A (▲)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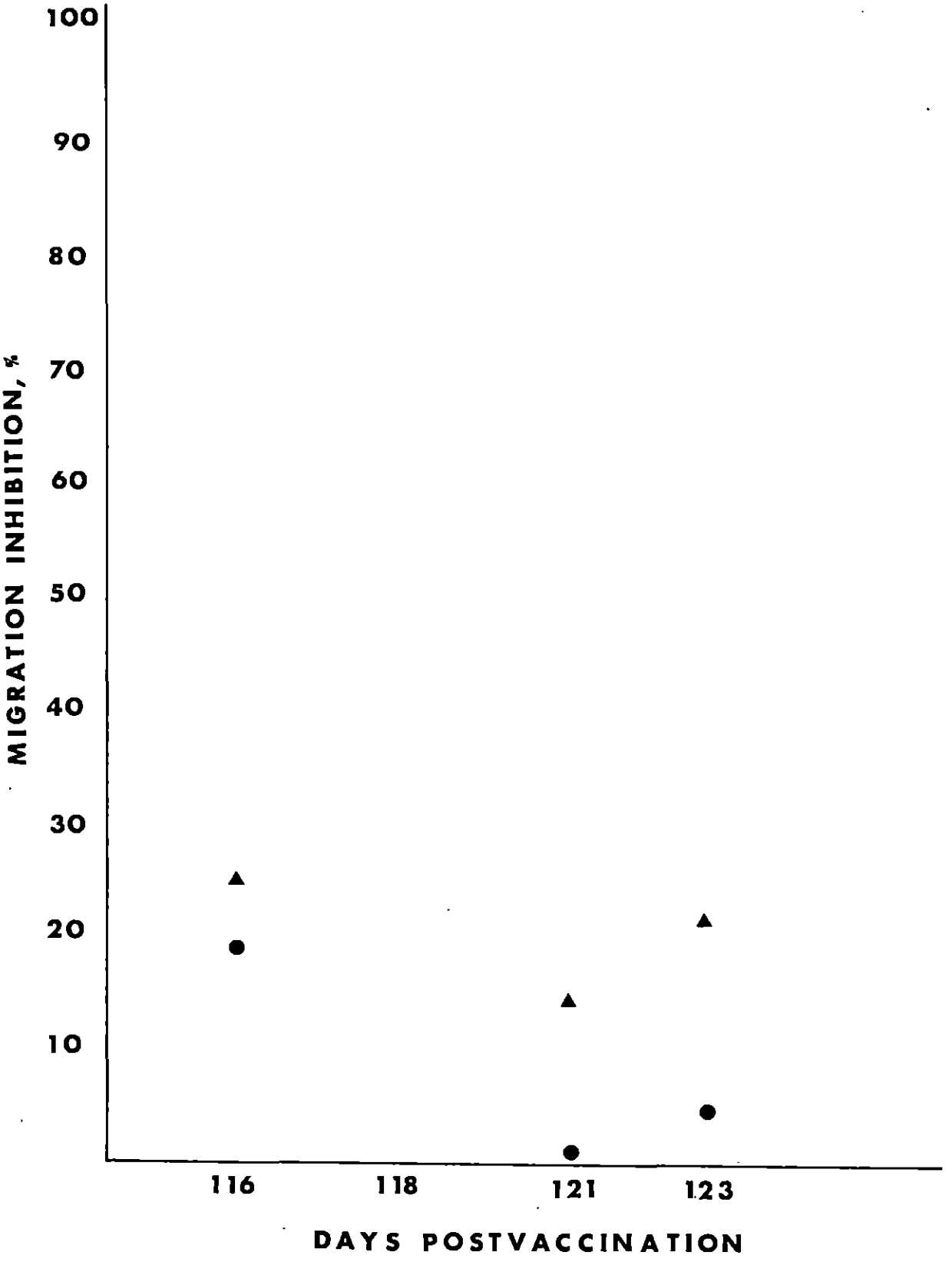
MIGRATION INHIBITION, %
116
118
121
123
DAYS POSTVACCINATION

Graph 14. Percent migration inhibition using PMNS/L obtained from cow No. 3396 (not exposed to B. abortus) on dates corresponding to 116, 121 and 123 days postvaccination with B. abortus strain 19. The PMNS/L were incubated with Brucella antigen (●) or Con A (▲)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MIGRATION INHIBITION, %
116
118
121
123
DAYS POSTVACCINATION

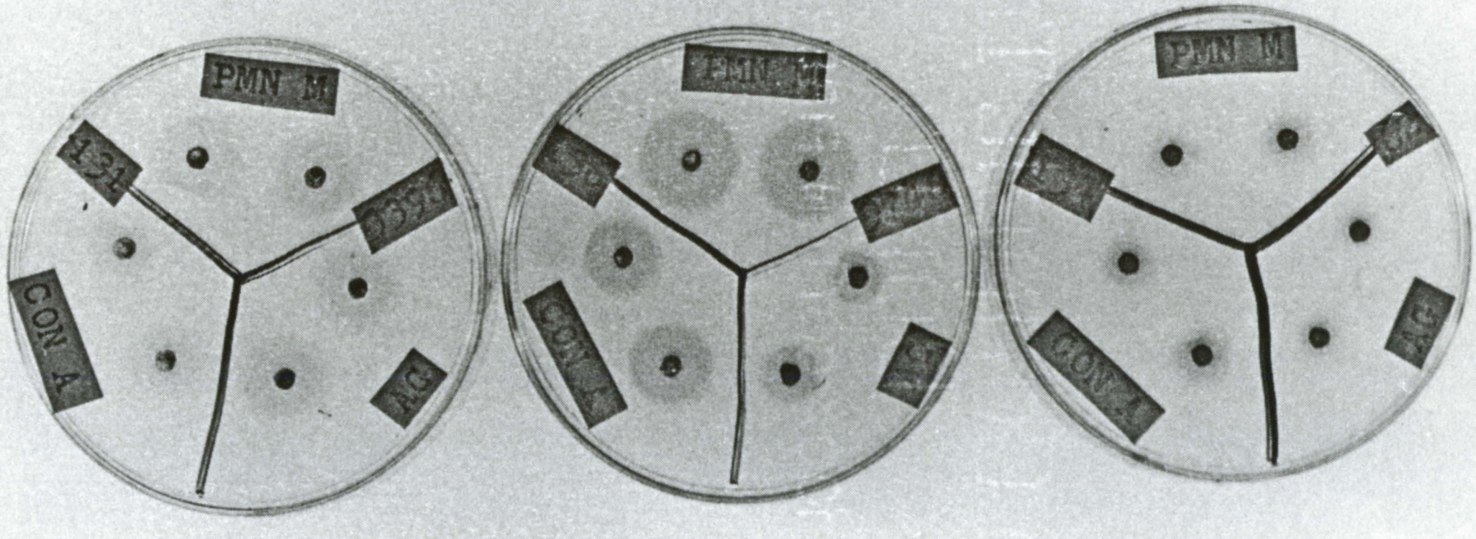
using PMNS/L incubated with Con A were 28.4, 48.5, 21.0 and 51.4.

The patterns of migration of PMNS/L obtained from cow No. 62 (B. abortus strain 2308 was isolated), cow No. 3746 (vaccinated with B. abortus strain 19) and cow No. 3396 (not exposed to B. abortus) are shown in Figure 2.

The areas of migration (group means) of PMNS/L obtained from 2 cows (Nos. 1329 and 3396) not exposed to B. abortus, 2 cows (Nos. 1280 and 3746) vaccinated with B. abortus strain 19, and 6 cows (Nos. 26, 40, 53, 54, 61 and 62) from which B. abortus strain 2308 was isolated are shown in Graph 15. A significantly greater area of migration (group mean) was observed for PMNS/L obtained from cows (Nos. 1329 and 3396) not exposed to Brucella abortus as compared to the area of migration of PMNS/L obtained from cows (Nos. 26, 40, 53, 54, 61 and 62) from which B. abortus strain 2308 was isolated ($p<0.005$). A greater area of migration (group mean) was observed using PMNS/L obtained from cows (Nos. 1280 and 3746) vaccinated with B. abortus strain 19 as compared to the area of migration of PMNS/L obtained from cows (Nos. 26, 40, 53, 54, 61 and 62) from which B. abortus was isolated ($p<0.01$).

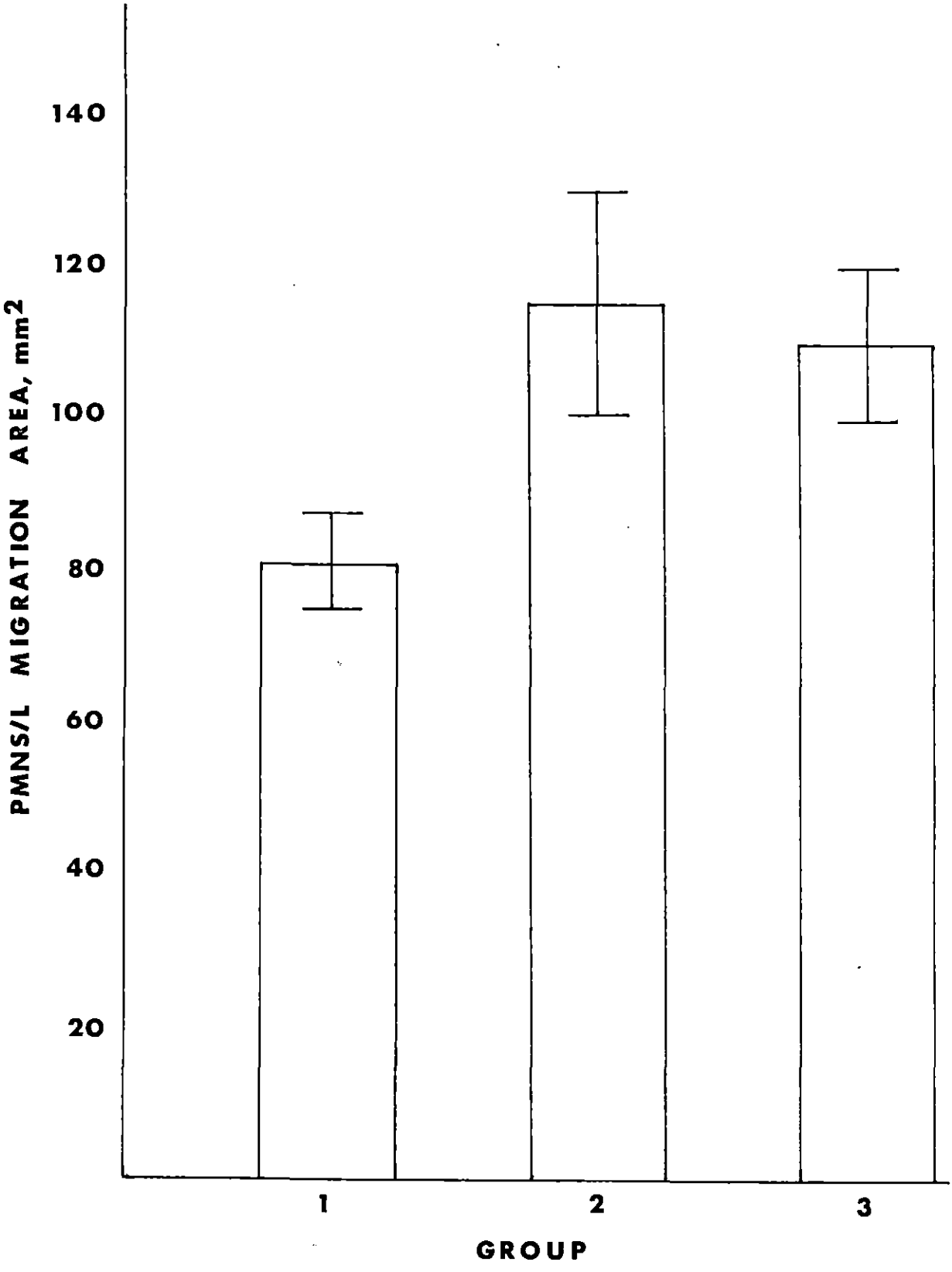
The %MI (mean) for 3 groups (2 cows not exposed to B. abortus, 2 cows vaccinated with B. abortus strain 19, and 6 cows from which B. abortus strain 2308 was isolated) using cultures of PMNS/L incubated with Brucella antigen are shown in Graph 16. The %MI (mean) for 3 groups using PMNS/L incubated with Con A are shown in Graph 17.

Figure 2. Pattern of migration, under agarose, of PMNS/L obtained from cow No. 62 from which B. abortus strain 2308 was isolated (group 1), from cow No. 3746 vaccinated with B. abortus strain 19 (group 2), and from cow No. 3396 not exposed to B. abortus (group 3). The migration of PMNS/L (PMN M) not incubated with Brucella antigen or Concanavalin A and the migration of PMNS/L following incubation with Brucella antigen (Ag) or Concanavalin A (Con A) are shown



3396 - Group 3 3746 - Group 2 62 - Group 1

Graph 15. Area of migration (mm^2) of PMNS/L (group mean ± standard error) obtained from cows (Nos. 26, 40, 53, 54, 61 and 62) from which B. abortus strain 2308 was isolated (group 1), from cows (Nos. 1280 and 3746) vaccinated with B. abortus strain 19 (group 2), and from cows (Nos. 1329 and 3396) not exposed to B. abortus (group 3)


140
120
100
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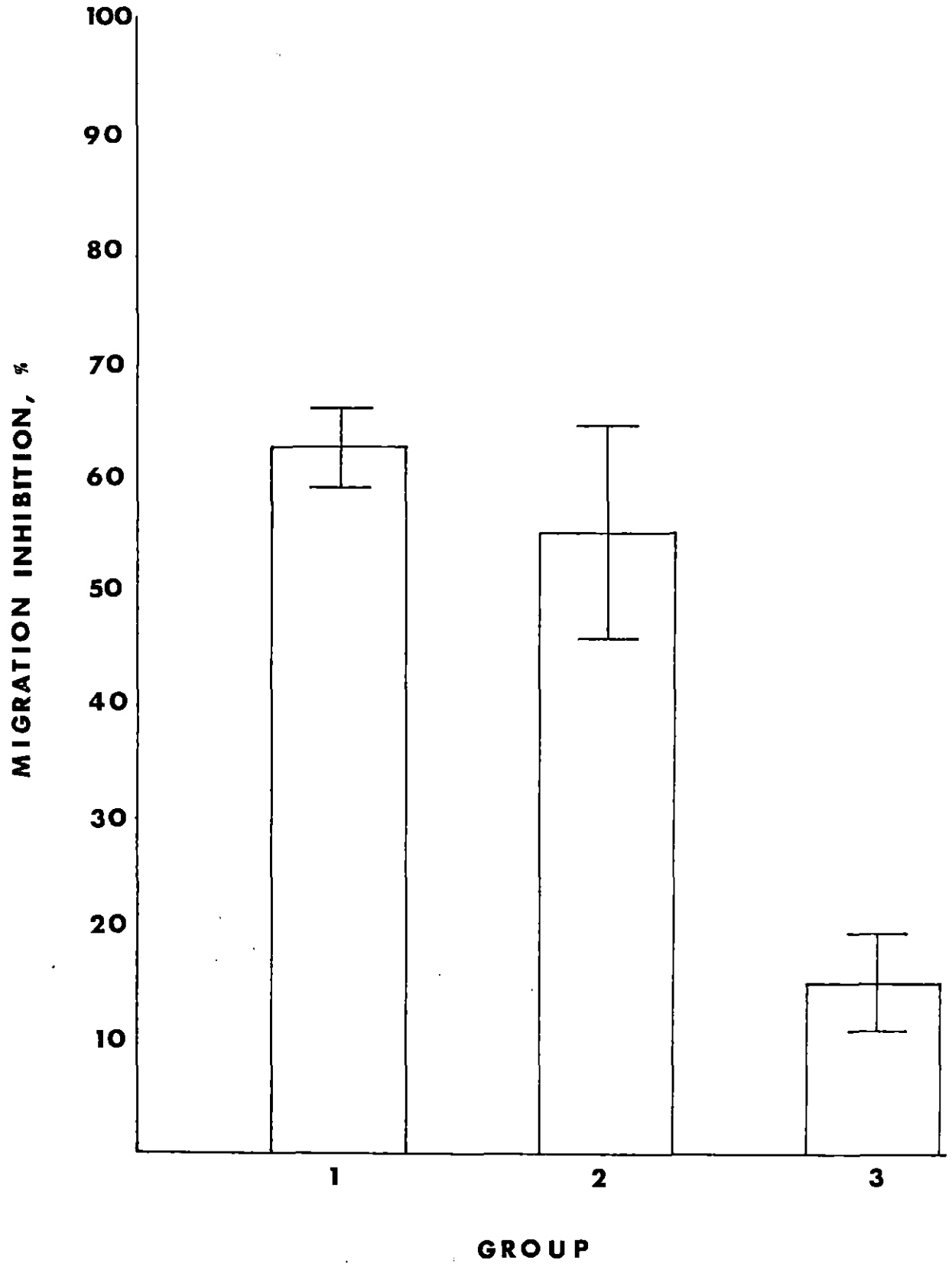
20
PMNS/L MIGRATION AREA, mm2
1
2
3
GROUP

Graph 16. Percent migration inhibition of PMNS/L (group mean ± standard error) obtained from cows (Nos. 26, 40, 53, 54, 61 and 62) from which B. abortus strain 2308 was isolated (group 1), from cows (Nos. 1280 and 3746) vaccinated with B. abortus strain 19 (group 2), and from cows (Nos. 1329 and 3396) not exposed to B. abortus (group 3). The PMNS/L were incubated with Brucella antigen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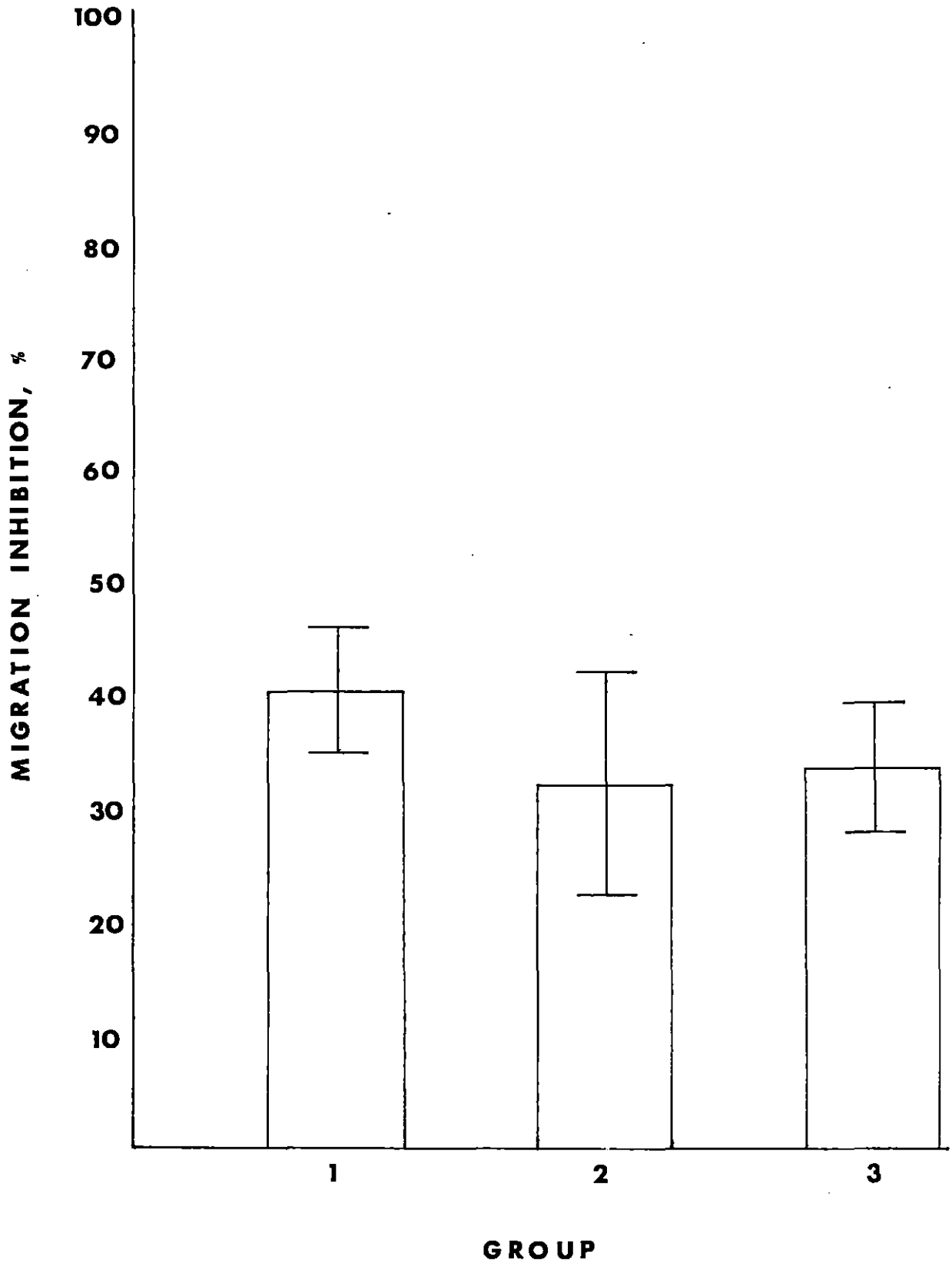
20
10
MIGRATION INHIBITION, %
1
2
3
GROUP

Graph 17. Percent migration inhibition of PMNS/L (group mean ± standard error) obtained from cows (Nos. 26, 40, 53, 54, 61 and 62) from which B. abortus strain 2308 was isolated (group 1), from cows (Nos. 1280 and 3746) vaccinated with B. abortus strain 19 (group 2), and from cows (Nos. 1329 and 3396) not exposed to B. abortus (group 3). The PMNS/L were incubated with Con A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MIGRATION INHIBITION, %
1
2
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GROUP

VII. DISCUSSION

An objective of this study was to examine some variables of a leukocyte migration inhibition assay for detecting cell-mediated immune responses of cows exposed to B. abortus. The variables examined included: serum used to supplement cell culture media, concentration of agarose, titration of Brucella antigen and Con A. Reproducibility of the assay was also examined.

Serum is used in cell culture media to maintain cellular viability and to support cellular proliferation in response to antigenic or mitogenic stimulation (73, 74). It has been reported that lymphocyte responsiveness varied when serums from various animal species and different lots of sera were examined (75, 76). Serum has been reported to stimulate, inhibit or kill lymphocytes (77, 78, 79, 80, 81 and 82). In this study, the areas of migration of PMNS obtained from cows not exposed to B. abortus differed when serums from various species and different lots of newborn calf sera were examined. The migration of PMNS (mean area) observed with fetal calf serum was 27 mm^2; the migration of PMNS (mean area) observed with horse serum was 50 mm^2. The migration of PMNS (mean area) observed with newborn calf serum was 78 mm^2. Considerable differences in the migration of PMNS obtained from cows not exposed to B. abortus were observed when different lots of newborn calf sera were used to supplement the cell culture media. The migration of PMNS (mean area) ranged from less than 5 mm^2 (Lot 27K1202) to 108.9 mm^2 (Lot 30N2902). It is not known what contributed to the variability. However it has been suggested some serum preparations contain

heat-stable factors with chemotactic properties; other preparations containing high concentrations of albumin may inhibit PMN migration (83). These results illustrate the importance of examining different serums for use in a leukocyte migration inhibition assay.

Various concentrations of agarose (ranging from 1% to 0.8%) have been reported for use in leukocyte migration inhibition assays; however none of the reports indicate why a particular concentration was selected (12, 13, 15, 70). To determine an optimum concentration of agarose for a bovine leukocyte migration inhibition assay, three concentrations of agarose (0.8%, 0.7% and 0.6%) were examined. Greater migration (mean area) of PMNS obtained from cows not exposed to B. abortus was observed in plates containing 0.7% agarose as compared to plates containing 0.8% agarose (10-15 mm^2). Plates containing 0.6% agarose were too soft to provide suitable results. The area of migration of PMNS or PMNS/L in plates containing 0.7% agarose could be measured without using microscopy, eliminating the need for expensive microscopic or projection equipment used in some studies (12, 13, 70). A possible explanation for the larger area of migration observed when a lower concentration of agarose was used may be that the matrix formed by the lower concentration of agarose had a larger pore size and less effect on the migration of cells as compared to the matrix formed by a higher concentration of agarose.

It was observed that low concentrations of Con A (2.5 µg/ml) did not inhibit migration of PMNS/L obtained from cows exposed to B. abortus or obtained from cows not exposed to B. abortus; low concentrations of

Brucella antigen (1:32 dilution of a McFarland nephelometer tube 1) did not inhibit migration of PMNS/L obtained from cows exposed to B. abortus or obtained from cows not exposed to B. abortus. These results are in agreement with previous reports suggesting that the commitment of lymphocytes to activation was dependent on the concentration of antigen or Con A (84). The concentration of Con A used in this study was 5 µg/ml; the concentration of Brucella antigen was equivalent to a 1:8 dilution of a McFarland nephelometer tube 1 (O.D. = 0.055 at 525 nm).

Results reported in this study indicate that suitable assay reproducibility was obtained when duplicate samples from cows were processed simultaneously. However samples collected from the same cow on different sample dates provided variable results. Variability of response for different sample dates has been reported previously (13, 15, 85, 86). A variety of in vivo factors including low molecular weight inhibitors, monosaccharides, immunoglobulins, ketone bodies, corticosteroids and transfer factor have been reported to influence responses of lymphocytes and PMNS measured in vitro, including PMNS/L migration (87, 88, 89, 90, 91, 92, 93, 94). It should be emphasized that the in vivo concentrations of these factors may vary on different sample dates thereby influencing the responses measured in vitro (88, 90, 93, 94).

A second objective of this study was to examine the applicability of the assay for detecting and differentiating cellular responses of cattle following exposure to virulent B. abortus strain 2308 or to attenuated B. abortus strain 19. The importance of comparing experimental groups directly to control groups without using arbitrary values to distinguish positive

from negative results has been emphasized (11). A significantly greater %MI (group mean) was observed using PMNS/L obtained from cows (Nos. 26, 40, 53, 54, 61 and 62) from which B. abortus strain 2308 was isolated as compared to using PMNS/L obtained from cows (Nos. 1329 and 3396) not exposed to B. abortus when PMNS/L were incubated with Brucella antigen ($p<0.002$). The %MI (group mean) was greater using PMNS/L obtained from cows (Nos. 1280 and 3746) vaccinated with B. abortus strain 19 as compared to the %MI using PMNS/L obtained from cows (Nos. 1329 and 3396) not exposed to B. abortus when PMNS/L were incubated with Brucella antigen ($p<0.04$). These results indicate that a leukocyte migration inhibition assay as described herein using Brucella antigen may be useful in differentiating responses of cows infected with B. abortus strain 2308 or cows vaccinated as adults with B. abortus strain 19 from cows not exposed to B. abortus. Responses of PMNS/L, following incubation with Con A, obtained from cows from which B. abortus strain 2308 was isolated, cows vaccinated with B. abortus strain 19, and cows not exposed to B. abortus were not significantly different ($p>0.05$).

Significant differences were observed when the area of migration of PMNS/L obtained from cows from which B. abortus strain 2308 was isolated was compared to the area of migration of PMNS/L obtained from cows vaccinated with B. abortus strain 19 ($p<0.01$) or was compared to the area of migration of PMNS/L obtained from cows not exposed to B. abortus ($p<0.005$). However, bovine PMNS have been reported to have large differences in their ability to migrate; therefore, it is possible the differences observed in the areas of migration are not related to exposure to B. abortus (86). No significant difference was observed when %MI (group mean) of PMNS/L

obtained from cows (Nos. 26, 40, 53, 54, 61 and 62) from which B. abortus strain 2308 was isolated was compared to %MI (group mean) of PMNS/L obtained from cows (Nos. 1280 and 3746) vaccinated with B. abortus strain 19 when PMNS/L were incubated with Brucella antigen ($p>0.05$). These results indicate that cellular responses of cows exposed to B. abortus strain 2308 and responses of cows vaccinated as adults with B. abortus strain 19 observed in a leukocyte migration inhibition assay using Brucella antigen were not different.

VIII. SUMMARY

Some variables affecting a leukocyte migration inhibition assay for detecting cell-mediated immune responses of cows exposed to B. abortus were examined. It was observed that areas of migration of PMNS obtained from cows not exposed to B. abortus were greater (78 mm^2) when newborn calf serum was used to supplement cell culture media as compared to the areas of migration of PMNS when either horse serum (50 mm^2) or fetal calf serum (27 mm^2) were used. Considerable differences in the migration of PMNS obtained from cows not exposed to B. abortus were observed when different lots of newborn calf sera were used to supplement cell culture media (range <5 mm^2 to 108.9 mm^2). To determine an optimum concentration of agarose for a bovine leukocyte migration inhibition assay, three concentrations of agarose (0.6%, 0.7% and 0.8%) were examined. Greater migration (mean area) of PMNS obtained from cows not exposed to B. abortus was observed in plates containing 0.7% agarose as compared to plates containing 0.8% agarose (10-15 mm^2). Plates containing 0.6% agarose were too soft to provide suitable results. Low concentrations of Con A (2.5 µg/ml) did not inhibit migration of PMNS plus lymphocytes (PMNS/L) obtained from cows exposed to B. abortus or cows not exposed to B. abortus; low concentrations of Brucella antigen (1:32 dilution of a McFarland nephelometer tube 1) did not inhibit migration of PMNS/L obtained from cows exposed to B. abortus strain 2308 or to B. abortus strain 19. The concentration of Con A used in this study was 5 µg/ml; the concentration of Brucella antigen was equivalent to a 1:8 dilution of a McFarland nephelometer tube 1 (O.D. = 0.055 at 525 nm).

Suitable assay reproducibility was obtained when duplicate samples were processed simultaneously.

Applicability of the assay for detecting and differentiating cellular responses of cows following exposure to virulent B. abortus strain 2308 or to attenuated B. abortus strain 19 was examined. Significantly greater percent migration inhibition (%MI) was observed using (PMNS/L) from cows from which B. abortus strain 2308 was isolated as compared to %MI using PMNS/L obtained from cows not exposed to B. abortus when PMNS/L were incubated with Brucella antigen ($p<0.002$). The %MI was greater using PMNS/L obtained from cows vaccinated with B. abortus strain 19 as compared to the %MI using PMNS/L obtained from cows not exposed to B. abortus when PMNS/L were incubated with Brucella antigen ($p<0.04$). Responses of PMNS/L, following incubation with Con A, obtained from cows from which B. abortus strain 2308 was isolated, cows vaccinated with B. abortus strain 19, and cows not exposed to B. abortus were not significantly different ($p<0.05$). Significant differences were observed when area of migration of PMNS/L obtained from cows from which B. abortus strain 2308 was isolated was compared to area of migration of PMNS/L obtained from cows vaccinated with B. abortus strain 19 ($p<0.01$) or was compared to area of migration of PMNS/L obtained from cows not exposed to B. abortus ($p<0.005$). No significant differences were observed when %MI using PMNS/L obtained from cows from which B. abortus strain 2308 was isolated was compared to %MI using PMNS/L obtained from cows vaccinated with B. abortus strain 19 when PMNS/L were incubated with Brucella antigen ($p>0.05$).

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