In vitro Lymphocyte Proliferative Responses and gamma-Interferon Production as Measures of Cell-Mediated Immunity of Cattle Exposed to Pasteurella haemolytica

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Abstract
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Antileukotoxin antibody titers were determined by a serum neutralization assay, and protection against pneumonic lesions was more closely correlated with antileukotoxin antibody responses than with lymphocyte proliferation or gamma-interferon responses.

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ABSTRACT

Cell-mediated immune mechanisms may play a role in the pathogenesis and prevention of pneumonia in cattle caused by Pasteurella haemolytica serotype A1. To determine the circumstances required to stimulate and identify cell-mediated immune responses, calves were vaccinated with a commercial P. haemolytica bacterin or a live commercial P. haemolytica vaccine, or were infected intratracheally with virulent P. haemolytica. All calves were challenged-exposed intratracheally with P. haemolytica 31 d after vaccination or prior infection. Peripheral blood mononuclear cells and mediastinal and superficial cervical lymph node cells were stimulated with antigens prepared from P. haemolytica to evaluate in vitro proliferative responses and γ-interferon production as measures of cell-mediated immunity. Strong proliferative responses and γ-interferon production were detected in lymph node cells from calves vaccinated with the live vaccine and from infected calves, especially in response to stimulation with an outer membrane protein preparation from P. haemolytica. Greater proliferative responses and γ-interferon production were associated with the lymph node nearer the site of bacterin administration (superficial cervical lymph node) or the site of infection (mediastinal lymph node), whereas greater proliferative responses and γ-interferon production were associated with the more distant lymph node (mediastinal lymph node) in calves vaccinated with the live vaccine. Neither proliferative responses nor γ-interferon production were detected in peripheral blood mononuclear cells from calves that were vaccinated for or infected with P. haemolytica. Antileukotoxin antibody titers were determined by a serum neutralization assay, and protection against pneumatic lesions was more closely correlated with antileukotoxin antibody responses than with lymphocyte proliferation or γ-interferon responses.

RÉSUMÉ

L’immunité à médiation cellulaire pourrait avoir un rôle à jouer dans la pathogénèse et la prévention de la pneumonie à Pasteurella haemolytica sérotype A1 chez les bovins. Afin de déterminer les facteurs requis pour stimuler et identifier la réponse immunitaire à médiation cellulaire, des veaux ont été vaccinés avec une bactérine commerciale de P. haemolytica ou un vaccin vivant commercial de P. haemolytica, ou ont été infectés par voie intra-trachéale avec une souche virulente de P. haemolytica. Tous les veaux furent inoculés par voie intra-trachéale avec P. haemolytica 31 jours après la vaccination ou la première infection. Des cellules mononucléées du sang périphérique et des cellules des noyaux lymphatiques médiastinaux et cervicaux superficiels furent stimulées avec des antigènes préparés à partir de P. haemolytica dans le but d’évaluer les réponses prolifératives in vitro et la production d’interféron-γ, deux mesures de l’immunité cellulaire. De fortes réponses prolifératives et une production élevée d’interféron-γ furent détectées dans les cellules des noyaux lymphatiques des veaux vaccinés avec le vaccin vivant et ceux des veaux pré-infectés, en réponse à une stimulation avec une préparation d’une protéine de la membrane externe de P. haemolytica. Les réponses prolifératives et la production d’interféron-γ étaient plus fortes dans le noyau lymphatique le plus proche du site d’administration de la bactérine (noyau lymphatique cervical superficiel) ou du site d’infection (noyau lymphatique médiastinal), alors que les réponses prolifératives et la production d’interféron-γ les plus fortes étaient observées dans le noyau lymphatique le plus éloigné (noyau lymphatique médiastinal) chez les veaux vaccinés avec le vaccin vivant. Aucune réponse proliférative ni production d’interféron-γ ne furent détectées dans les cellules mononucléées du sang périphérique chez les veaux vaccinés contre ou infectés avec P. haemolytica. Les titres d’anticorps anti-leucotoxine furent déterminés par une épreuve de séronéutralisation, et la protection contre les lésions de pneumonie était plus en corrélation avec le titre d’anticorps anti-leucotoxine qu’avec la prolifération lymphocytaire ou la production d’interféron-γ.

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INTRODUCTION

*Pasteurella haemolytica* biotype A serotype 1 causes severe pneumonia in cattle. Experimental and commercial vaccines, including killed bacterins and live vaccines have been developed for immunization of cattle against *P. haemolytica*. In earlier reports, immunization with *P. haemolytica* bacterins provided poor protection of cattle against natural or experimental disease (1–3), and has been associated with enhancement of experimental and natural disease (4,5). Immunization of calves with bacterins in Freund’s adjuvant (6), or live *P. haemolytica* (7–10), however has been demonstrated to provide protection against challenge.

*Pasteurella haemolytica* produces a potent leukotoxin that is inhibitory or lethal to bovine neutrophils, mononuclear phagocytes, and lymphocytes (11–13). Investigators have attributed the protection afforded by live vaccines to their induction of leukotoxin-neutralizing antibodies (14). Calves protected against *P. haemolytica* challenge by immunization with a bacterin in Freund’s adjuvant, however, did not produce leukotoxin-neutralizing antibodies, indicating that aspects of the immune response other than antileukotoxin antibody can afford protection (6). Subsequently, it was demonstrated that immunization of calves with a commercial *P. haemolytica* culture supernatant vaccine (Presponse, Langford, Guelph, Ontario) containing leukotoxin and other soluble antigens protected against experimental infection (15), while recombinant *P. haemolytica* leukotoxin did not result in protective immunity (16), also supporting that effective humoral immune protection probably requires antibody responses to somatic antigens of *P. haemolytica* in addition to antileukotoxin antibodies. Others (9,17) have suggested that cell-mediated immune (CMI) responses may play a role in immunity to *P. haemolytica*-induced pneumonia. The authors of the present study were unable to find reports of attempts to measure CMI to *P. haemolytica* antigens in cattle after vaccination or recovery from infection.

The purpose of this study was to measure aspects of CMI responses of cattle to *P. haemolytica*, as well as to identify *P. haemolytica* antigens that can be used to measure this response. Calves infected with *P. haemolytica* and calves vaccinated with a live or killed *P. haemolytica* vaccine were used to evaluate CMI responses to *P. haemolytica*. Peripheral blood mononuclear cells (PBMC) and lymph node cells were harvested and stimulated with 5 antigen preparations from *P. haemolytica*. Lymphocyte proliferative responses and γ-interferon production were used as measures of CMI.

To assess the role of CMI versus humoral immunity as protective factors against pneumonic pasteurellosis, all calves were challenge-exposed to *P. haemolytica*, and lung lesions and bacterial concentrations in lung tissues were assessed for correlation with lymphocyte proliferative responses, γ-interferon production, and antileukotoxin antibodies.

MATERIALS AND METHODS

BACTERIAL CULTURE

*Pasteurella haemolytica* biotype A serotype 1 (strain L101, kindly provided by G.H. Frank, Ames, Iowa) was isolated on blood agar from a pneumatic bovine lung. Tryptose broth cultures were incubated at 37°C for 6 h, dispensed into vials in 1 mL aliquots, and stored at −70°C until used for antigen or inoculum preparations.

ANTIGEN PREPARATION

Capsular polysaccharide (CP), lipopolysaccharide (LPS), lipopolysaccharide-associated protein (LAP), and a bacterial cell sonicate (SON) were prepared from *P. haemolytica* strain L101 for use as antigens to stimulate PBMC and lymph node cell cultures. An outer membrane protein preparation (OMP) of *P. haemolytica* serotype 1 was kindly provided by A.W. Confer, Stillwater, Oklahoma, prepared as previously described (18).

Strain L101 was grown on dextrose starch agar for 24 h at 37°C, and harvested in distilled water. Bacteria were pelleted by centrifugation and the supernatant was saved. Cells were dehydrated in 50% ethanol, washed twice in acetone, twice in ethyl ether, and then air-dried.

The supernatant was placed in dialysis bags (12–14, 500 nominal mol. wt. cutoff, Spectrum Medical Industries, Los Angeles, California) and covered with carboxymethylcellulose (Aquacide I, Calbiochem Corp, La Jolla, California). The CP was extracted from the concentrated culture supernatant as previously described (19). Alpha-amylase (Sigma Chemical Co, St. Louis, Missouri) was added to the extracted CP and the mixture was incubated for 2 h at 37°C and 18 h at 4°C to remove residual starch from the growth medium. The CP was resuspended in a buffer (pH 7.2) containing 10 Mm Tris, 145 Mm NaCl, 0.2% SDS and 500 μg (16 U)/mL proteinase K (Amresco, Solon, Ohio). The CP contained less than 0.25% protein (20) and 0.01% LPS, as determined by a chromogenic limulus amebocyte assay (QCL-1000, Whittaker Bioproducts, Walkersville, Maryland).

Lipopolysaccharide was extracted from dried cells with a phenol, chloroform, and petroleum ether solution, as previously described (21). The LPS was treated with proteinase K similarly as the CP, after which it contained 0.7% protein (20).

Lipopolysaccharide-associated protein was extracted from the phenol supernatant of the LPS extraction as previously described (22). The LAP contained 0.02% LPS.

The SON was prepared by collecting *P. haemolytica* cells from 18 h of growth on sheep blood agar plates that were incubated at 37°C. Cells were collected in distilled water and sonicated on ice with an ultrasonic cell disrupter (Microson, Heat Systems Ultrasoins, Farmingdale, New York) for 1 min at the maximum power setting. The preparation was centrifuged at 5900 x g for 20 min, and the supernatant was filtered through a 0.22 μm filter.

After extraction procedures, the CP, LPS, LAP and SON antigens were lyophilized. Prior to use, these antigens were resuspended in Hanks balanced salt solution in concentrations based on dry weight of the antigen.
preparation. The OMP antigen was resuspended based on μg of protein/μl.

CALVES

Twelve colostrum-deprived, male Holstein and Holstein crossbred calves were obtained at birth and housed in isolation barns in American Association for Accreditation of Laboratory Animal Care (AAALAC) accredited facilities. Calves were fed a complete pelleted ration twice daily, and water was available ad libitum. The calves were 3–7 mo old when used in the experiment.

VACCINATION AND INFECTION OF CALVES

Calves were divided into 4 groups of 3 calves, and were vaccinated or infected with P. haemolytica on day 0. Calves of one group (bacterin group) were vaccinated with a Pasteurella haemolytica-Pasteurella multocida bacterin (Pasteurella haemolytica-Pasteurella multocida bacterin, Lot No. 923, Colorado Serum Co., Denver, Colorado). Calves in a 2nd group (live vaccine group) were vaccinated with a live P. haemolytica vaccine (Respirvac, Lot No. 7671, Beecham Laboratories, Bristol, Tennessee). Vaccines were administered in the subcutis of the left cervical area. Calves in a 3rd group (convalescent group) were intratracheally infected with 10 mL of an inoculum containing 3.0 × 10^8 CFU of P. haemolytica strain L101. The inoculum was prepared by inoculating one aliquot of strain L101 into 30 mL of tryptose broth and incubating at 37°C for 3 h. Bacteria were pelletted by centrifugation at 5900 × g for 5 min, resuspended in tryptose broth to 80% of transmittance at 600 nm wavelength, then further diluted in tryptose broth to approximately 1 × 10^8 CFU/mL for inoculation of calves. Bacterial concentration was verified by plate counts. Calves in the convalescent group were treated with gentamicin (Gentocin, Schering Corp, Kenilworth, New Jersey) and ceftiofur (Naxcel, Upjohn Co, Kalamazoo, Missouri) on days 3–6 to reduce the morbidity following infection with P. haemolytica. A 4th group (unvaccinated group) consisted of calves that received no exposure to P. haemolytica. Body temperatures of all calves were monitored daily for 3 d after initial vaccination or infection. Calves in the bacterin group were revaccinated on day 15 of the experiment.

ANTILEUKOTOXIN ANTIBODY TITERS

Serum samples were collected from all calves on days 1, 4, 8, 11, 15, 18, 22, and 25 for determination of antileukotoxin antibody titers to P. haemolytica, using a serum leukotoxin neutralization assay (23) with minor modifications. BL-3 bovine lymphoma cells were used as target cells, and 100 μl of dimethylsulfoxide was added to each well just prior to the measurement of optical density at a dual wavelength (550–650 nm). Antileukotoxin titers were determined at the serum dilution where 30–70% of the BL-3 cells were killed. For standards, 100% of cells killed was determined by adding sonicated BL-3 cells to wells, and 0% of cells killed was determined by including no toxin in the wells. Positive control wells on each plate, contained toxin added to cells in the absence of serum. Titers were converted to log2 prior to statistical analyses.

LYMPHOCYTE PROLIFERATION ASSAYS

Blood was collected from calves on days 1, 4, 11, 15, 18, 22, and 25 for isolation of PBMC for lymphocyte proliferation assays. Fifty mL of venous blood were collected into 5 mL of acid citrate dextrose, centrifuged at 600 × g for 25 min, and buffy coats were collected. Erythrocytes in buffy coats were lysed with phosphate buffered water, followed by restoration of osmolarity with hypertonic phosphate buffered saline. Peripheral blood mononuclear cells were washed twice and resuspended in complete medium (RPMI, L-glutamine, 5 X 10^-5 M 2-mercaptoethanol, 10% bovine fetal serum, and 200 IU penicillin, 200 μg streptomycin and 0.5 μg amphotericin B/mL. Two-hundred μl of complete medium containing 2 × 10^5 PBMC were delivered to each well of flat-bottom microtiter plates. Antigens (CP, LPS, LAP, and OMP), in 25 μl of RPMI, were added to wells in triplicate at concentrations of 1.0 and 0.1 μg/well. The SON antigen was added at 0.1 and 0.01 μg/well. Cell cultures were incubated for 52–54 h at 37°C in a humidified atmosphere with 5% CO₂, and pulse-labelled with [3H]-thymidine (Amersham Corp, Arlington Heights, Illinois) at 0.4 μCi/well. Antigen concentrations and incubation periods were based on preliminary studies. Cells were incubated an additional 18–20 h, harvested onto fiberglass filters, and radioactivity was measured by liquid scintillation counting. Mean counts per minute from antigen-stimulated wells were divided by the mean counts per minute of the background wells (no antigen) and the quotient was designated as the stimulation index (SI).

For lymph node cell assays, lymph nodes (collected on days 35 or 36) were minced and cells were passed through #60 mesh stainless steel screens. Lymphocytes were isolated using Histopaque 1083 (Sigma Chemical Co), washed twice in 0.1 M phosphate-buffered saline, pH 7.2, and resuspended at 2 × 10^6 cells/mL in complete media. Lymph node cell preparations were thereafter treated in the same manner as PBMC preparations.

Υ-INTERFERON ASSAYS

Peripheral blood mononuclear cell and lymph node cell suspensions were added to plates and stimulated with antigens in the identical manner as that used for the lymphocyte proliferation assays. After 46–48 h incubation, plates were centrifuged at 100 × g for 5 min, and supernatants were collected and immediately frozen and stored at −20°C. Υ-interferon was measured using a commercial ELISA kit (Mycobacterium paratuberculosis gamma-interferon test kit, IDEXX Laboratories, Westbrook, Maine), with modifications. Culture supernatants were substituted for plasma samples, and bovine recombinant Υ-interferon (IDEXX Laboratories) was used as a standard for quantitative determinations. Supernatants from PBMC cultures from days 4, 11, and 18 that were stimulated withOMP at 0.1 μg/well and supernatants from lymph node cell cultures that were stimulated with OMP at 0.1 μg/well were analyzed for Υ-interferon. Υ-interferon concentrations in supernatants of background wells were subtracted
from those of supernatants of antigen-stimulated wells and the difference was designated IFN.

CHALLENGE AND NECROPSY

On day 31 of the experiment, all 12 calves were challenge-exposed intratracheally to 10 mL of inoculum containing 2.0 $\times$ $10^8$ CFU of $P. \text{haemolytica}$ strain L101. Body temperatures were monitored daily for 3 d after the challenge. Calves were euthanized and necropsied on days 35 (6 calves) and 36 (5 calves). One calf from the unvaccinated group died on day 33 with severe pneumonic lesions as a result of the challenge.

The caudal mediastinal and superficial cervical lymph node (ipsilateral to site of vaccination) were removed at necropsy for lymph node cell preparations in the lymphocyte proliferation and $\gamma$-interferon assays. The volume of acute pneumonic parenchyma was estimated by visual observation and palpation for each calf and recorded as % pneumonia. Approximately 10 gm of pneumonic lung was collected from each animal for quantitative bacterial culture, and the CFU of $P. \text{haemolytica}/g$ of lung were converted to $\log_{10}$ prior to statistical analyses. The lung section was collected from a severely pneumonic area, or from the right middle lobe if pneumonia was not present.

STATISTICAL ANALYSES

Analysis of variance (SAS Institute Inc, Cary, North Carolina) was used to determine significant differences between groups for IFN, body temperature, % pneumonia, and CFU of $P. \text{haemolytica}/g$ of lung. Separate analyses were performed for each combination of antigen, concentration, and lymph node location. When the overall $F$ test was significant ($P \leq 0.05$), $t$-tests (LSD) were used to determine specific differences between groups ($P \leq 0.05$).

For proliferative assay results, exploratory analysis indicated that the best measure of activity for statistical purposes was the logarithm of SI, which is biologically interpretable as a measure of the difference in rates of mitotic activity between stimulated and background wells. Each antigen's log-transformed SI were analyzed in a single mixed linear model (SAS proc mixed) using random effect terms as appropriate to specify a covariance structure compatible with the repeated measurements on animals (two lymph nodes per animal) and lymph nodes within animals (two antigen concentrations per node). Experiment wise significance levels for comparisons between groups were controlled by the stagewise modified Bonferroni procedure of Hommel (24), using logical relations between hypothesis.

Linear regression analysis was used to correlate immune responses (serum antileukotoxin antibody titer on day 25, IFN and SI (from mediastinal lymph node cells stimulated with OMP at 0.1 $\mu$g/well) to the severity of pulmonary disease (% pneumonia) (Fig. 4). Correlation coefficients were also determined for IFN versus SI (from mediastinal lymph node cells stimulated with OMP at 0.1 $\mu$g/well) to correlate $\gamma$-interferon responses with proliferative responses. In addition, SI and IFN from mediastinal lymph node cells stimulated with OMP at 0.1 $\mu$g/well were correlated with serum antibody response on day 25 to determine relationships between CMI and the humoral immune response to leukotoxin.

RESULTS

CLINICAL RESPONSES OF CALVES

By 20 h after vaccination or initial infection, calves in the live vaccine and convalescent group had significantly elevated body temperatures ($P \leq 0.05$) compared to calves in the unvaccinated group. Body temperatures in these two groups remained significantly elevated until approximately 96 h after vaccination or initial infection (data not shown). All 3 calves in the live vaccine group developed extensive swelling at the site of vaccination, which extended ventrally to the sternum in one calf.

Calves in the convalescent group had clinical evidence of pneumonia after initial infection on day 0, which included hyperpnea, anorexia, and coughing. However, signs subsided by day 5.

ANTILEUKOTOXIN ANTIBODY RESPONSES

Serum antileukotoxin antibody titers peaked for the live vaccine and convalescent group on day 15 (Fig. 1). Calves in the bacterin group, which were revaccinated on day 15, had a maximum mean antibody titer on day 25. After vaccination, calves in the bacterin and live vaccine group had
similar antibody titers, while the calves in the convalescent group had much higher antileukotoxin titers.

PROLIFERATIVE RESPONSES

Fixed effects considered for inclusion in the analysis of log SI included treatment group, node location, antigen concentration, and the possible interactions of subsets of these main effects. In the case of CP, there was no strong evidence of differences between groups or node locations, and in fact the antigen appeared to be ineffective in stimulating activity above background levels. In the case of LPS, LAP, OMP, and SON, antigen concentration was not observed to interact with the other 2 main effects. The main effect of concentration was significant only in the case of LAP and OMP; in both cases greater activity was observed at the higher concentration. Concentration was retained as a covariate in all 4 analyses to reduce possible confounding, even when not achieving significance.

For all antigens except CP, evidence for the presence of interaction between treatment and node location was strong (P values ranged from 0.0007 to 0.011). This statistical interaction corresponds to the biological observation that the relationship between the 2 node locations is different in different treatment groups. As Figure 2 shows, which is typical for all antigens excluding CP, greater proliferative activity was observed in the superficial cervical lymph node cells in the control and bacterin groups, while the reverse was true in the live vaccine and convalescent groups.

For each antigen, statistical tests were performed comparing mean log SI between lymph node locations in each treatment group, and between treatment groups at each node location. In addition, the difference in log SI between the 2 lymph node locations was computed for each treatment group and between-group comparisons of the differences were performed. To avoid spurious findings of apparently significant results due merely to the large number of statistical tests conducted rather than to real differences in the quantities being compared, Hommel's modified Bonferroni procedure was used to insure that in the absence of any differences, the probability of rejecting none of the hypotheses tested would be at least 0.95. In our data, the procedure required an individual comparison to achieve approximately P < 0.003 to be declared significant. Under this stringent standard, the difference in mean log SI between superficial cervical and mediastinal node cells was found to be unequal between the bacterin and convalescent treatment groups for all antigens except CP. In the case of LAP, the same statement can be made regarding the control and convalescent groups.

Log SI was found to differ between the 2 lymph node locations in the bacterin group (for LPS and LAP) and in the convalescent group (for SON). For OMP, log SI of mediastinal lymph node cells was higher for the convalescent group than for the bacterin group.

A large number of comparisons achieved individual P values that were less than 0.05, but not small enough for hypothesis rejection under the modified Bonferroni procedure. Because of the likelihood that some of these are statistical type I errors, they cannot be regarded as individually reliable. As a group, however, they are consistent with the results already presented, in that they suggest that: (1) in the convalescent and bacterin groups, proliferative activity levels differed between superficial cervical node cells and mediastinal node cells; (2) in the bacterin group, superficial cervical lymph node cells had higher proliferative responses than mediastinal cells, while the reverse was true in the convalescent group; and (3) mediastinal lymph node cells had higher proliferative responses in the convalescent group than in the bacterin group.

Results for the control and live vaccine groups are less clear. There is no real evidence that the results observed in the control group differ from those in the bacterin group, but because of the loss of one control animal, the sample sizes in the control group are smaller, and statistical power is reduced. In the live vaccine group, the observed results are intermediate between the patterns exhibited in the bacterin and convalescent groups, and we are unable to make statistical distinctions in this study.

Proliferative responses for PBMC stimulated with CP, LPS, LAP, OMP and SON were unremarkable when compared to background wells, with CPM generally below $2 \times 10^5$ CPM.

![Figure 2. Proliferative responses of superficial cervical and mediastinal lymph node cells stimulated with outer membrane protein (OMP) from \textit{P. haemolytica}. Calves were vaccinated or infected with \textit{P. haemolytica} 35 or 36 d prior to stimulation of lymph node cells. Values shown correspond to estimated mean log stimulation index (SI) by experimental group, converted back to the SI scale by taking antilogarithms. Error bars correspond in the same way to values one standard error above estimated log SI.](image-url)
γ-INTERFERON RESPONSES

Differences between experimental groups in γ-interferon production in culture supernatants from mediastinal lymph node cells stimulated with OMP were present (P ≤ 0.002) (Fig. 3). The highest γ-interferon production occurred in the live vaccine group, which was significantly higher than the γ-interferon production of lymphocytes from the unvaccinated, bacterin or convalescent groups. γ-interferon production for the convalescent group was also significantly higher than the γ-interferon production of either the unvaccinated or bacterin groups.

Significant differences between experimental groups were not present for γ-interferon production by superficial cervical lymph node cells stimulated with OMP (Fig. 3). Significant differences between experimental groups were not present for γ-interferon of culture supernatants from PBMC stimulated with OMP for any of the days analyzed (Days 11, 18, and 25) (P > 0.05) (data not shown).

NECROPSY FINDINGS

Acute pneumonia lesions consisted of consolidated parenchyma of ventral portions of the lobes, often accompanied by hemorrhage and fibrinous pleuritis. The mean % pneumonia for each group was: unvaccinated — 25.1, bacterin — 10.5, live vaccine — 16.5, and convalescent — 0.1 (SEM = 9.6), with no significant differences between the experimental groups (P > 0.05) due to the small number of animals in each group and the variability between animals within groups.

Chronic lung lesions were present in two calves of the convalescent group, characterized by abscessation of ventral portions of all lung lobes. These lesions were consistent with pneumonia of several weeks duration, as a result of infection of the calves with P. haemolytica on day 0.

Bacterial cultures of lungs revealed pure populations of P. haemolytica. Mean CFU log$_{10}$/gm of lung by experimental group were: unvaccinated group — 4.9, bacterin group — 5.0, live vaccine group — 7.6, and convalescent group — 5.8 (SEM = 1.6). There were no significant differences (P > 0.05) between the means.

CORRELATIONS

Lymphocyte proliferation and γ-interferon production by OMP-stimulated mediastinal lymph node cells were not significantly correlated with % pneumonia (Fig. 4A, B) or with bacterial concentration in pneumatic lesions. Serum antileukotoxin antibody titer on day 25 and % pneumonia were significantly correlated (P = 0.01) (Fig. 4C).

The log SI of OMP-stimulated mediastinal lymph node cells (on day 35 or 36) was significantly correlated with the antileukotoxin antibody titer on day 25 (r = 0.61; P = 0.05), however the γ-interferon production by OMP-stimulated mediastinal lymph node cells did not correlate significantly with antileukotoxin antibody titer on day 25 (data not shown). γ-interferon production by OMP-stimulated mediastinal lymph node cells did not correlate significantly with the log SI of OMP-stimulated mediastinal lymph node cells (data not shown).

DISCUSSION

The results reported here indicate that lymphocytes from calves exposed to live P. haemolytica react to fractionated antigens of P. haemolytica and a whole cell sonicate by undergoing mitosis and producing γ-interferon. These responses are indications that the calves have developed cell-mediated immunity to P. haemolytica. The lymphocyte responses were detected in lymph node lymphocytes collected 4 or 5 d after intratracheal challenge with P. haemolytica in calves that had been exposed to virulent P. haemolytica 31 d before challenge, with no evidence of responses in calves that had not been previously exposed. Previous vaccination with 2 doses of a killed alum adjuvanted vaccine also gave indications of stimulating lymphocyte proliferation and γ-interferon production, but the response was lower and was not statistically significant.

The lymphocyte responsiveness to P. haemolytica antigens was found only in lymph node lymphocytes collected at necropsy and not in PBMC collected prior to intratracheal challenge on day 31. Peripheral blood mononuclear cells were not tested on the day of necropsy because sufficient personnel weren’t available to test both lymph node and blood lymphocytes at the same time. The results indicate that either the lymphocytes from the blood do not develop
Since the convalescent group had recovered from a previous challenge and still did not develop detectable PBMC reactivity to \textit{P. haemolytica} antigens, it seems more likely that the reactive lymphocytes were not present in the blood and were partitioned in the lymph nodes. It is also possible that in the in vitro assays, the antigen presenting cells in the lymph nodes were more effective than those in the peripheral blood. The lymphocyte reactivity was detected when four of the five \textit{P. haemolytica} antigen preparations were used to stimulate the lymphocytes in vitro, with the capsular polysaccharide being the exception.

The highest proliferative responses for mediastinal lymph node cells occurred in calves in the live vaccine group and the convalescent group. There was clinical evidence of pneumonia in all calves of the convalescent group and chronic pneumonia was present in 2 of 3 calves at necropsy. In cattle, the caudal mediastinal lymph node receives lymphatic flow from the lung, therefore abundant \textit{P. haemolytica} antigen from pneumonic parenchyma should have been received by the caudal mediastinal lymph node of calves in the convalescent group.

Calves in the bacterin group had higher proliferative responses in superficial cervical than in mediastinal lymph node cells, indicating that the immunologic responses were localized near the site of administration of the bacterin. Conversely, calves in the live vaccine group had higher $\gamma$-interferon responses (and higher, but not statistically significant proliferative responses) in mediastinal than superficial cervical lymph node cells, indicating that abundant antigen from \textit{P. haemolytica} was delivered to mediastinal lymph node cells, indicating that abundant antigen from \textit{P. haemolytica} was delivered to mediastinal lymph nodes for sensitization of T-lymphocytes. It is possible that the live vaccine replicated in the lung or that blood-borne antigens were removed in the lung and transported to mediastinal lymph nodes. \textit{Pasteurella haemolytica} may cause pneumonia when inoculated intravenously into animals (26,27) and it is possible that the live vaccine replicated at sites other than the site of vaccination. Administration of live \textit{P. haemolytica} vaccine has been associated with \textit{P. haemolytica} septiceemia and meningitis in cattle (28). In the present study, calves vaccinated with the live vaccine had febrile responses after vaccination, as well as extensive tissue swelling at the site of vaccination; either may be associated with septicemia, but blood cultures were not performed on calves to determine if bacteremia occurred.

The titer of antileukotoxin antibody in the serum on day 25 correlated significantly with protection from challenge, whereas the lymphocyte proliferation and $\gamma$-interferon production did not correlate with protection. This again confirms the important role of antileukotoxin antibody in protecting cattle from pneumonia caused by \textit{P. haemolytica}. $\gamma$-interferon is known to activate neutrophils from cattle to have enhanced bactericidal activity (29). Even though there was no significant correlation between either lymphocyte proliferation or $\gamma$-interferon production, and extent of pneumonia after challenge, it is still possible that CMI may contribute to protection of cattle from \textit{P. haemolytica} induced pneumonia. Further studies will be needed to determine the importance of the observation that lymph node lymphocytes from cattle exposed to live \textit{P. haemolytica} respond to \textit{P. haemolytica} antigens by proliferating and secreting $\gamma$-interferon.

**REFERENCES**

6. CONFER AW, PANCIERA RJ, GENTRY MJ, FULTON RW. Immunologic


