Effects of injectable trace minerals on the immune response to *Mannheimia haemolytica* and *Pasteurella multocida* following vaccination of dairy calves with a commercial attenuated-live bacterin vaccine

J. H. J. Bittar,* D. J. Hurley,* A. R. Woolums,† N. A. Norton,‡ C. E. Barber,* F. Moliere,‡ L. J. Havenga,§ and R. A. Palomares*1

*Department of Population Health, University of Georgia, Athens 30602-2771; †Department of Pathobiology and Population Medicine, College of Veterinary Medicine, Mississippi State University, Mississippi State 39762; ‡Department of Large Animal Medicine, College of Veterinary Medicine, University of Georgia, Athens 30602-2771; and §Multimin USA Inc., Fort Collins, CO 80528

**ABSTRACT**

The objective was to evaluate the effects of an injectable trace mineral (ITM) supplement containing Zn, Mn, Se, and Cu on the humoral and cell mediated immune responses to vaccine antigens in dairy calves receiving an attenuated-live bacterin vaccine containing *Mannheimia haemolytica* and *Pasteurella multocida*. Thirty 3-mo-old dairy calves received 2 doses (21 d apart) of an attenuated-live *M. haemolytica* and *P. multocida* bacterin vaccine (Once PMH, Merck Animal Health, Summit, NJ), and a 5-way modified-live-virus vaccine (Express 5, Boehringer Ingelheim, Vetmedica, St. Joseph, MO). On the day of primary vaccination, animals were randomly assigned to 1 of the 2 treatment groups (n = 15 per group): ITM (ITM administration) or control (sterile saline injection). Treatments were administered concurrently with vaccinations. Blood samples were collected for determination of antibody titers against *M. haemolytica* and *P. multocida* and of antigen-induced proliferation and interferon-γ secretion by peripheral blood mononuclear cells. Serum Se and Mn concentrations were greater (*P* < 0.05) in the ITM group than the control group after ITM use. Serum end-point antibody titers against both bacteria and interferon-γ secretion by peripheral blood mononuclear cells were not different (*P* > 0.05) between groups. The use of ITM with bovine respiratory disease vaccines enhanced (*P* < 0.01) antibody titer fold-change to *M. haemolytica*. Proliferation of peripheral blood mononuclear cells after *P. multocida* stimulation was increased (*P* = 0.03) in the ITM group on d 21 relative to baseline value. In conclusion, ITM administration concurrently with bacterin vaccination improved the immune response to *M. haemolytica* and *P. multocida* and might be a valuable tool to enhance dairy calves’ response to bovine respiratory disease vaccination.

**Key words:** trace minerals, dairy calf, bovine respiratory disease, attenuated-live bacterin vaccine, immune response

**INTRODUCTION**

Bovine respiratory disease complex (BRDC) is considered a major illness that affects North America’s cattle industry resulting in substantial economic losses (> $1 billion/yr; Griffin, 1997; McVey, 2009). The complexity of BRDC can be attributed to several factors that potentiate its pathogenesis, including its poly-microbial etiology, stress, immune suppression, failure of passive transfer, weather extremes, or poor biosecurity. The infectious agents commonly involved in BRDC include bovine viral diarrhea virus (BVDV), bovine herpes virus-1, bovine respiratory syncytial virus (BRSV), parainfluenza 3 virus, *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis*. The typically commensal relationship of *M. haemolytica* and *P. multocida* with cattle makes prevention and control of BRDC more difficult (Filion et al., 1984; Confer, 2009). These 2 bacteria are considered normal flora of the cattle upper respiratory tract and on occasion, when animals are immunosuppressed (especially during stress), they colonize the bronchi and lungs causing mild to fatal illness (Dabo et al., 2007; Rice et al., 2007; Confer, 2009). The damage caused by these bacteria in the lower respiratory tract is usually due to the excessive influx of neutrophils and accumulation of fibrin in the lungs, which result in acute respiratory disease and occasionally in death (Dabo et al., 2007; Rice et al., 2007; Confer, 2009).

---

The authors declare no conflicts of interest, with the exception of L. J. Havenga, who is an employee of Multimin USA.

*Corresponding author. palommr@uga.edu*
Appropriate biosecurity measures and vaccination programs are crucial to prevent and control BRDC. The use of bacterins against *M. haemolytica* and *P. multocida* plays an important role in the prevention of BRDC by reducing the level of colonization and decreasing the likelihood of negative effects on cattle health and performance (Aubry et al., 2001; Larson and Step, 2012). Serum antibodies against bacterial leukotoxin and to specific bacterial surface antigens are considered core in the defense against these pathogens (Shewen and Wilkie, 1988; Crouch et al., 2012), and high concentration of *P. multocida* antibodies in serum at feedlot entry has been correlated with improved beef cattle performance (Fulton et al., 2002).

Trace minerals play an important role in adequate health, performance, and immune response to viral BRDC pathogens in dairy calves (Teixeira et al., 2014; Palomares et al., 2016). Some trace minerals, including Se, Zn, Cu, and Mn, are fundamental elements in the structure and function of several proteins that participate in general homeostatic processes essential for an adequate immune function. These include the pathways regulating energy production, DNA replication and transcription, and modulators of reactive oxygen species (ROS; Failla, 2003; Genther and Hansen, 2014). The use of injectable trace minerals (ITM) combined with vaccination demonstrated a positive effect in beef cattle by increasing serum neutralizing antibodies against bovine herpes virus-1 (Arthington and Havenga, 2012; Arthington et al., 2014), reducing morbidity and antibiotic treatment and costs, and increasing ADG in shipping-stressed calves (Richeson and Kegley, 2011). Additionally, in dairy calves with adequate mineral status, administration of ITM has been proven to enhance health status by increasing neutrophil function, glutathione peroxidase activity (Teixeira et al., 2014), as well as serum neutralizing antibody titers and leukocyte proliferation against common bovine respiratory disease (BRD) pathogens (Palomares et al., 2016).

In a previous study, we reported that administration of ITM concurrent with a modified-live-virus (MLV) vaccine in dairy calves resulted in earlier and more robust antibody titers against bovine herpes virus-1 (Arthington and Havenga, 2012; Arthington et al., 2014), reducing morbidity and antibiotic treatment and costs, and increasing ADG in shipping-stressed calves (Richeson and Kegley, 2011). Additionally, in dairy calves with adequate mineral status, administration of ITM has been proven to enhance health status by increasing neutrophil function, glutathione peroxidase activity (Teixeira et al., 2014), as well as serum neutralizing antibody titers and leukocyte proliferation against common bovine respiratory disease (BRD) pathogens (Palomares et al., 2016).

In a previous study, we reported that administration of ITM concurrent with a modified-live-virus (MLV) vaccine in dairy calves resulted in earlier and more robust antibody titers against BVDV1 and in more robust peripheral blood mononuclear cell (PBMC) proliferation after stimulation with BVDV1 and BRSV antigen than for the control group (Palomares et al., 2016). In the present study, we tested the hypothesis that administration of ITM can also improve the antibody and cell mediated immune responses to vaccine bacterial antigens in dairy calves receiving an attenuated-live bacterin vaccine containing *M. haemolytica* and *P. multocida*.

**MATERIALS AND METHODS**

This trial is part of a larger study performed at the University of Georgia, where we investigated the effects of ITM on the immune response to viral antigens following MLV vaccination (Palomares et al., 2016). The calves were cared for in accordance with acceptable practices as for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 2010). The research protocol was approved by the University of Georgia Institutional Animal Care and Use Committee.

**Experimental Design, Animals, and Treatments**

A total of 30 weaned dairy bull calves (3.5 mo of age) received 2 mL of an attenuated-live *M. haemolytica* and *P. multocida* bacterin (Once PMH, Merck Animal Health, Summit, NJ) and 5 mL of a MLV vaccine containing bovine herpes viruses-1, BVDV1 and 2, BRSV, and parainfluenza 3 virus (Express 5, Boehringer Ingelheim, Vetmedica, St. Joseph, MO). Both vaccines were given subcutaneously as recommended by the manufacturer. On the day of primary vaccination, calves were randomly assigned to 1 of 2 groups (15 calves per group) using a software (Research randomizer, V3.0, Social Psychology Network, Middletown, CT): (1) ITM, administration of injectable trace minerals (1 mL/45 kg of BW; Multimin 90, Multimin USA Inc., Fort Collins, CO) subcutaneously concurrently with vaccination, or (2) control, injection of sterile saline (1 mL/45 kg) subcutaneously at the time of vaccination. Three weeks after initial vaccination, calves received a booster of the same vaccines and a second dose of ITM or saline according to previous group assignment. Administration of ITM provided 15 mg/mL of Cu, 60 mg/mL of Zn, 5 mg/mL of Se, and 10 mg/mL of Mn. Calves were grazing in bermudagrass (*Cynodon dactylon*) and fescue (*Festuca arundinacea*) with ad libitum access to hay and water. A commercial cattle ration (Bulk Cattleman’s Special; Godfrey’s Warehouse, Madison, GA) containing energy, protein, minerals, and vitamins was offered twice daily as a supplement (around 2.7 kg/d per calf).

**Sample Collection**

Blood was collected from all the calves via jugular venipuncture into vacuum tubes (Vacutainer, BD Diagnostic, Franklin Lakes, N.J) with and without anticoagulant (acid citrate dextrose) to obtain whole blood and serum, respectively. Blood was collected on d −7, 0 ( enrollment), 7, 14, 21, 28, 42, 56, and 90 relative to primary vaccine and ITM administration, for determination of serum trace mineral concentration, serum neutralizing antibody titers to *M. haemolytica* and *P. multocida*, antigen-induced PBMC proliferation, and interferon (IFN)-γ production upon stimulation with *P. multocida*. Additionally, trace mineral status was assessed in liver biopsy samples collected from each calf on d −7, 21, and 56 relative to the day of primary vaccination (d 0) as previously described (Palomares et al., 2016).

**Blood Sample Processing and PBMC Preparation**

Within 2 h after collection, blood samples were transported on ice to the laboratory and tubes without an-
ticoagulant (Vacutainer, BD Diagnosis) were centrifuged (650 × g for 12 min at room temperature). The serum was collected and stored in microcentrifuge-tube (Fisherbrand Premium Microcentrifuge Tubes, Fisher Scientific, Waltham, MA) aliquots at −80°C until analysis for antibody titers against *M. haemolytica* and *P. multocida*. Mineral concentrations in liver and serum were determined at the Diagnostic Center for Population and Animal Health (Michigan State University, Lansing, MI). Blood samples with anticoagulant were used to obtain PBMC for antigen-induced proliferation and IFN-γ production assays. Peripheral blood mononuclear cells were isolated and suspended as previously described (Palomares et al., 2016).

**Serum Antibody Titers Against *M. haemolytica* and *P. multocida***

Serum IgG antibodies binding to *M. haemolytica* and *P. multocida* antigens were determined via an in-house ELISA at the research laboratory of the Department of Large Animal Medicine of the College of Veterinary Medicine, University of Georgia (building 11). Briefly, 96-well plates coated with *M. haemolytica* antigen (1:200 dilution in coat buffer of a clinical isolate from the University of Georgia Athens Diagnostic Laboratory, Athens, GA) were incubated at 4°C for 18 to 24 h and washed 3 times with wash buffer (phosphate buffered saline with 0.5% Tween-20 added) before use. Serial dilutions in wash buffer of each calves’ serum and a 1:40 dilution of positive control serum and 1:100 dilution of negative control serum in wash buffer were plated in quadruplicate wells and incubated at room temperature for 1 h. The plates were washed 3 times with wash buffer, and then 100 μL of a commercial anti-bovine IgG (heavy and light chain specific whole serum from rabbit) conjugated with horseradish peroxidase (1:1000 dilution in wash buffer; Sigma-Aldrich, St. Louis, MO) was added to each well. The plates were incubated for 30 min at room temperature and then washed 4 times. Next, 100 μL of ABTS (2,2′-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid], Sigma-Aldrich) substrate solution containing saturating amounts of hydrogen peroxide was added to each well. The plates were incubated at room temperature in the dark for 15 to 60 min (until the positive control serum reached saturation color) to then be read at 405 nm wavelengths (GEN5 Epoch Microplate Spectrophotometer, BioTek Instruments Inc., Highland Park, Winooski, VT), and mean optical density (OD) for each sample was recorded. A similar ELISA protocol was used for detection of *P. multocida* IgG antibody in the calf serum, except the plates were coated with *P. multocida* antigen at 1:500 dilution (using a clinical isolate from the University of Georgia Athens Diagnostic Laboratory, Athens, GA). The endpoint titers for both bacteria were determined by finding the highest dilution of serum that had a mean OD that was twice or greater than the mean OD of the negative control sample on each plate, and titer was expressed as the inverse of the dilution of the serum. Plates were considered valid if the positive control samples yielded an average OD of 0.8 to 1.1 and the negative control yielded an average OD of −0.6 to 0.01.

**PBMC Proliferation in Response to *P. multocida***

Procedures for PBMC proliferation were performed as previously described (Palomares et al., 2016). For PBMC proliferation assay, 100 μL of the PBMC suspension (6 × 10⁶ cells/mL) from each calf was aliquoted into wells of a 96-well round bottom plate in triplicate. To each set of triplicate wells, 100 μL of *P. multocida* killed antigen suspension (prepared from a clinical isolate of the University of Georgia Athens Diagnostic Laboratory, Athens, GA, using 2.9 × 10¹⁰ cfu killed at 60°C for 1 h before freezing) and staphylococci enterotoxin SEB (Toxin Technology Inc., Sarasota, FL; 0.5 μg/mL as positive control), or Roswell Park Memorial Institute medium + 10% gamma-irradiated fetal calf serum + 2 mM of L-glutamine and 1% penicillin-streptomycin (negative control), were added. Results were expressed as the specific 3H-thymidine (7.4 × 10¹⁰ Bq per well, stock 2.48 × 10²¹ Bq per mM, from MP Inc., Indianapolis, IN) incorporation calculated as the mean counts per minute for PBMC cultured with *P. multocida*, or mitogen, divided by the mean counts per minute for PBMC cultured in cell culture media alone.

**IFN-γ Secretion by PBMC in Response to *P. multocida***

A total of 600 μL of PBMC suspension (6 × 10⁶ cells/mL) was aliquoted into each well of a 24-well flat bottom plate. To each set of triplicate wells, 600 μL of *P. multocida* killed antigen was added as previously described for other pathogens (Palomares et al., 2016). Secretion of IFN-γ by PBMC in response to stimulation with *P. multocida* was measured by ELISA using a commercially available antibody reagent pair and recombinant standard (R&D Systems, Minneapolis, MN) at dilutions optimized in our laboratory as previously described (Palomares et al., 2016). All PBMC supernatant samples designated for the IFN-γ assay were stored at −80°C until sample collection was completed, so that all the samples could be analyzed at the same time.

**Statistical Analysis**

Data were analyzed using SAS (SAS version 9.3; SAS Institute Inc., Cary, NC). Statistical assumptions of normality and constant variance were tested through Shapiro Wilk’s and Levene’s tests, respectively. A logarithmic base 2 transformation was applied to the antibody titer and stimulation index values. For calculation and comparison of geometric mean antibody titers, back-transformed antibody titers were calculated for each group at d 0, 7, 14, 21, 28, 42, 56, and 90 after vaccination. Stimulation index triplicates for PBMC proliferation were Q-tested (n = 3, CI = 0.941) for outlier identification and elimination.
A 2-independent-sample t-test was performed to compare antibody titers, PBMC proliferation, IFN-γ secretion, and trace mineral concentrations between treatment groups for each day. The model was defined as follows:

\[ y_{ij} = \mu + \tau_i + \beta_j + \varepsilon_{ij}, \]

where \( y_{ij} \) is the respective response (antibody titers, IFN-γ secretion, or stimulation index) of subject \( j \) to treatment \( i \) (ITM or saline) at 0, 7, 14, 21, 28, 42, 56, and 90 d after treatment; \( \mu \) is the mean; \( \tau_i \) is the effect of day \( i \) (ITM or saline); \( \beta_j \) is a variable associated with subject \( j \) on d 0 (baseline); and \( \varepsilon_{ij} \) is the random error.

Additionally, a repeated measure analysis was done to compare the antibody titers, IFN-γ secretion, and PBMC proliferation during the experimental period (7, 14, 21, 28, 42, 56, and 90 d) with the baseline on d 0. The mixed generalized linear model was defined by the following equation:

\[ y_{ij} = \mu + \tau_i + \beta_j + \varepsilon_{ij}, \]

where \( y_{ij} \) is the response (antibody titer, IFN-γ secretion or stimulation index) of subject \( j \) on day \( i \) for each treatment group; \( \mu \) is the mean; \( \tau_i \) is the effect of day \( i \) after ITM or saline treatment (d 7, 14, 21, 28, 42, 56, and 90), compared with the effect on d 0; \( \beta_j \) is a variable associated with subject \( j \) on d 0 (baseline); and \( \varepsilon_{ij} \) is the error. We assumed that days after treatment were fixed and that the calves used were a random sample of calves from a larger population of calves. Thus, the calves collectively represented a random effect, so we assumed that the mean of \( \beta_j \) was zero and that the variance of \( \beta_j \) was \( \sigma^2 \). Because the term \( \beta_j \) was common on all days after inoculation of the same calf, the covariance between \( y_{ij} \) and \( y_{ij'} \) was not zero but constant across all days and subjects.

Nonparametric analysis was also applied using Mann-Whitney U and Friedman tests for comparisons between treatment groups and over time, respectively. For all analyses, values of \( P \leq 0.05 \) were considered significant, and \( 0.05 < P \leq 0.10 \) was considered a tendency.

**RESULTS AND DISCUSSION**

**Trace Mineral Concentration in Serum and Liver**

Calves in both groups had similar liver concentrations of Se, Zn, Cu, and Mn on \( d -7 \) (\( P > 0.05 \); Palomares et al., 2016), which were within adequate reference values (Herdt and Hoff, 2011) during the experimental period, indicating that there were no Se, Zn, Cu, and Mn deficiencies in the animals used in this study. Administration of ITM resulted in increased concentrations of liver Se (on d 21 and 56; \( P < 0.001 \)), Cu (on d 56; \( P < 0.01 \)), and Mn (on d 56; \( P < 0.05 \)) compared with saline injected calves (Palomares et al., 2016). In the present study, supplementation with injectable trace minerals in dairy calves was able to increase or mitigate the abrupt decay in hepatic concentrations of the studied trace minerals after vaccination compared with the control calves, which agrees with previous reports (Droke and Loerch, 1989; Arthington and Havenga, 2012; Teixeira et al., 2014).

Serum Se, Cu, Zn, and Mn concentrations were also similar in both groups before vaccination and treatment administration (d \( -7 \); \( P > 0.05 \)). Selenium concentration in serum increased dramatically between d 14 and 90 after primary vaccination and ITM administration (\( P < 0.05 \)). These values were significantly greater than those observed in the control group during the same time points (\( P < 0.05 \); Figure 1A). Serum concentration of zinc, copper, and manganese increased in both groups after primary and secondary vaccination (\( P < 0.05 \); Figure 1B, 1C, and 1D). Zinc concentration was numerically greater in the ITM calves on d 21 and 42 after vaccination compared with the saline-injected calves (Figure 1B) but without statistical significance (\( P > 0.05 \)). There was no significant difference in serum copper levels between groups during the experimental period (\( P > 0.05 \); Figure 1C). The injectable trace mineral group had greater manganese concentration on d 21 compared with control group (\( P < 0.05 \); Figure 1D).

Previous studies have shown an increase in serum copper and zinc concentrations in newly received beef heifers after ITM administration (Richeson and Kegley, 2011). It is possible that in the present study, the Zn and Cu levels did not show an accurate indication of mineral status; these concentrations might have been augmented due to physiological stress (Richeson and Kegley, 2011).

**Antibody Titers Against M. haemolytica and P. multocida**

The antibody response was evaluated through absolute values (end point antibody titers) and normalized values relative to the day of the primary vaccinations (fold change antibody titers). Group assignment was not stratified based on the levels of antibody titers against \( P. multocida \) and \( M. haemolytica \). Therefore, there were numerical differences in antibody titers against \( M. haemolytica \) between groups before vaccination. Fold change antibody titer was calculated to normalize the antibody titers to the baseline values on d 0 in an attempt to better understand the effects of treatments on the dynamics of antibody production.

A strong humoral immune response to \( M. haemolytica \) or \( P. multocida \) was not observed after the primary vaccination. However, both the ITM and control group had a significantly increased antibody response (measured as end point antibody titer) to both antigens after secondary vaccination (\( P < 0.01 \); Figure 2A and 2C). In this study, the low antibody response (evaluated either as end point antibody titer or as fold change titer) observed after primary vaccination might be associated with the presence of passive (maternally derived antibodies) and active humoral immune responses interfering with vaccine stimulation (Brar et al., 1978; Aubry et al., 2001). The initial dose of...
vaccine apparently primed the immune system; therefore, the animals were able to respond more effectively to secondary vaccination, even in the presence of maternally derived antibodies (Aubry et al., 2001). Similar results were previously observed in another study using a similar M. haemolytica or P. multocida vaccine, in which antibody titers were the greatest 2 wk after the second dose of vaccine was administered (Srinand et al., 1996).

Significant differences were not observed for the end point serum antibody titers against M. haemolytica between groups at any time point ($P > 0.05$; Figure 2A). The antibody titers to M. haemolytica changed differentially in both groups during the experimental period. In the control group, antibody titers decreased after primary vaccination and rose on d 28, 42, and 56, but a significant increase relative to the baseline levels was only observed on d 90 after primary vaccination ($P < 0.01$). In contrast, in the ITM group, the end point antibody titers significantly increased earlier and more consistently (from d 28 to 90) compared with the baseline titers on d 0 ($P < 0.01$). Accordingly, the ITM group had a greater fold change of antibody titers on d 14 ($P = 0.06$) and 56 ($P = 0.02$) compared with the control group (Figure 2B). Antibody titers against P. multocida increased similarly in both groups during the experimental period relative to d 0 ($P < 0.01$; Figure 2C). However, there were no differences in fold change antibody titers against P. multocida between groups ($P > 0.05$) at any time point (Figure 2D).
Although a slight increase was observed after the primary vaccination (approximately 1-fold change), the secondary vaccination induced a strong anamnestic immune response with almost 3-fold increase in titers in both treatment groups (Figure 2D).

The poor antibody response after primary vaccination observed in this study reinforces the necessity of booster vaccination by 2 to 3 wk after primary vaccination to reac(h a significant humoral immunity to *P. multocida* and *M. haemolytica*, which is particularly important before commingling with other calves (Aubry et al., 2001; Fulton et al., 2002; Larson and Step, 2012). The presence of neutralizing antibodies against *P. multocida* is considered a major arm of defense that contributes to the prevention of BRD and has been positively correlated with enhanced performance at feedlot entry (Fulton et al., 2002). However, a previous study to evaluate the performance of vaccinated calves in a scenario of greater natural disease challenge or submitted to *M. haemolytica* and *P. multocida* challenge is warranted.

**Proliferation and IFN-γ Secretion by PBMC to *P. multocida* Stimulation**

There was an augmented PBMC proliferation upon *P. multocida* stimulation only in the ITM group on d 21 after primary vaccination compared with d 0 (*P* = 0.03; Figure 3). Conversely, control calves did not show any significant change in PBMC proliferation to *P. multocida* over time when compared with d 0 (*P* > 0.05). The increased PBMC proliferation in the ITM group on d 21 to *P. multocida* stimulation might indicate that ITM enhanced the immune priming induced by the initial vaccine dose, so that PBMC in this group were more responsive to ex vivo bacterial antigen stimulation, suggesting a potential im-
Injectable trace minerals and postvaccination immune response

Figure 3. Peripheral blood mononuclear cell (PBMC) proliferation response to *Pasteurella multocida* in dairy calves treated or not with injectable trace minerals (ITM) concurrently with an attenuated-live bacterin vaccine. Errors bars represent the SEM. No significant difference existed between groups in any day (P > 0.05), and no difference from d 0 existed on any day for the control group (P > 0.05). Significant difference compared with d 0 for the ITM group (P = 0.03).

administration could have contributed to enhance the immune response in an attempt to prevent respiratory disease in the dairy cattle raising system.

ACKNOWLEDGMENTS

The authors thank the University of Georgia and MultiMin USA Inc. and for the financial support to perform this research. The authors appreciate the valuable contribution of Mark Chastain and Troupe Tabb from the University of Georgia Oconee County farm.

LITERATURE CITED


FASS. 2010. Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching. 3rd ed. FASS Inc., Champaign, IL.


