IMMUNOMODULATORS IN SWINE: SODIUM DIETHYLDITHIOCARBAMATE IN WEANLING PIGS, AND DECREASED LYMPHOCYTE FUNCTION IN PSEUDORABIES VIRUS INFECTED FEEDER PIGS: INFLUENCE OF ISOPRINOSINE.

by

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LITERATURE REVIEW

Introduction

Most swine produced for market in the United States are raised in large, intensively managed, total confinement operations. Farrow to finish operations with hundreds to thousands of sows are common. Because large numbers of animals are being closely confined, disease is always a major concern. An outbreak of an acute infectious disease could have disastrous consequences under these conditions, consequently herd health and disease prevention have become keys to the producer's success. Because disease is often the culmination of many factors, an effective herd health program must be multifaceted. Environment, nutrition, genetics, infectious disease, and parasitism must all be managed in an integrated fashion to maintain a successful operation. Many factors influence the immune system and hence the animals susceptibility to disease. Several recent reviews have discussed the effects of various stressors on the immune system.[1-4] Herd health programs work primarily to minimize stressors on the immune system, alternatively a current area of intense research is the search for exogenous substances that can mitigate suppressive influences on the immune system, or perhaps, even boost the immunological status of the animal. These types of drugs are often referred to as immunomodulators: broadly interpreted to refer to any drug that influences the immune system, either enhancing or

suppressing, but commonly used to refer only to those substances that potentiate immune responses- the definition used in this thesis.

Immunomodulation

Immunomodulation may someday become a routine part of production management in the swine industry. The growing concern by consumers over the use of antibiotics as feed additives may make immunomodulators a wise marketing choice for pork producers.[5] Immunomodulators may also prove very beneficial if they could be administered to mitigate anticipated problem times in the production life of an animal: e.g. weaning, transport, the neonatal period, during disease outbreaks (especially viral disease), and during inclement weather. Immunomodulators may also become valuable in the treatment of several clinical syndromes where an acquired immunodeficiency may be the underlying problem. This literature review will briefly discuss some immunomodulators that have been examined in swine.

Dietary Factors

Producers have realized the effects of diet on the efficiency of production by food animals for quite some time, however, recent investigations have revealed an influence of diet on the immune system. Normal dietary constituents have been shown to have an effect on the immune response. Not

suprisingly, amino acid supplementation in gestating sows fed a low protein diet has been shown to increase antibody production to sheep red blood cells, as well as increase transfer of passive immunity to piglets.[6] Vitamin E and selenium have also been investigated for their effect on the immune system. Ellis and Vorheis demonstrated an increase in whole cell agglutinin responses to an Escherichia coli whole cell bacterin in animals fed supplemental vitamin E.[7] Subsequently, Peplowski, et.al., showed increased antibody titers to sheep red blood cells in weanling swine associated with either feeding or injecting vitamin E or selenium with an additive effect when both were administered.[8] In a similar manner proliferative responses to phytohemagglutinin, a T cell mitogen, were found to be augmented by feeding either vitamin E or selenium, or both.[9] There is some evidence that vitamin C may also be an effective immunomodulator in some species. Roth and Kaberle reported enhanced neutrophil functions when vitamin C was injected into cattle,[10] but recent reports on the use of vitamin C in swine have shown no effect on humoral or cellular immune responses.[11,12] We are just now beginning to understand the profound effects diet has on health, certainly more information will come to light in this field in the future.

Cytokines

The understanding of the role of cytokines in the

regulation of the immune system combined with recombinant DNA technology is opening an exciting area of potential immunomodulators. Interferons are just one of the cytokines under investigation as a possible immunomodulator of clinical significance. Interferons are part of the hosts nonspecific immune response to viral invaders; these cytokines, synthesized by virally infected host cells, act to produce an interferon-induced antiviral state that prevents viral replication in the induced cell.[13] A recent study sought to determine if bovine recombinant a-1 interferon (BoIFN) could protect newborn pigs against transmissible gastroenteritis virus (TGE) infection. Initial in vitro work showed that BoIFN was effective in inhibiting replication of both vesicular stomatitis virus and TGE virus in cultures of swine testicular cells. However, when newborn pigs were administered BoIFN orally at 12 hour intervals from 6 hours of age and subsequently challenged with TGE, disease and mortality were similar for both control and BoIFN treated animals. Further data indicated that the BoIFN was inactivated within one minute of entering the small intestine; the authors concluded that BoIFN might be effective in preventing TGE infection if a method could be found to prevent enzymatic degradation in the small intestine.[14]

Interleukin-2 (IL-2) is another cytokine that through recombinant DNA technology has become available in large

enough quantities to allow clinical trials. This lymphokine, secreted by T cells, is capable of stimulating T cell clonal expansion, activation of cytotoxic T cells, and induction of natural killer cell activity.[15] Pigs administered human recombinant IL-2 and a Haemophilus pleuropneumoniae vaccine had higher antibody titers to H. pleuropneumoniae and a lower incidence of lung lesions after challenge.[16,17] Interleukin-2 administration was also shown to increase natural cytotoxicity in pigs when administered in vivo.[18] In a vaccination and challenge experiment calves that recieved bovine recombinant IL-2 had increased antibody titers, increased cytotoxic responses, and decreased clinical disease; however, there were significant clinical side effects associated with IL-2 administration.[19] These experiments indicate that there are some exciting possibilities for the future use of IL-2, yet more work must be done before this lymphokine is useful in a clinical setting.

Levamisole

Levamisole was the first in a new class of immunomodulators to be described. Originally marketed as an anthelmintic drug, levamisole came under intense scrutiny after a 1971 report that described an enhanced response by mice to a Brucella vaccine when given levamisole.[20] Levamisole quickly became perhaps the most widely tested immunoactive drug. The extensive literature about levamisole

has been well reviewed.[21-23] Our laboratory has reported some work that demonstrated levamisole to be an effective immunomodulator in artificially reared pigs. Pigs were removed from the sow within two days of parturition and artifically reared for 21 days. Levamisole and isoprinosine, another immunomodulator discussed below, were administered to separate groups of artificially reared pigs. Lymphocyte proliferation and phytohemagglutinin skin responses were evaluated in drug treated groups, artificially reared pigs, and sow reared controls. Those pigs that were artificially reared had decreased responses when compared to pigs that remained with the sow. Both levamisole and isoprinosine were effective in returning the immunologic parameters of artificially reared pigs to sow reared control levels.[24] Other workers demonstrated an increased secondary antibody response to antigen after levamisole treatment.[25] Clearly, levamisole can alter immune responses in mammals; but generally this drug is only effective in enhancing suppressed immune systems. The drug works primarily on phagocytes and T lymphocytes, with no direct action on B cells. The exact mode of action of levamisole remains unclear. The timing and dosage of the drug appear to be very important in enhancing the immune response. No alteration or actual suppression has been observed as a consequence of levamisole administration.[21-23] A recent review in a clinical journal outlined some guidelines for the clinical use of levamisole. The authors suggested that

levamisole only be used in immunosuppressed individuals at the recommended dosage regimen, and only be used where immune parameters could be monitored for improvement or adverse reactions. In addition some of the problems associated with the use of levamisole were outlined. The effects of the drug appear to be inconsistant, nonresponsive individuals appear to be present in most populations; increased inflammation and gingivitis have been observed as adverse side effects. The most severe side effect noted after levamisole administration is the development of an autoimmune like reaction that results in a severe agranulocytosis.[22] Unfortunatly, levamisole has suffered from faddish popularity; a fact which Renoux commented on in a 1978 review, advice that probably should be applied to all new drugs in this field. "The random and uncritical use of levamisole may produce inconsistent, uninterpretable results and unjustifiably dampen the enthusiasm for a rational, scientifically-based immunopharmacology in the future."[26]

Imuthiol

The ambivalent responses to levamisole led the Renoux team to search for a compound devoid of the inhibitory effects of levamisole, (thought to be due to cholinergic properties of the imadazole moity) while retaining the positive aspects of its immunomodulation ability. Sodium diethyldithiocarbamate (also known as DTC and imuthiol) was the compound finally

selected that fit the desired criteria. Imuthiol is a chelating agent used in chemistry, agriculture, and in the treatment of nickel poisoning.[27] This drug is nonmutagenic, noncarcinogenic, nonpyrogenic, and nonantigenic.[28] Like its predecessor imuthiol was found to act primarily on T-cells. The compound is not active in vitro, but becomes an effective immunoenhancer when administered in vivo. Imuthiol posseses direct antibiotic effects, is protective against the effects of ionizing radiation and can act as a detoxifier of chemicals such as chloroform, carbon tetrachloride, and bromobenzene. Unlike levamisole, imuthiol appears to be effective over a wide dose range without any deleterious side effects. B cells are unaffected by imuthiol, however the drug has been shown to speed the shift from IgM to IgG production, a T cell mediated event.[27,29] Differentiation of T cell precursors, increased natural killer cell activity, increased lymphocyte proliferation to mitogens, and increased IL-2 production have all been shown after in vivo imuthiol administration.[30-34] Imuthiol has been used in several laboratory animals and humans. Individuals regained age suppressed T cell responses after imuthiol administration.[30] Offspring of female mice that received imuthiol during pregnancy had enhanced T cell responses and neonatal growth rates.[35,36] Because of the above observations we sought to evaluate the use of imuthiol in the weanling pig. Pigs at weaning have decreased T cell responses[37] and are often afflicted with disease, thereby

making them good candidates for immunotherapy. The experiment is fully described in the second part of this thesis. Unfortunately, imuthiol was not effective in weaned pigs at the dosage rates used. Perhaps, further experimentation will illucidate an effective treatment regimen for imuthiol use in the pig.

Isoprinosine

First investigated in 1972 as an antiviral drug by Gordon and Brown,[38] isoprinosine was soon recognized as an immunoenhancing drug.[39] Isoprinosine, a compound formed from a 1:3 molar ratio of inosine and N,N-dimethylamino-2-propanol, is thought to affect purine metabolism, either by providing substrate or perhaps through competetive inhibition of enzymes, in such a way as to increase intracellular cGMP, thereby stimulating T cells and macrophages.[40] Isoprinosine has been shown to augment a number of T cell mediated functions: increased lymphocyte blastogenesis, increased cytotoxic T lymphocyte activity, increased IL-2 production, as well as increased natural killer cell and macrophage activity.[41,42] Clinically, isoprinosine has been shown to be efficacious in treating human herpesvirus and chronic lung infections. Positive clinical results have also been reported in a number of other disease states.[41,42] Our laboratory previously reported that isoprinosine successfully abrogated the suppressive effect of artificial rearing in neonatal

pigs.[24] However, in a more recent experiment, described in part III of this thesis, isoprinosine had no effect on pseudorabies virus induced immunosuppression in feeder pigs. Perhaps, the mode of immunoenhancement associated with isoprinosine is able to compensate for the suppression caused by management stressors (i.e. artificial rearing) but is not able to reverse viral induced suppression.

Conclusions

Immunomodulators are not yet the silver bullet of medicine, now or maybe ever. Yet, the new drugs offer new ways to study, explore, and understand the immune system. A better understanding of the regulation and action of the immune system during normal and disease states should lead to improved disease management and intervention regimens.

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Influence of sodium diethyldithiocarbamate (imuthiol) on lymphocyte function and growth in weanling pigs

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Footnotes

[a]Gibco Laboratories, Grand Island, NY.

[b]Sigma Chemical Co., St. Louis, MO.

[c]Schering Laboratories, Maplewood, NJ.

[d]Hybri-sure, Hazeleton Dutchland, Denver, PA.

[e]Catalog No. 76-042-05 Flow Labs, McLean, VA.

[f]Pharmacia Fine Chemicals, Piscataway, NJ.

SUMMARY

Mitogen-stimulated lymphocyte proliferation, delayed-type hypersensitivity (DTH) reactions, interleukin-2 (IL-2) production, and growth performance were evaluated in 3-week-old pigs treated with imuthiol. Lymphocyte proliferative responses to Con A and PWM were reduced ($P<0.05$) in pigs treated with imuthiol at 25 mg/kg; PHA proliferative responses were not influenced by imuthiol treatment. Imuthiol at 2.5 mg/kg and 25 mg/kg lowered IL-2 production when compared to saline-treated controls. Delayed-type hypersensitivity responses to PHA were higher in 25 mg/kg imuthiol-treated pigs; however, 2.5 mg/kg imuthiol-treated pigs had lower DTH reactions. Imuthiol at 2.5 mg/kg and 25 mg/kg reduced ($P<0.05$) average daily feed intake. These data suggest that in vivo imuthiol treatment in pigs lowers lymphocyte proliferative responses, IL-2 production, and growth performance.

Introduction

Immunotherapeutic agents have been evaluated in cattle and pigs as a means of stimulating nonspecific, natural immunity.[1-5] Pigs that are weaned at an early age are more affected than older animals by the stress of weaning and, subsequently, are more susceptible to several disease syndromes.[6,7] Thus, early weaned pigs could potentially benefit from biologic response modifier immunoenhancement. Immunoenhancement at this critical time for the young pig would most likely be reflected in decreased morbidity and mortality, with concomitant financial gains for the producer. One immunotherapeutic agent that has been shown to be immunostimulatory in mammals is sodium diethyldithiocarbamate (imuthiol).

Imuthiol has a marked influence on T-cell function in vivo. Under the influence of imuthiol, T-cell precursors are induced to differentiate, natural killer cell cytolytic activity is increased, proliferative responses to mitogens are heightened, and interleukin-2 production is increased.[8-12] Imuthiol has been shown to speed the shift from immunoglobulin M production to immunoglobulin G in response to a foreign antigen; this change is considered to be T-cell mediated.[13,14] Immunosuppressed individuals regained normal T-cell function after being treated with imuthiol[10] and when imuthiol was administered to pregnant mice, T-cell dependent responses in the offspring were enhanced along with neonatal growth rates;

birth rates remained unaffected.[15,16] Toxicity studies have shown imuthiol to be nonmutagenic, noncarcinogenic, nonpyrogenic, and nonantigenic.[14,17] The immune-stimulating properties of imuthiol suggested to us that this compound might be an effective immunotherapeutic agent in weanling pigs. Therefore, the objective of the present study was to evaluate the influence of imuthiol on in vivo and in vitro immune parameters in weaned pigs.

Materials and Methods

Animals and experimental design -- Twenty-one crossbred, 3-week-old (6.5 kg) pigs were weaned and assigned to one of three treatment groups (7 pigs per treatment) on the first day of the experiment (day 0). Pigs from the same litter were allotted to each treatment group. All pigs received a single subcutaneous injection on day 0, of either: imuthiol at 25 mg/kg body weight; imuthiol at 2.5 mg/kg body weight; or sterile physiologic saline. Pigs were housed by treatment groups in 1.2 m x 1.5 m raised pens with plastic-coated expanded-metal floors throughout the 19 days of the experiment. Feed and water were provided ad libitum; ambient temperature was maintained at 35C.

Lymphocyte transformation assay -- Lymphocyte transformation assays were performed with a procedure similar to that described by Blecha et al.[7] on days 0,3,6,9, and 18 of the experiment. Briefly, heparinized whole blood, obtained by

jugular venipuncture, was centrifuged (10 min at 700 x g) to allow collection of the white blood cell layer. The buffy coat was diluted with an equal volume of medium (Dulbecco's Modified Eagle Medium[a], 25 mM HEPES[b], 100 U/ml penicillin[a], 100 µg/ml streptomycin[a], 250 µg/l amphotericin-B[a], 50 µg/ml gentamicin[c], essential and nonessential amino acids and vitamins[a]), layered onto 4 ml of a density gradient (Histopaque 1077[b]), and subsequently centrifuged for 40 min at 950 x g. The cells at the medium-Histopaque interface were collected, mixed with medium, and again layered over Histopaque, then centrifuged for 30 min at 700 x g. The cells were collected from the interface and washed three times in medium, with the final wash in medium plus 10% (v/v) fetal bovine serum[d] (FBS). Triplicate assays, with three mitogens and medium controls, were performed in U-bottom microtiter plates[e] using 2 x 10^5 cells/well in a 200 µl final volume of medium + 10% FBS. Mitogens were used at the following concentrations: concanavalin A[f] (Con A) 20 µg/ml, phytohemagglutinin[a] (PHA) 100 µl/ml, and pokeweed mitogen[a] (PWM) 100 µl/ml. Cultures were incubated for 48 hours in a 93% air, 7% CO_2 humidified atmosphere at 37C, then pulsed with 1 µCi/well of [3H]-thymidine and incubated for an additional 18 hours, before being harvested onto glass-fiber discs. After liquid scintillation counting, results were reported as the mean counts per minute of triplicate assays.

Interleukin-2 assay -- Interleukin-2 determinations were

performed in conjunction with the lymphocyte transformation assays on days 0,3,6,9, and 18. Isolated lymphocytes (2×10^6 cells) were incubated in a 93% air, 7% CO_2, humidified atmosphere at 37C in 2 ml of medium +10% FBS containing 10 µg/ml Con A. After 24 hours, supernatants were harvested and frozen (-20C) until assayed. Interleukin-2 was measured using the IL-2 dependent cell line, CTL-FD.[18,19] Briefly, 2×10^5 washed CTL-FD cells were added to a series of 5 twofold serial dilutions of supernatant in medium plus a medium control (RPMI 1640[a], 5×10^{-5} M 2-mercaptoethanol[b], 5% v/v FBS[d], 100 U/ml penicillin[a], 100 µg/ml streptomycin[a]). Cultures were incubated for 48 hours, pulsed with 1 µCi/well of [^{3}H]thymidine, incubated for an additional 12 hours, and harvested onto glass-fiber discs for liquid scintillation counting. Data were analyzed using a logit data analysis program with all samples being compared to an inhouse standard.[20]

Delayed-type hypersensitivity -- Phytohemagglutinin-induced, delayed-type, hypersensitivity (DTH) tests were performed on days 7 and 14 of the experiment. Phytohemagglutinin[b] (250 µg) in 0.5 ml sterile saline was injected intradermally in the skin fold of the flank. Skin-fold thickness was measured 24 hours later, using a constant-pressure dial micrometer.[7]

Growth performance -- Pigs were individually weighed on days 0,7,14, and 18 of the experiment; feed weights were

determined on the same days on a per pen basis (pigs within a treatment were housed in the same pen). Average daily gain and average daily feed intake were calculated, using the totals for each housing unit.

Statistical analysis -- Data were analyzed as a randomized complete block design by the General Linear Models Procedure of the Statistical Analysis System.[21]

Results

Lymphocyte blastogenesis and IL-2 production -- Lymphocyte proliferative responses of medium controls for all treatments were not different on any of the assay days (Table 1); this uniformity suggests that imuthiol is not mitogenic in vivo in pigs. however, in mitogen-stimulated cultures (day 9 with Con A, day 3 with PHA, and days 3 and 6 with PWM), one or both of the imuthiol-treated groups had a significantly lower degree of proliferation than the control animals. This pattern was much more obvious when the data were pooled over all days of the experiment (Fig. 1). When the lymphocyte proliferation data were averaged over all sampling days, Con A- and PWM-stimulated cultures from 25 mg/kg imuthiol-treated animals had lower ($P<0.05$) proliferation than controls. Lymphocytes from 25 mg/kg imuthiol-treated pigs also tended to have a lower proliferative response to PHA than controls. In all cases, 2.5 mg/kg imuthiol-treated pigs were not significantly different from either control or the 25 mg/kg imuthiol group.

Interleukin-2 production, among the three treatment groups, did not vary ($P<0.05$) on any single day; however, when pooled over all sampling days (Fig. 2) a pattern similar to that of the lymphocyte proliferation data was observed. Both imuthiol-treated groups had lower ($P<0.05$) IL-2 production when compared to control values.

Delayed-type hypersensitivity -- The delayed-type hypersensitivity test results showed some divergence from the above pattern (Table 2). On both test days, the 2.5 mg/kg imuthiol-treated animals had a significantly lowered response to PHA injection than the controls. However, on day 14, animals receiving the 25 mg/kg dose of imuthiol showed a greater ($P<0.05$) response than the control group.

Growth performance -- Feed intake data were similar to both the proliferation and IL-2 results (Table 3); imuthiol treatment lowered the response. The data show a stepwise progression, with the control animals consuming the most feed, followed by the 2.5 mg/kg imuthiol-treated animals, and the 25 mg/kg imuthiol group consuming the least amount of feed. Average daily gain values paralleled the feed intake data numerically but were not statistically different.

Discussion

These data evaluating the use of imuthiol in pigs are the first of which we are aware. Most of the current literature suggests that imuthiol causes an increase in both lymphocyte

blastogenesis and IL-2 production in mice and humans[8-11,14]; however, stimulation of lymphocyte blastogenesis has been reported to be delayed or to follow an initial period of suppression.[14] Our data support previous reports[13,14] that imuthiol has no, or a slightly inhibitory, effect on PWM-induced blastogenesis. Additionally, imuthiol treatment in 3-week-old pigs depressed IL-2 production and Con A-induced lymphocyte blastogenesis. The feed intake data and average daily gain both reflect an increasingly negative response to increased imuthiol dosage. The results of the PHA-induced, delayed-type, hypersensitivity test are more difficult to interpret. Imuthiol has been reported to increase delayed-type, hypersensitivity responses in mice.[10,13] In support of this observation, the high-dose animals exhibited an increased DTH response over control animals on day 14, yet on both test days, the low-dose animals had the lowest DTH response. Collectively, these results suggest several possibilities. Perhaps the dose or route of administration for imuthiol treatment that we used was incorrect. Even though these factors were consistent with published data for mice and humans[9,11,13,14,16,22,23], different species often differ widely in the correct dosage for a given drug. Another possibility for the lack of an immunostimulatory effect by imuthiol in pigs may be that the parameters we evaluated were not adequate measure of the efficacy of imuthiol; perhaps an evaluation of T-cell subsets and/or natural killer cell

activity would have been more revealing. However, a final consideration, especially in light of the growth performance data, is that imuthiol may not be immunoenhancing in the weanling pig. Further study will enable a better understanding of the immunoregulating properties of imuthiol in the pig.

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Table 1. Mitogen-stimulated lymphocyte blastogenesis in 3-week-old pigs administered imuthiol.

Day	Control	Imuthiol 2.5 mg/kg	Imuthiol 25 mg/kg	SEM
		Concanavalin A		
0	274.6	249.3	222.0	20.9
3	311.0	265.7	278.6	42.8
6	335.8	345.6	278.1	32.8
9	395.9a	373.4ab	328.3b	22.7
18	330.4	314.6	219.4	46.8
		Phytohemagglutinin		
0	572.8	617.1	537.0	48.1
3	623.7a	505.9b	584.1ab	27.7
6	606.3	619.0	465.9	76.2
9	539.5	613.1	484.7	52.7
18	476.1	481.7	305.6	55.2
		Pokeweed Mitogen		
0	210.0	214.7	154.2	34.8
3	267.6a	194.1b	150.4b	20.9
6	245.4a	208.5a	138.0b	34.6
9	214.1	222.7	173.3	24.0
18	193.0	186.2	112.1	40.3
		Control		
0	8.1	8.9	11.1	1.4
3	9.9	10.5	8.7	1.8
6	5.4	4.6	7.5	1.1
9	21.0	16.3	17.2	2.4
18	20.2	17.9	16.2	3.4

ab Means within the same row with different superscripts differ ($P<0.05$). Values are least squares means, cpm x 10^3. Pigs were injected sc with imuthiol on day 0.

Table 2. Phytohemagglutinin skin-test reactions in 3-week-old pigs administered imuthiol.

Day	Control	Imuthiol 2.5 mg/kg	Imuthiol 25 mg/kg	SEM
7	7.4[a]	6.3[b]	7.8[a]	.35
14	4.9[a]	4.3[b]	5.6[c]	.14

[abc]Means within the same row with different superscripts differ (P<0.05). Values are least squares means, mm. Pigs were injected sc with imuthiol on day 0.

Table 3. Average daily gain (ADG) and average daily feed intake (ADFI) in 3-week-old pigs administered imuthiol.

Item	Control	Imuthiol		SEM
		2.5 mg/kg	25 mg/kg	
ADG, kg	.31	.24	.23	0.6
ADFI, kg	.45[a]	.35[b]	.31[c]	0.01

[a][b]Means within the same row with different superscripts differ ($P<0.05$).

Figure Legends

Fig. 1. The effect of imuthiol on mitogen-stimulated lymphocyte proliferation. Bars represent data that are pooled over the 5 sampling days of the experiment. Bars with different superscripts differ ($P<0.05$).

Fig. 2. The effect of imuthiol on IL-2 production. Bars represent data that are pooled over the 5 sampling days of the experiment. Bars with different superscripts differ ($P<0.05$).

Figure 1

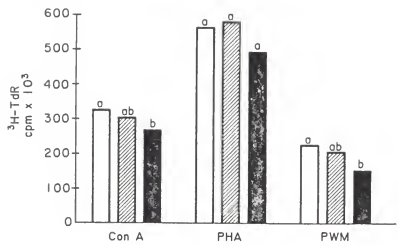
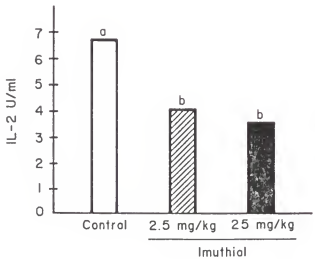


Figure 2



Decreased lymphocyte function associated with pseudorabies virus infection in feeder pigs: influence of isoprinosine

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Footnotes

[a] Gibco Laboratories, Grand Island, NY

[b] Schering Laboratories, Maplewood, NJ

[c] Sigma Chemical Co., St. Louis, MO

[d] Hazleton Dutchland, Denver, PA

[e] Flow Labs, McLean, VA

[f] Pharmacia Fine Chemicals, Piscataway, NJ

[g] ICN Radiochemicals, Irvin, CA

[h] Coulter Electronics, Inc. Hialeah, FL

[i] T. Molitor, Univ. of Minn. personal communication

SUMMARY

Pseudorabies is a porcine herpesvirus of major importance in the swine industry. Isoprinosine is an immunomodulating drug that has been shown to be beneficial in treating herpesvirus infections. Twenty-four, 7-week-old pigs were allotted within litters to one of four groups: control (CONT), isoprinosine (ISO), pseudorabies virus (PRV), or isoprinosine and pseudorabies (ISO-PRV). Isoprinosine was administered daily for 16 days (75 mg/kg/day, orally). Pigs were challenged with pseudorabies virus (10^5 TCID50) on day 4. Rectal temperatures and viral excretion were monitored daily; total and differential leukocyte counts, lymphocyte response to mitogens, and interleukin-2 production were monitored every 4 days. Pigs challenged with pseudorabies virus became very ill, with the ISO-PRV group most severely affected. Rectal temperatures were elevated ($P < 0.05$) in virally challenged pigs on days 5 to 12 and 14 to 16; isoprinosine did not alter this effect. Pseudorabies virus-infected pigs displayed a leukocytosis ($P < 0.05$) on days 12 and 16, primarily caused by a neutrophilia. Concanavalin-A-stimulated lymphocyte proliferation was decreased ($P < 0.06$) in both PRV and ISO-PRV groups on day 12 compared to control animals, but only in the PRV group on day 16. Pokeweed mitogen-stimulated lymphocyte proliferation was decreased ($P < 0.02$) in ISO-PRV pigs on day 8. Interleukin-2 concentrations, pooled over all sampling days, were decreased ($P < 0.03$) in pseudorabies virus-infected

pigs. Viral excretion was not altered by isoprinosine treatment. These data suggest that pseudorabies virus infection decreased lymphocyte proliferative responses and IL-2 production in pigs, and that isoprinosine did not mitigate these effects.

INTRODUCTION

Pseudorabies, a porcine herpesvirus, is a major disease of swine, annually causing losses of millions of dollars for United States swine producers.[1] A recent review of pseudorabies vaccines indicates a need for new, more effective vaccines to aid in controlling this disease, most probably exploiting subunit technology.[2] Another disease intervention modality currently receiving attention is the use of immunomodulators. For example, Pirtle et al.[3] 1986, reported that the effects of pseudorabies virus infection in young pigs were mitigated by feeding butylated hydroxytoluene. Isoprinosine is another drug that has shown some immunoenhancing activity. First investigated for its antiviral activity in 1972 by Gordon and Brown[4], isoprinosine was later found to act primarily through stimulation of the immune system.[5] Subsequent research has shown that isoprinosine is effective in restoring immune function in several immunosuppressive conditions and is also beneficial in the treatment of herpesvirus infections in humans.[6,7] Previous research in our laboratory has shown isoprinosine to

be immunoenhancing when administered in vivo to artifically reared piglets.[8] In this experiment, we sought to evaluate the effect of isoprinosine in pseudorabies virus-infected, feeder pigs on the clinical course of disease as well as various immune parameters.

MATERIALS AND METHODS

Animals and Experimental Design -- Isoprinosine and pseudorabies virus (PRV) were applied as treatments to 24, 7-week-old, crossbred pigs (10.7 kg $\pm$ 2.3 sd obtained from a PRV-free herd) with a 2x2 factorial arrangement of treatments in a randomized, complete block, experimental design. Pigs from the same litter were blocked across the four treatment groups: control, isoprinosine (ISO), pseudorabies virus (PRV), and isoprinosine and pseudorabies virus (ISO-PRV). Each treatment group of six pigs was housed in a single room within the Animal Health Research Facility of the Department of Veterinary Science, University of Nebraska, Lincoln. Animals were placed in their respective isolation rooms 1 week before the experiment began to allow them to adjust to the new environment.

Isoprinosine (a gift from Newport Pharmaceuticals International, Inc. Newport Beach, CA) was administered orally once a day to the ISO and ISO-PRV groups throughout the experiment (beginning on day 0) at a rate of 75 mg/kg body weight/day in a distilled water solution of 100 mg/ml. The

remaining 12 pigs received daily doses of water in a similar manner. Pseudorabies virus challange was performed on day 4. The PRV and ISO-PRV groups received 10^5 tissue culture, infective dose $_{50}$ ($TCID_{50}$) units of Shope strain pseudorabies virus in aerosol form (half intranasally, half conjunctivally). Rectal temperatures and clinical signs of disease were evaluated daily throughout the experiment.

Lymphocyte transformation -- Lymphocyte transformation assays were performed on days 0, 4, 8, 12, and 16 of the experiment. Whole blood, collected into heparinized blood collection tubes via jugular venipuncture, was centrifuged (10 min at 700 x g) to allow collection of the white blood cell layer. This buffy coat was added to 3 ml of medium (RPMI 1640[a], 100 U/ml penicillin,[a] 100 ug/ml streptomycin,[a] 250 ug/l amphotericin-B,[a] 50 ug/ml gentamicin,[b]) then layered onto 4 ml of a density gradient solution (Histopaque 1077,[c]), and subsequently centrifuged for 40 min at 950 x g. The cells at the medium-Histopaque interface were collected, mixed with medium, and again layered on Histopaque for a second centrifugation of 30 min at 700 x g. Cells were collected from the interface and washed three times in medium, with the final wash in medium containing 10% (v/v) fetal bovine serum (FBS, Hybri-sure,[d]). Triplicate assays, with three mitogens and medium controls, were performed in U-bottom microtiter plates[e] using 2 x 10^5 cells/well in a 200 ul final volume of medium + 10% FBS. Mitogens were used at the following final

concentrations: concanavalin A[f] (Con A) 20 ug/ml, phytohemagglutinin[a] (PHA) 100 ul/ml, and pokeweed mitogen[a] (PWM) 100ul/ml. Assay cultures were incubated for 48 hours in a 93% air, 7% CO2, humidified atmosphere at 37 C. Cultures were then pulsed with 1 uCi/well of [3H]-thymidine[g] (6.7 Ci/mmole) and incubated for an additional 18 hours before being harvested onto glass-fiber discs. After liquid scintillation counting, results were reported as the mean counts per minute of triplicate assays.

Interleukin-2 assay -- Interleukin 2 (IL-2) determinations were performed together with the lymphocyte transformation assays on days 0, 4, 8, 12, and 16. Isolated lymphocytes (2 x 10^6 cells from the above procedure) were incubated in 24-well plates with 2 ml of medium + 10% FBS containing 10 ug/ml Con A for 24 hours in a 37 C, 93% air, 7% CO 2, humidified atmosphere. Supernatants then were collected and frozen (-20 C) until assayed. Interleukin-2 was measured using the IL-2 dependant cell line, CTL-FD.[9,10] Assays were conducted by adding 2.5 x 10^5 washed CTL-FD cells to a series of 5, twofold serial dilutions of supernatant in medium plus one medium control (RPMI 1640,[a]; 5 x 10^-5 M 2-mercaptoethanol,[c]; 5% v/v FBS; 100 U/ml penicillin; 100 ug/ml streptomycin). Cultures were incubated for for 48 hours, pulsed with 1 uCi/well of [3H]-thymidine, incubated a final 12 hours, and harvested onto glass-fiber discs for liquid scintillation counting. Logit analysis was performed

on the raw data, comparing all samples to an in-house standard.[11]

Total and differential leukocyte counts -- Blood samples in sodium EDTA also were obtained on days 0, 4, 8, 12, and 16 to determine total leukocyte counts and differentials. Leukocyte counts were performed using an electronic particle counter.[h] Differential counts were performed on Wrights' stained blood smears.

Pseudorabies virus titers -- Pseudorabies virus serum neutralization titers were performed in the manner described by Hill et al.[12]

Nasal virus shedding -- Nasal swabs were obtained on a daily basis from all pigs. Swabs were frozen at -70 C until assayed by a cytopathic effect assay on vero monkey kidney cells.[12] Titers were determined using the Reed and Muench method.[13]

Statistical Analysis -- The data were analyzed as a randomized complete block design, blocking on litter, using the General Linear Models procedure of Statistical Analysis System.[14] Mean separations were performed by protected least significant differences comparisons.

RESULTS

Pigs challenged with pseudorabies had severe clinical symptoms. All pigs challenged exhibited signs consistent with a pseudorabies virus infection, including: depression,

anorexia, conjunctivitis, and dyspnea. Additionally, some animals demonstrated central nervous system signs of ataxia and head tilt, and the more severely affected animals died. Animals receiving both virus and isoprinosine were clinically more severely affected than those that received virus alone. Four of six animals died in the ISO-PRV group, whereas only one of six died in the group receiving virus alone (Fig 1). All animals that were not challenged with virus remained clinically normal throughout the experiment. Rectal temperatures were elevated ($P < 0.05$) from day 5 through day 16 (excluding day 13; Fig 1) in animals that received virus. Isoprinosine treatment did not affect rectal temperature in either infected or noninfected groups.

Pseudorabies challenge was associated with an absolute increase in total leukocytes (Fig 2). This increase was due primarily to a marked increase in circulating neutrophils (Fig 3). Lymphocyte numbers showed an initial drop at 4 days post-infection but rebounded 4 days later to above initial values (Fig. 4). Overall, the ISO-PRV and PRV groups had similar leukocyte responses (except for neutrophil levels on day 12 post-infection), whereas both the ISO and control groups maintained a steady baseline level. Isoprinosine treatment did not appear to have an effect on leukocyte numbers either in the presence or absence of viral challange.

Lymphocyte blastogenic responses to Con A (Fig 5) were initially the same for all treatment groups. Pigs receiving

isoprinosine did not differ from control animals throughout the experiment. However, suppression of lymphocyte blastogenesis was associated with pseudorabies virus challange ($P < 0.06$ on days 8 and 12 postinfection for PRV animals, day 8 for ISO-PRV animals). Isoprinosine may have reversed the lowered Con A-induced lymphocyte proliferative responses on day 12; however, only two animals were alive in the ISO-PRV group by 8 days postinfection. Lymphocyte blastogenesis induced by PHA (Fig 5) was similar to the pattern observed with Con A, and significant differences were not found. The ISO and control groups did not differ greatly; a decreasing proliferative response in the PRV and ISO-PRV groups was present following viral challange, yet again at 12 days postinfection, the ISO-PRV group returned to near control values of proliferation. Lymphocyte blastogenesis with PWM showed little change associated with isoprinosine treatment (Fig 5). However, at 4 days postinfection, a decrease ($P< 0.02$) in PWM-induced lymphocyte proliferation occurred in the ISO-PRV group. After this initial drop, PWM-induced proliferative responses in the ISO-PRV group returned to near control levels. The PRV group had numerically decreased proliferation on all assay days post-infection.

Interleukin-2 levels did not differ significantly on any sample day; however, some interesting observations were made when the pooled data were analyzed (Table 1). Interleukin-2 production was significantly decreased ($P < 0.03$) in all

animals receiving viral challenge compared to animals not being challenged. Isoprinosine treatment did not alter interleukin-2 production. Data for individual treatment groups numerically reflect the viral associated suppression but have no significant differences.

Serum neutralization titers were less than 1:2 in all animals on the day of viral challange. At 12 days post-infection, all animals not receiving virus maintained a titer of less than 1:2, whereas those animals that did receive virus had titers ranging from >1:4 to >1:32.

Nasal viral shedding was not affected by isoprinosine treatment. No virus was detected from challenge control animals. The group-average viral shedding did not differ significantly between the PRV and ISO-PRV groups on any sample day.

DISCUSSION

Viral-induced immunosuppression has been reported for both bovine and human herpesvirus infections.[7,15] Necrosis of lymphoid tissue has been described subsequent to PRV infection in pigs.[1,16] Additionally, Lee et al.[16], have reported a greatly suppressed immune response to a Bordetella bronchiseptica bacterin after recovery from PRV infection. Our data also support viral-induced immunosuppression caused by PRV infection.

Mitogen-stimulated lymphocyte blastogenesis was

consistently decreased after viral challenge. Additionally, interleukin-2 production was also lower in the viral challenged animals. Collectively, these data suggest a decrease in lymphocyte function. Other investigators have reported no change in blastogenesis values after PRV infection in assays using the same mitogens, but there are several reports of increases in blastogenesis after infection when PRV antigens were used as the stimulants.[17-19] This may suggest a general suppression of T cell function within the framework of a specific immune response to the infective agent. Recent data have indicated that PRV may decrease lymphocyte proliferative responses by inhibiting the monocyte-macrophage system.[1,20] A decrease in macrophage function may be responsible for lowered T-helper function (e.g., by decreasing IL-1 production), which would decrease IL-2 production and cause a concomitant drop in proliferation. Further work is needed to examine these possibilities.

The neutrophilia and initial drop in circulating lymphocytes that we observed would be expected responses to a viral infection with subsequent tissue damage. Similarly, serum neutralization titers indicate a susceptible population of animals with subsequent seroconversion in pigs that were infected. The viral shedding data (not shown) were unremarkable; no differences were associated with isoprinosine administration.

Isoprinosine has been shown to increase lymphocyte

proliferative responses, increase IL-2 production, and alter the clinical course of a herpesvirus infection when administered in vivo to laboratory animals and humans. The data from our experiment do not reflect these previous observations. Overall, isoprinosine administration did not have any marked effects on the paramaters observed in either infected or noninfected animals. Our observation that Con A-induced blastogenesis in the ISO-PRV group returned to control levels on day 12 postinfection after initial suppression should be judged critically. Animals in the ISO-PRV group were more severely affected by the disease than those in the PRV group, and 4 animals had died by 8 days postinfection. Therefore, the data for the final 2 assay days were based on only two animals in the ISO-PRV group: a small sample from which to make inferences. Similar scrutiny should be applied to the data from the other two mitogens.

There is evidence that the effects of pseudorabies virus infection can be altered in the pig[3], however, we were unable to discern any benefits from isoprinosine administration to feeder pigs. Additionally, the observations of decreased lymphocyte blastogenesis and decreased IL-2 production support the conclusion that suppression of cell-mediated immunity occurs during pseudorabies virus infections.

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Table 1. Mean IL-2 production. Data pooled from all assay days.

Statistical grouping	Treatment PRV	ISO	IL-2/ml
Viral challenge effects (pooled data)	+		1.61
	-		2.27*
Isoprinosine effects (pooled data)		+	1.83
		-	2.05
Individual treatment groups	-	-	2.37
	-	+	2.17
	+	-	1.74
	+	+	1.49

* = $P < 0.05$

FIGURE LEGENDS

Fig 1 - Mean rectal temperatures for all treatment groups, and a plot of mortality in PRV challenged animals.

Fig 2 - Mean total leukocyte numbers for all treatment groups.

Fig 3 - Mean neutrophil counts for all treatment groups.

Fig 4 - Mean lymphocyte counts for all treatment groups.

Fig 5 - Lymphocyte blastogenesis values (group means) as a percent of the control group mean on individual assay days. (* = $P < 0.05$).

Figure 1

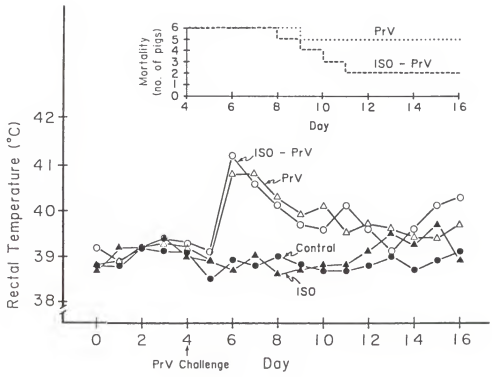

Mortality (no. of pigs)
6
5
4
3
2
1
0
4
6
8
10
12
14
16
Day
PrV
ISO - PrV
Rectal Temperature (°C)
42
41
40
39
38
ISO - PrV
PrV
Control
ISO
0
2
4
6
8
10
12
14
16
PrV Challenge
Day

Figure 2

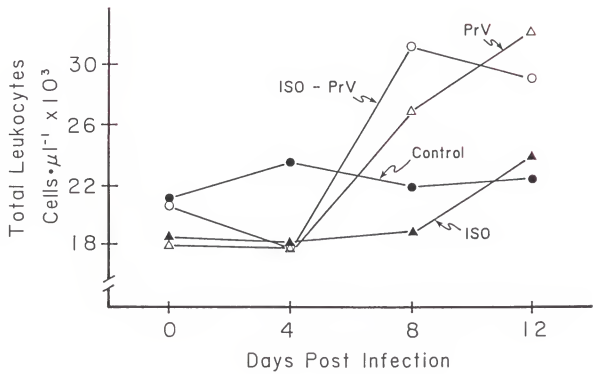

Total Leukocytes
Cells • µl⁻¹ x 10³
30
26
22
18
0
4
8
12
Days Post Infection
PrV
ISO - PrV
Control
ISO

Figure 3

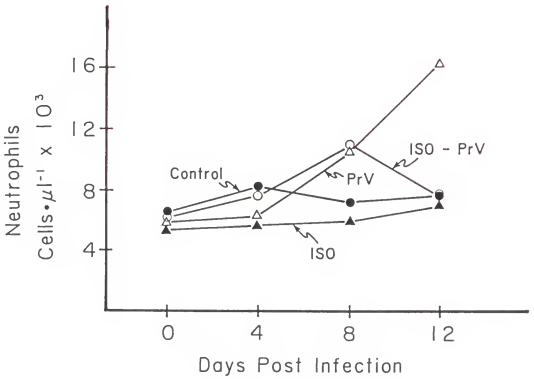

Neutrophils
Cells•μl⁻¹ x 10³
16
12
8
4
0
4
8
12
Days Post Infection
Control
PrV
ISO - PrV
ISO

Figure 4

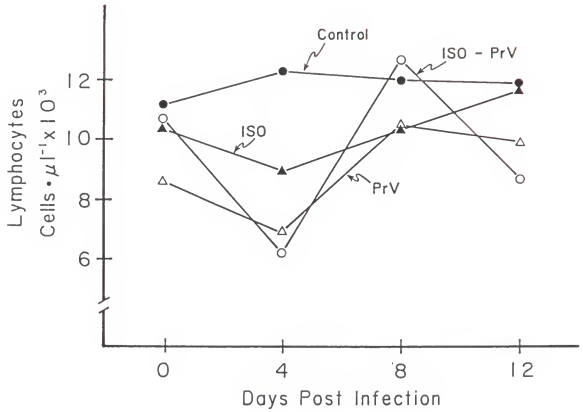
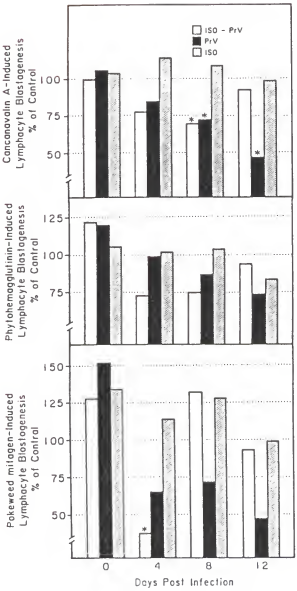

Control
ISO - PrV
ISO
PrV
Lymphocytes
Cells • μl⁻¹ x 10³
12
10
8
6
0
4
8
12
Days Post Infection

Figure 5


ISO - PrV
PrV
ISO
Concanavalin A-Induced Lymphocyte Blastogenesis % of Control
Phytohemagglutinin-Induced Lymphocyte Blastogenesis % of Control
Pokeweed mitogen-Induced Lymphocyte Blastogenesis % of Control
100
75
50
125
150
0
4
8
12
Days Post Infection

FUTURE RESEARCH

The area of immunomodulators will continue to be a growing and exciting area of research for years to come. The work in the field must proceed along two lines simultaneously. As newly discovered immunoactive substances become available they should be screened for their effectiveness and possible clinical use. Secondly, the mechanisms of immune regulation, immune function, and immune dysfunction should be pursued so that the "hows" and "whys" of immunomodulation will become clear. This two pronged approach seems to be the most efficient; allowing the clinical use of immunomodulators as they become available while using those same substances and other methods to further illucidate the molecular interactions of the immune system.

I feel the most significant conclusion to be drawn from my research is that PRV is immunosuppressive. Pseudorabies virus infection may prove to be a good immunosuppression model to further evaluate other influences on the immune system. As usual further work is needed, for our knowledge is only partial.

IMMUNOMODULATORS IN SWINE: SODIUM DIETHYLDITHIOCARBAMATE IN WEANLING PIGS, AND DECREASED LYMPHOCYTE FUNCTION IN PSEUDORABIES VIRUS INFECTED FEEDER PIGS: INFLUENCE OF ISOPRINOSINE.

by

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AN ABSTRACT OF A THESIS

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Immunomodulators are becoming the focus of a large group of researchers today. The possibility of manipulating the immune system to further understand its regulation and to treat or prevent disease poses an almost irresistable lure. Many types of immunomodulators have been described and tested, and much of the information concerning immunomodulators in swine is discussed. In addition, two experiments involving the use of immunomodulators in young pigs are presented.

I. Sodium diethyldithiocarbamate (imuthiol) is an immunoactive drug that has been shown to be an effective T cell stimulant. Consequently, mitogen-stimulated lymphocyte proliferation, delayed-type hypersensitivity (DTH) reactions, interleukin-2 (IL-2) production, and growth performance were evaluated in 3-week-old pigs treated with imuthiol. Lymphocyte proliferative responses to Con A and PWM were reduced ($P<0.05$) in pigs treated with imuthiol at 25 mg/kg; PHA proliferative responses were not influenced by imuthiol treatment. Imuthiol at 2.5 mg/kg and 25 mg/kg lowered IL-2 production when compared to saline-treated controls. Delayed-type hypersensitivity responses to PHA were higher in 25 mg/kg imuthiol-treated pigs; however, 2.5 mg/kg imuthiol-treated pigs had lower DTH reactions. Imuthiol at 2.5 mg/kg and 25 mg/kg reduced ($P<0.05$) average daily feed intake. These data suggest that in vivo imuthiol treatment in pigs lowers lymphocyte proliferative responses, IL-2 production, and growth performance.

II. Pseudorabies is a porcine herpesvirus of major importance in the swine industry. Isoprinosine is an immunomodulating drug that has been shown to be beneficial in treating herpesvirus infections. Twenty-four, 7-week-old pigs were allotted within litters to one of four groups: control (CONT), isoprinosine (ISO), pseudorabies virus (PRV), or isoprinosine and pseudorabies (ISO-PRV). Isoprinosine was administered daily for 16 days (75 mg/kg/day, orally). Pigs were challenged with pseudorabies virus (10^5 TCID 50) on day 4. Rectal temperatures and viral excretion were monitored daily; total and differential leukocyte counts, lymphocyte response to mitogens, and interleukin-2 production were monitored every 4 days. Pigs challenged with pseudorabies virus became very ill, with the ISO-PRV group most severely affected. Rectal temperatures were elevated ($P < 0.05$) in virally challenged pigs on days 5 to 12 and 14 to 16; isoprinosine did not alter this effect. Pseudorabies virus-infected pigs displayed a leukocytosis ($P < 0.05$) on days 12 and 16, primarily caused by a neutorphilia. Concanavalin-A-stimulated lymphocyte proliferation was decreased ($P < 0.06$) in both PRV and ISO-PRV groups on day 12 compared to control animals, but only in the PRV group on day 16. Pokeweed mitogen-stimulated lymphocyte proliferation was decreased ($P < 0.02$) in ISO-PRV pigs on day 8. Interleukin-2 concentrations, pooled over all sampling days, were decreased ($P < 0.03$) in pseudorabies virus-infected pigs. Viral

excretion was not altered by isoprinosine treatment. These data suggest that pseudorabies virus infection decreased lymphocyte proliferative responses and IL-2 production in pigs, and that isoprinosine did not mitigate these effects.