

Studies on the effect of human recombinant interleukin 2 on  
the porcine immune response to a pseudorabies subunit vaccine

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by

Kazunori Kawashima

A Thesis Submitted to the  
Graduate Faculty in Partial Fulfillment of the  
Requirements for the Degree of

MASTER OF SCIENCE

Department: Veterinary Microbiology and Preventive Medicine  
Major: Veterinary Microbiology

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Iowa State University  
Ames, Iowa  
1987

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## GENERAL INTRODUCTION

Pseudorabies or Aujeszky's disease is caused by a herpesvirus and is considered one of the most economically important swine diseases in the world.<sup>15,20</sup> The virus causes systemic infection in a broad range of mammals, but swine are recognized as the primary host. Clinical characteristics of pseudorabies virus (PRV) infection in swine include: 1) respiratory and central nervous system disturbances in growing pigs which lead to high mortality and reduction in growth rates; and 2) reproductive failure in pregnant sows and gilts. Clinical and sub-clinical infection also lead to the establishment of latent infections. According to a recent survey,<sup>75</sup> the annual loss attributed to pseudorabies in the United States of America has been estimated to exceed \$ 30 million. The magnitude of this economic loss has generated widespread support for the initiation of a campaign for the control and eventual eradication of pseudorabies. It is anticipated that subunit vaccines will play an important role in control and eradication programs.<sup>51</sup> These vaccines will be particularly useful because their defined antigen composition will make it possible to identify virus infected vaccinated pigs by using non-vaccine viral proteins as diagnostic test antigens.<sup>50</sup>

Recently, an effective subunit vaccine has been developed at Iowa State University, Ames, IA.<sup>49,50</sup> However, the cost of producing this vaccine may prevent it from being used by swine producers.<sup>51</sup> Enhancing

the immunogenicity of the vaccine will reduce the cost because less antigen per dose will be required. Recent studies have shown that interleukin 2 can enhance the immune response of mice to vaccine antigen.<sup>31</sup> These findings suggest that IL-2 may have a similar effect in swine. The following thesis was conducted to test this hypothesis.

## REVIEW OF THE LITERATURE

## Introduction

Interleukin 2 (IL-2) is a T cell-secreted glycoprotein which regulates immune responses.<sup>1,34,61</sup> It was first described in 1976 by Morgan et al.<sup>41</sup> who detected its presence in the supernatant of lectin-stimulated human peripheral leukocyte cultures. Interleukin 2 was initially termed T cell growth factor (TCGF) because of its mitogenic effect on T cells.<sup>17,41,60</sup> Recent studies utilizing recombinant IL-2 (rIL-2)<sup>67</sup> have demonstrated more definitive properties for this lymphokine. These properties include the ability to induce: a) the proliferation of lymphocytes,<sup>4,10,17,27,43,72,76</sup> b) the differentiation of lymphocytes,<sup>16,19,21,30,33,45,65</sup> and c) the production of additional lymphokines.<sup>14,29,54,69</sup> As a result of these studies, there has been increasing interest in the use of IL-2 as an immune enhancing agent.<sup>2,6,7,23,26,31,57,59,64,69,71,72</sup> The following review will first discuss the role that IL-2 plays in the immune response. Subsequently, the review will focus on the clinical application of IL-2.

## Biochemical Characteristics of IL-2

Human IL-2 is a glycosylated protein consisting of 133 amino acids<sup>67</sup> with a molecular weight of 15,000.<sup>13,20,61</sup> The isoelectric point is 6.5. Interleukin 2 retains its biological activity following exposure to 70°C for 15 min.<sup>12,18</sup> It is also stable at a pH of 2.2 to 10.0.<sup>12,18</sup>

## Role of IL-2 in the Immune Response

### Production of IL-2

Pfizenmaier et al.<sup>47</sup> demonstrated that helper T lymphocytes ( $T_h$ ) are the primary source of IL-2. Production of IL-2 is believed to be initiated by at least two sequential events that take place on the surface of  $T_h$ .<sup>54,61</sup> Initially, antigen is processed and presented to  $T_h$  by an antigen presenting cell (APC). This interaction is major histocompatibility complex (MHC) restricted. Subsequently the APC releases interleukin 1 (IL-1). Antigen and IL-1 are thought to interact with specific receptors present on the surface of  $T_h$ .<sup>11,44,61</sup> The signals that are generated from this interaction are transmitted by protein kinase C to the nucleus which results in IL-2 production following gene expression.<sup>9,32</sup>

The effect of IL-2 on specific lymphocyte populations

Studies utilizing flow cytometry and radio-labeled IL-2 have revealed that IL-2 binds to specific receptors on the surface of T lymphocytes,<sup>3,37,46,55,56,66</sup> B lymphocytes,<sup>5,37,40,46</sup> NK cells<sup>45</sup> and macrophages.<sup>22,28</sup> To date no IL-2 receptors have been demonstrated on suppressor T lymphocytes. Several investigators<sup>37,46,55</sup> have demonstrated by competitive binding studies that a high affinity receptor plays a significant role in cell activation. However, the high affinity receptors are expressed and stable only when the cells are activated. This requirement has workers to speculate that the mechanism of IL-2 receptor expression may be regulated by other factors.<sup>21,66</sup> Some investigators have proposed that the signal from the interaction of IL-2 and its receptor is generated and transmitted by protein kinase C to the nucleus inducing cell activation.<sup>13</sup>

Initially, many studies on lymphocyte-IL-2 interaction focused on cytotoxic T lymphocytes, ( $T_C$ ).<sup>17,35,36,47</sup> Lutz et al.<sup>38</sup> demonstrated that both alloantigen and IL-2 were necessary to induce primary  $T_C$  to proliferate and to activate their cytotoxic function. Later, Andrew et al.<sup>3</sup> reported similar observations and further demonstrated that removal of alloantigen resulted in the cessation of  $T_C$  proliferation and loss of cytotoxic activity even though rIL-2 was present. In contrast, Erard et al.<sup>10</sup> demonstrated that  $T_C$  proliferation and cytotoxic activation could be induced by rIL-2 alone. However, several groups of investigators<sup>16,21</sup> have speculated from their experiments that antigen-induced monokines and

lymphokines other than IL-2 are necessary for the induction of  $T_C$  proliferation and the activation of cytotoxic activity.

The role of IL-2 in the reactivation of  $T_C$  memory cells has been studied by Lefrancois et al.,<sup>36</sup> who found that reactivation of small resting  $T_C$  memory cells occurs following exposure to either highly purified IL-2 or antigen. The finding of this work was supported by Hamuro,<sup>21</sup> who restored cytotoxic activity to  $T_C$  memory cells by exposing them to human rIL-2. Hamuro's work was particularly valuable because the recombinant nature of the IL-2 used in his study eliminated the possibility that other co-factors were involved. Such co-factors could have been present in trace amounts in the non-recombinant IL-2 preparations used by earlier workers.

Studies have also demonstrated that IL-2 plays an important role in inducing cytotoxic activity of NK cells. Initially, activation of NK cells was thought to be due to the effect of gamma interferon (IFN-r) alone.<sup>8,74</sup> Subsequently, Kuribayashi et al.<sup>33</sup> demonstrated that either IL-2 or IFN-r could independently activate cytotoxic NK cell activity. Svedersky et al.<sup>65</sup> conducted a more definitive study using rIL-2 and demonstrated that IL-2 was a more effective NK cell activator than IFN-r. These workers and others<sup>30</sup> reported that cytotoxic activity was directly proportional to the dose of IL-2. Grimm et al.<sup>19</sup> described that LAK cells could also be activated by IL-2. Recent studies have suggested that LAK cells and NK cells might belong to same population because they are similarly activated by IL-2 and have similar surface markers.<sup>24,30</sup>

Studies by several investigators with IL-2 have shown that this



lymphokine plays a critical role in the induction of clonal expansion of B cells and in their differentiation into immunoglobulin secreting cells.<sup>29,39,40,42,43,46,72,76</sup> Initially, Howard et al.<sup>29</sup> demonstrated that IL-2 induced  $T_h$  to produce B cell growth factor (BCGF). These workers were not able to demonstrate that IL-2 had a direct effect on B cell proliferation. However, Zubler et al.<sup>76</sup> demonstrated that IL-2 could directly induced proliferation of B cells which had been activated with lipopolysaccharide and anti-Ig M. The results reported by Zubler et al.<sup>76</sup> have subsequently been confirmed by other workers.<sup>5,40,42,43</sup>

Although there is ample evidence that IL-2 directly induces B cell proliferation, there is no current evidence to indicate that IL-2 plays a role in the differentiation of B cell.<sup>27</sup> However, several investigators<sup>29,39,40,43,54</sup> have suggested that IL-2 has an indirect role in B cell differentiation because of its ability to induce  $T_h$  to secrete IFN-r, BCGF, and B cell differentiation factors (BCDF). These lymphokines have been shown to directly affect B cell differentiation.<sup>27,43</sup>

## Clinical Application

Interleukin-2 has been evaluated in-vivo by several investigators to determine its effectiveness as a therapeutic and immune enhancing agent. Many of these studies have focused on the ability of IL-2 to enhance the anti-tumor property of LAK cells. This property was clearly characterized in mice by Hinuma et al.,<sup>26</sup> who demonstrated that daily doses of rIL-2 resulted in increased killer cell activity against a variety of tumor cells. This cytotoxic activity decreased to undetectable levels within 3 days of IL-2 withdrawal. The dose of IL-2 required for sustaining this activity was extremely large and produced toxic side effects, which led these investigators to question the practicality of using IL-2 to treat human cancer patients. Rosenberg et al.<sup>57</sup> have also used IL-2 to successfully treat human cancer patients. These investigators were able to use lower doses of IL-2 and reduce toxic side effects by treating lymphocytes from cancer patients with IL-2 and tumor cells in-vitro.

Enhancement of the in-vivo anti-tumor activity of  $T_C$  has also been demonstrated by Hefeneider et al.,<sup>23</sup> who reported that tumor specific  $T_C$  cytotoxic activity was induced in mice by inoculating a mixture of allogeneic tumor cells and IL-2. Cheever et al.<sup>7</sup> demonstrated in a similar study that the degree of  $T_C$  activity was proportional to the dose of IL-2 inoculated.

The use of IL-2 to reduce the severity of viral infection has also been studied. Rouse et al.<sup>59</sup> were able to increase virus clearance in

herpes simplex virus type 1 (HSV-1) infected mice by treating these mice with IL-2 and IL-2 treated antigen-sensitized lymphocytes collected from the spleen of syngeneic mice. They were not able to demonstrate enhanced virus clearance by treating mice with IL-2 alone. However, Weinberg et al.<sup>71</sup> reported that the use of IL-2 by itself did have an anti-viral effect. This effect was demonstrated by challenging IL-2 treated and non-treated guinea pigs with HSV-2. Both infection rates and the severity of the clinical response were significantly lower in the treated guinea pigs. These investigators did not speculate on the possible anti-viral mechanisms that were enhanced. However, Rouse et al.<sup>59</sup> proposed in their study that the most likely anti-viral mechanism affected was the cell-mediated immune response.

One of the first studies to suggest that IL-2 could be used to enhance the immune response of animals to vaccine antigen was conducted by Kawamura et al.<sup>31</sup> These workers reported that the incorporation of rIL-2 with antigen in Freund's complete adjuvant induced higher antibody levels in mice than adjuvanted antigen alone. Recently Anderson and Urban<sup>2</sup> reported that human rIL-2 enhanced vaccine immunity to Hemophilus pleuropneumoniae in swine when IL-2 was given separately from the vaccine over five consecutive days beginning on the day of vaccination. Their conclusion was based only on clinical observations.

## MATERIALS AND METHODS

## Medium and Reagents

Cell growth medium (GM) consisted of Dulbecco's modified Eagle's minimum essential medium (MEM) with Earle's salts and supplemented with 5 % fetal calf serum (FCS). Maintenance medium (MM) was GM with 2 % FCS. Hank's balanced salts solution (HBSS), without calcium and magnesium, was used for lymphocyte processing. Medium 199 containing 25 mM Hepes, L-glutamine, and Earle's salts was supplemented with 15 % FCS. All media contained 100 units/ml of penicillin G, 100 ug/ml of streptomycin sulfate, 10 ug/ml of gentamycin sulfate and 1.65 ug/ml fungizone.

Human recombinant interleukin 2 (rIL-2) was supplied in the lyophilized state by Cetus Corp, Emeryville, CA. It was rehydrated to stock concentration with 1.2 ml of sterile deionized double distilled water and extended to the desired concentration with 10 mM sodium phosphate buffer without potassium (pH 7.5), containing 0.01 % SDS. Reconstituted interleukin 2 was stored at 4°C and used within 10 h of preparation. The solubilized state of rIL-2 was verified by demonstrating the presence of protein in solution and failure to reduce optical density readings at 280 nm after centrifugation of the IL-2 preparation at 50 K x g for 60 min.

## Virus and Cells

Virulent pseudorabies virus (PRV) strain Be was used for subunit vaccine preparation, antigen for lymphocyte blastogenesis assays and virus challenge studies. Virus was propagated in PK 15 cells for vaccine production and in Madin-Darby bovine kidney (MDBK) cells for use in challenge studies and blastogenesis assays.

## Vaccine Preparation

The pseudorabies virus subunit vaccine consisted of viral glycoproteins that were extracted from solubilized virus infected PK 15 cells by lectin affinity chromatography as described by Platt.<sup>49</sup> In brief, PK 15 cells were inoculated with strain Be at a multiplicity of 10. Virus infected cells were harvested 18 to 20 h post infection, washed 3 times with serum free medium and solubilized with 0.025 M tris/tricine (TT) buffer pH 8.0, containing 1 % v/v triton-X 100. Solubilized glycoprotein was then extracted by lectin affinity chromatography using Lens culinaris agglutinin (LCA) covalently linked to agarose beads. Glycoprotein was eluted from the lectin column with 1 % w/v mannose in TT buffer. The protein concentration was determined by the dye binding assay as described by Read and Northcote.<sup>52</sup> Protein was diluted to desired concentration and suspended in Freund's incomplete adjuvant.

### Serum Virus Neutralization Assay

Serum virus neutralizing (SN) titers were determined by the microtiter serum neutralization test as described by Hill et al.<sup>25</sup> with modifications. In brief, serum samples were heat-inactivated at 56°C for 45 min. Duplicate serial two-fold dilutions were made with 50 ul of MEM in 96 well flat-bottomed microtiter plates. Subsequently, 300 TCID<sub>50</sub> of virus suspended in 50 ul of MEM was placed into each well and incubated for 60 min. at 37°C. Following incubation, 4 x 10<sup>4</sup> MDBK cells contained in 150 ul of GM were added to each well and the preparations were incubated for 48 h at 37°C in a 5 % CO<sub>2</sub> humidified atmosphere. The cell-virus preparations were then fixed with 10 % formalin and stained with crystal violet. Serum titers were expressed as the geometric mean (log<sub>2</sub>) of the reciprocal of the highest serum dilution that prevented CPE in duplicate wells. Positive and negative serums were run for each assay.

### Enzyme Linked Immunosorbent Assay

The enzyme linked immunosorbent assay (ELISA) used in this study was a modification of the procedure described by Snyder and Erickson.<sup>62</sup> Test and control antigens were produced by solubilizing virus infected and non-infected PK 15 cells with 0.025 M TT buffer, pH 8.0, containing 1 % v/v triton-X 100. Antigen preparations were optimally diluted in 0.02 M sodium carbonate/bicarbonate (CB) buffer, pH 9.6 and added at the rate of

100 ul to individual wells of Immulon I microtiter plates (Dynatech Lab. Inc., Chantilly, VA). The plates were incubated for 37°C and for at least 18 h at 4°C. Unreacted sites in wells were blocked with 2 % gelatin in CB buffer for 1 h at 37°C. Plates were then washed with 0.01 M phosphate buffered saline (PBS), pH 7.2 containing 0.05 % Tween 20 (WB) and stored at 4°C until used.

Test and control serums were diluted 1:20 in 0.05 M Tris buffer pH 7.4 containing 150 mM sodium chloride, 0.01 mM EDTA, 0.05 % Tween 20 and 1.0 % gelatin. One hundred ul of each diluted serum was added in duplicate to antigen-coated wells. The preparations were incubated 30 min. at 37°C and washed eight times with WB and stained for 30 min. at 37°C with 100 ul of optimally diluted goat anti-porcine Ig G (H+L) conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD). All wells were again washed eight times with WB and dried.

One hundred ul of enzyme substrate solution consisting of 0.05 % v/v hydrogen peroxide in 0.1 M citric acid disodium phosphate buffer, pH 5.0 containing 0.034 % w/v O-phenylene diamine was added to all wells. Color was allowed to develop in the dark at 25°C for 25 min. and stabilized with 50 ul of 4.5 M sulfuric acid. Individual absorbance values were determined with a Dynatech MR 580 ELISA Microplate reader equipped with a test filter wavelength of 490 nm and a reference filter wavelength of 450 nm. The data were expressed as the mean of duplicate corrected optical density readings, which were calculated by subtracting the optical density (OD) of control antigen wells from the OD of test antigen wells.

Results of the above ELISA were confirmed with a commercially available ELISA (Agritech Inc., Portland, ME) at the Iowa State Veterinary Diagnostic Laboratory located at Iowa State University, Ames, IA.

### Lymphocyte Blastogenesis Assay

Lymphocytes were isolated from citrated blood by extending 9 ml of blood to 20 ml by adding 0.01 M PBS, pH 7.2 heated to 37°C. Diluted blood was then carefully layered over 10 ml of Histopaque-1077 (Sigma Chemical Co., St. Louis, MO) contained in a 25 X 150 mm siliconized glass tube. The preparations were then centrifuged at 400 X g for 45 min. Lymphocytes were collected from the Histopaque-plasma interphase into a 15 X 125 mm plastic culture tube. The cells were washed with 5-10 ml of warmed HBSS and collected by low speed centrifugation at 250 X g for 10 min. Residual red blood cells in the cell pellet were lysed by exposure to 0.87 % w/v ammonium chloride in distilled water for 5 min. Lymphocytes were washed twice with warmed HBSS and resuspended in Medium 199 to adjust the cell concentration to  $2.5 \times 10^6/\text{ml}$ .

Whole virus was used as test antigen in the lymphocyte blastogenesis assay. Virus was produced in PK 15 cells that were inoculated at a multiplicity of 5. Virus was harvested by 2 cycles of freeze-thawing when 100 % of cell monolayer showed CPE. The cell lysate was clarified by low speed centrifugation and heated at 56°C for 45 min. Heat-inactivated virions were concentrated by ultracentrifugation at 50 K x g



for 120 min. The resulting pellet was resuspended to 1/10 of the original volume in serum-free Medium 199. The antigen preparation was aliquoted and stored at  $-70^{\circ}\text{C}$  until used. Control antigen was produced in identical manner from non-infected cells. Concanavarin A (Con A) (EY Laboratories Inc., San Mateo, CA) was used as lymphocyte mitogenic control. Concanavarin A was rehydrated in distilled water, filtered through a 0.22  $\mu\text{m}$  pore size membrane and stored at  $-70^{\circ}\text{C}$ .

The lymphocyte blastogenesis assay was a modification of the procedure described by Roth et al.<sup>58</sup> Two hundred  $\mu\text{l}$  of individual lymphocyte suspensions were dispensed into individual wells of a flat-bottom 96 well culture plate and treated in triplicate with 25  $\mu\text{l}$  of optimum concentrations of either test antigen, control antigen, Con A or medium. The preparations were incubated at  $37^{\circ}\text{C}$ , in a 5 %  $\text{CO}_2$  humidified atmosphere for 96 h and pulsed with 0.2  $\mu\text{Ci}$  of  $^3\text{H}$ -thymidine (Amersham Corp., Arlington Heights, IL) for 18 h. Lymphocytes from each well were harvested with a Cell Harvester (Flow Lab. Inc., Rockville, MD) onto glass fiber filter paper (Skatron Inc., Sterling, VA) and measured for beta emissions with a liquid scintillation counter. Mean counts per minutes (cpm) were determined for each sample and stimulation indexes (SI) were calculated by dividing the mean cpm induced by test antigen by the mean cpm induced by control antigen.

## Experimental Design

The effect of IL-2 as an immune enhancing agent in pigs was evaluated by comparing the clinical and immune response of IL-2 treated and non-treated PRV subunit vaccinated pigs. Weanling, cross-bred pigs were randomly divided into 5 groups of 3 principals each. Two groups received 2 high vaccine doses of 25 ug each given s.c. 3 weeks apart. The other two groups received 2 low vaccine doses of 5 ug each given in the same manner. A fifth group served as unvaccinated controls. One high and one low vaccine dose group were treated two times with IL-2 given s.c. at the rate of  $10^5$  units/kg for five consecutive days beginning on the day of each antigen inoculation.<sup>2</sup> All pigs were housed together and challenged with  $10^{5.0}$  PFU of virus 3 weeks after the second vaccine inoculation.

The clinical response of pigs was evaluated by comparing survival rates, weight responses and the amount of virus recovery from nasal cavities following virus challenge. Weight was monitored every other day, beginning on the day of challenge and continuing through day 14 post challenge (p.c.). Nasal swabs for virus assay were collected on the day of challenge and on days 2, 4, and 6 p.c. The cell-mediated immune response was evaluated by the lymphocyte blastogenesis assay 2 days before challenge, the day of challenge (day 0) and days 3, 5, 8, and 10 p.c. Test control lymphocytes were collected on the same days from 2 separately housed pigs which were not challenged with virus. The humoral immune response was evaluated by the ELISA, and the SN using serums

collected on the day of the first and second vaccine inoculation, 7 and 2 days before virus challenge, and on days 0, 3, 5, 8, 10, 14, and 22 p.c.

Daily SN titers and ELISA corrected OD readings of IL-2 treated and non-treated groups were independently compared for each vaccine dose by the least significant difference (LSD) procedure<sup>63</sup> for: a) the pre challenge period extending from the day of the second vaccine inoculation (day -21) through the day of challenge (day 0); and b) the anamnestic period between day 0 and 10. Cell-mediated immune responses of each treatment group were expressed as transformed ( $\log_{10}$ ) stimulation indexes (SI) and independently compared among treatment groups by Tukey's w procedure<sup>63</sup> over three time periods: a) days -2 and 0; b) days 3 and 5; c) days 8 and 10. The SI values were also analyzed between vaccinated and non-vaccinated groups by the LSD procedure over the same periods.

## RESULTS

The Effect of Recombinant Interleukin 2 on the Clinical Response of  
Vaccinates to Nasal Challenge with PRV

No differences were observed in the clinical response of IL-2 treated and non-treated vaccinated pigs. All 12 vaccinated pigs survived virus challenge (100 %) while only 2 of 3 non-vaccinated controls survived (67 %). The weight response of treatment groups is summarized in Table 1 and illustrated in Figure 1. All treated and non-treated vaccinates gained weight during the first 2 days p.c. The mean daily gain during this period ranged from 0.35 to 0.95 kg. Thereafter vaccinated pigs lost weight during the next 4 days at a mean daily rate ranging from 0.55 to 0.90 kg. In contrast, the mean daily weight lost of non-vaccinated controls was 1.20 kg. Subsequently, all groups of vaccinates gained weight from day 6 through day 14 p.c. at mean daily rates ranging from 0.70 to 1.10 kg. In contrast, non-vaccinated controls gained weight at a mean daily rate of 0.20 kg.

Virus shedding patterns of IL-2 treated and non-treated pigs are summarized in Figure 2. No differences were observed between IL-2 treated and non-treated vaccinated pigs. The maximum amount of virus was recovered from 11 out of 12 vaccinated pigs on day 4 p.c. The mean amount of virus recovered from all vaccine groups at this time ranged from  $10^{3.2}$  to  $10^{4.2}$  PFU. By day 6 p.c. the mean amount of virus

recovered from vaccinates decreased to less than  $10^{3.2}$  PFU. In contrast, the mean amount of virus recovered from non-vaccinated controls exceeded  $10^{4.2}$  PFU on days 2, 4, and 6 p.c.

The Effect of Recombinant Interleukin 2 on the Cell-mediated Immune (CMI)  
Response of Vaccinates to Nasal Challenge with PRV

The CMI response as represented by transformed ( $\log_{10}$ ) stimulation indexes (SI) of IL-2 treated and non-treated vaccinates to whole virus antigen is summarized in Table 2 and Figure 3. No differences were observed between the CMI response of IL-2 treated and non-treated vaccinated groups before and after virus challenge. However, significant differences were observed between vaccinated and non-vaccinated controls. The mean SI of all vaccinated groups on days -2 and 0 were  $0.37 \pm 0.06$  compared to  $0.10 \pm 0.03$  for pigs of the non-vaccinated and test-control groups ( $P < 0.01$ ). Significantly different mean SI responses of  $1.02 \pm 0.10$  and  $0.44 \pm 0.08$  were observed for vaccinates and non-vaccinated controls on days 3 and 5 p.c. ( $P < 0.01$ ). However, significant differences between these groups were not observed on days 8 and 10.

Table 1. Weight<sup>a</sup> response of IL-2 treated or non-treated subunit-vaccinated pigs following nasal challenge<sup>b</sup>

Treatments <sup>c</sup>	Mean weights on day 0 ( <u>±</u> SE)	Mean weight gain day 0-14	Mean daily weight gain		
			day 0-2	day 2-6	day 6-14
25 ug/dose + IL-2	35.5 <u>±</u> 4.6	+ 6.3	+ 0.45	- 0.85	+ 1.10
25 ug/dose	31.0 <u>±</u> 3.9	+ 7.3	+ 0.70	- 0.55	+ 1.00
5 ug/dose + IL-2	27.2 <u>±</u> 2.8	+ 3.9	+ 0.95	- 0.90	+ 0.70
5 ug/dose	34.9 <u>±</u> 4.1	+ 7.0	+ 0.70	- 0.80	+ 1.00
Control	35.4 <u>±</u> 4.4	- 4.1	+ 0.30	- 1.20	+ 0.20

<sup>a</sup>Weight expressed in kg.

<sup>b</sup>Pigs were nasally challenged with  $10^{5.0}$  PFU of virulent PRV strain Be on day 0.

<sup>c</sup>Pigs were vaccinated twice s.c. with lectin extracted PRV glycoproteins at 3 week intervals. Recombinant interleukin 2 (rIL-2) was given s.c. at a rate of  $10^5$  units/kg during two 5 day periods beginning on the day of the first and second vaccination. N=3 for all groups.

Figure 1. Weight response of IL-2 treated and non-treated subunit-vaccinated pigs following nasal challenge with  $10^{5.0}$  PFU of virulent pseudorabies virus. Day 0 is the day of virus challenge. N=3 for all vaccinated groups. N=3 for control group through day 10, thereafter N=2

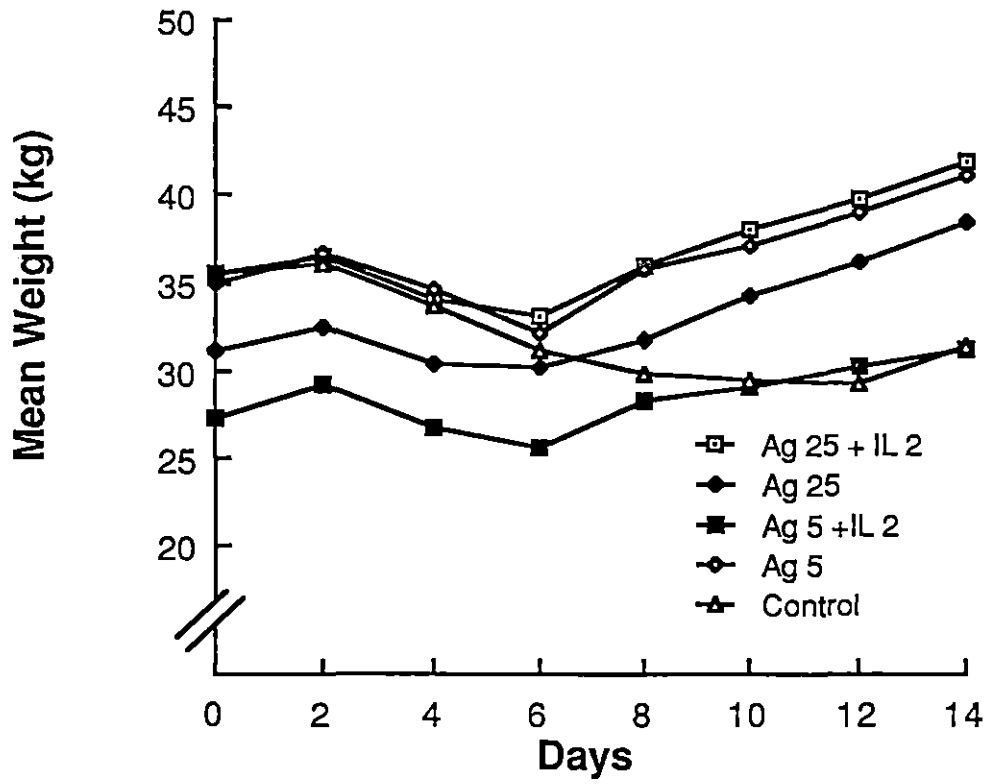




Figure 2. The effect of IL-2 on the clearance of virus from the nasal cavities of subunit vaccinated pigs following nasal challenge with  $10^{5.0}$  PFU of virulent pseudorabies virus. Day 0 is the day of virus challenge. N=3 for all groups

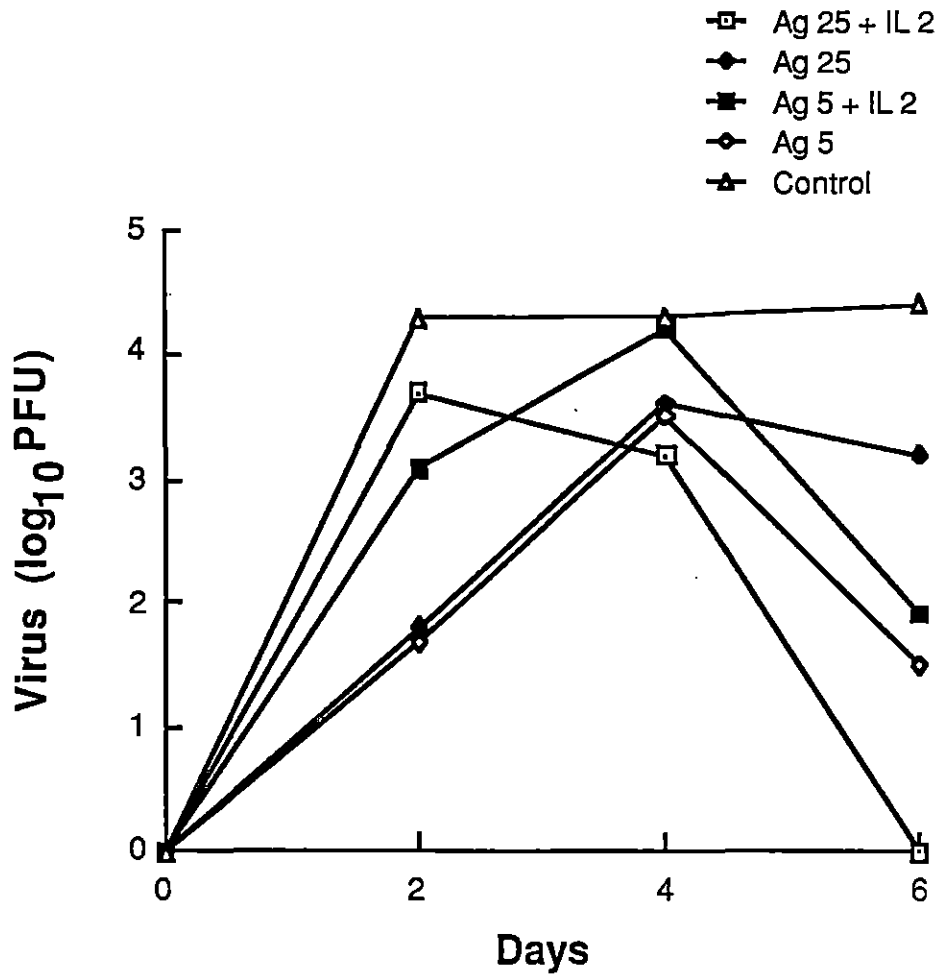


Table 2. Cell-mediated immune response of IL-2 treated and non-treated subunit-vaccinated pigs pre and post nasal challenge with virulent pseudorabies virus (PRV) as determined by the lymphocyte blastogenesis assay

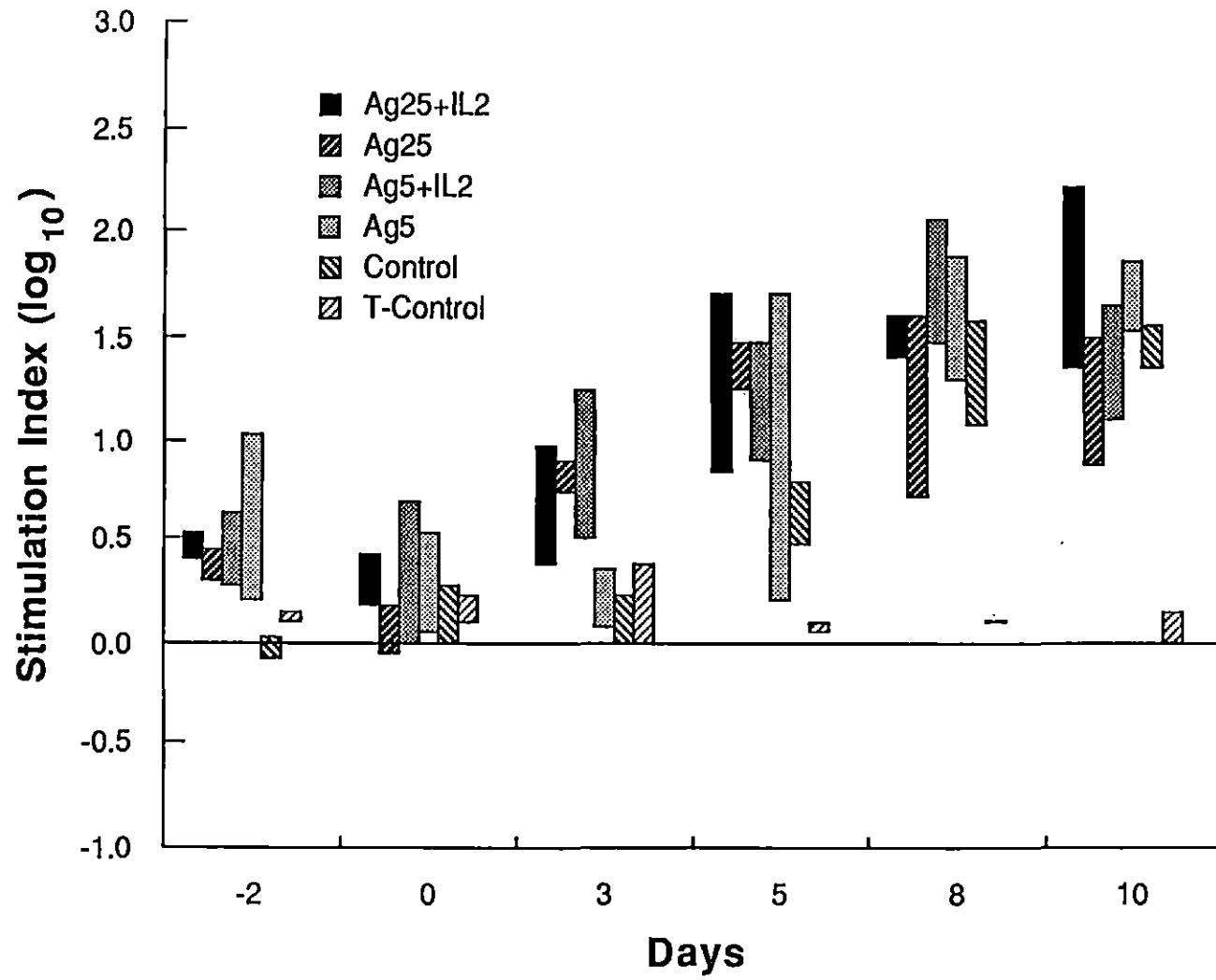
Treatments <sup>a</sup>	Mean stimulation index $\pm$ SE ( $\log_{10}$ ) <sup>b</sup>					
	Day pre and post nasal challenge <sup>c</sup>					
	-2	0	3	5	8	10
25 ug/dose + IL-2	0.49 $\pm$ 0.03	0.27 $\pm$ 0.08	0.64 $\pm$ 0.17	1.27 $\pm$ 0.44	1.53 $\pm$ 0.07	1.72 $\pm$ 0.26
25 ug/dose	0.37 $\pm$ 0.04	0.15 $\pm$ 0.11	0.84 $\pm$ 0.06	1.35 $\pm$ 0.07	1.31 $\pm$ 0.30	1.12 $\pm$ 0.19
5 ug/dose + IL-2	0.46 $\pm$ 0.10	0.29 $\pm$ 0.20	0.86 $\pm$ 0.21	1.10 $\pm$ 0.19	0.74 $\pm$ 0.17	1.36 $\pm$ 0.16
5 ug/dose	0.54 $\pm$ 0.25	0.31 $\pm$ 0.15	0.22 $\pm$ 0.08	1.05 $\pm$ 0.45	0.64 $\pm$ 0.18	1.68 $\pm$ 0.09
Control	-0.06 $\pm$ 0.03	0.16 $\pm$ 0.08	0.10 $\pm$ 0.07	0.63 $\pm$ 0.09	0.41 $\pm$ 0.18	1.35 $\pm$ 0.12
Test-control	0.12 $\pm$ 0.03	0.17 $\pm$ 0.06	0.19 $\pm$ 0.19	0.08 $\pm$ 0.04	0.11	0.08 $\pm$ 0.09

<sup>a</sup>Pigs were vaccinated twice s.c. with lectin extracted PRV glycoproteins at 3 week intervals. Recombinant interleukin 2 (rIL-2) was given s.c. at a rate of  $10^5$  units/kg during two 5 day periods beginning on the day of the first and second vaccination. N=3 for all groups except Test-control where N=2 for all days except day 8 when N=1.

<sup>b</sup>Stimulation index (SI) = cpm induced by viral antigen / cpm induced by control antigen.

<sup>c</sup>All groups except Test-control were challenged with  $10^{5.0}$  PFU of virulent PRV strain Be. Test-controls were separately housed.

Figure 3. Cell-mediated immune response of IL-2 treated and non-treated subunit-vaccinated pigs following nasal challenge with  $10^{5.0}$  PFU of virulent pseudorabies virus as determined by the lymphocyte blastogenesis assay. Day 0 is the day of virus challenge. N=3 for all groups except Test-control where N=2 for all days except day 8 when N=1



The Effect of Recombinant Interleukin 2 on the Humoral Immune Response of  
Vaccinates to Nasal Challenge with PRV

The humoral antibody response of IL-2 treated and non-treated vaccinated pigs was determined by the ELISA, and the SN assay. Results of these tests are summarized in Tables 3, 4, 5 and illustrated in Figures 4, 5, 6, 7, 8 respectively. The mean SN titers of treated high and low vaccine dose groups were consistently greater than SN titers of non-treated pigs throughout the test period (Table 3). These values were only significantly different among high dose vaccinates during the pre-challenge period ( $P < 0.05$ ).

The ELISA corrected OD values of IL-2 treated high and low dose vaccinates were also greater than the corresponding values of non-treated vaccinates throughout the test period (Tables 4 and 5). Highly significant differences were demonstrated between the treatment groups of high dose vaccinates by the commercial ELISA ( $P < 0.01$ ). No significant differences were observed between treatment groups of low dose vaccinates.

Table 3. Humoral antibody response of IL-2 treated and non-treated subunit-vaccinated pigs pre and post nasal challenge with virulent pseudorabies virus (PRV) as determined by the microtiter serum neutralization assay

Treatments <sup>a</sup>	Geometric mean $\pm$ SE ( $\log_2$ )									
	Day pre and post nasal challenge <sup>b</sup>									
	-21	-7	-2	0	3	5	8	10	14	22
25 ug/dose + IL-2	0.00 $\pm$ 0.00	0.83 $\pm$ 0.17	0.33 $\pm$ 0.17	0.50 $\pm$ 0.00	0.50 $\pm$ 0.29	0.67 $\pm$ 0.33	3.17 $\pm$ 0.17	4.67 $\pm$ 0.17	4.17 $\pm$ 0.17	4.17 $\pm$ 0.17
25 ug/dose	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	2.50 $\pm$ 0.50	3.50 $\pm$ 0.50	4.33 $\pm$ 0.17	4.17 $\pm$ 0.17
5 ug/dose + IL-2	0.00 $\pm$ 0.00	0.13 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	2.00 $\pm$ 0.50	4.33 $\pm$ 0.44	4.17 $\pm$ 0.17	4.17 $\pm$ 0.17
5 ug/dose	0.00 $\pm$ 0.00	0.13 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	1.50 $\pm$ 0.29	3.33 $\pm$ 0.33	3.50 $\pm$ 0.00	3.67 $\pm$ 0.33
Control	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.33 $\pm$ 0.17	1.70 $\pm$ 0.33	4.50 <sup>C</sup> $\pm$ 0.00	4.50 $\pm$ 0.50

<sup>a</sup>Pigs were vaccinated twice s.c. with lectin extracted PRV glycoproteins at 3 week intervals. Recombinant interleukin 2 (rIL-2) was given s.c. at a rate of  $10^{5.0}$  units/kg during two 5 day periods beginning on the day of the first and second vaccination. N=3 per group.



<sup>b</sup>Pigs were nasally challenged with  $10^{5.0}$  PFU of virulent PRV strain Be.

<sup>c</sup>One of 3 pigs died on day 11.

Figure 4. Humoral antibody response of IL-2 treated and non-treated, subunit-vaccinated pigs following nasal challenge with  $10^{5.0}$  PFU of virulent pseudorabies virus as determined by the microtiter serum neutralization assay. Day 0 is the day of virus challenge. N=3 for all vaccinated groups. N=3 for control group through day 10, thereafter N=2

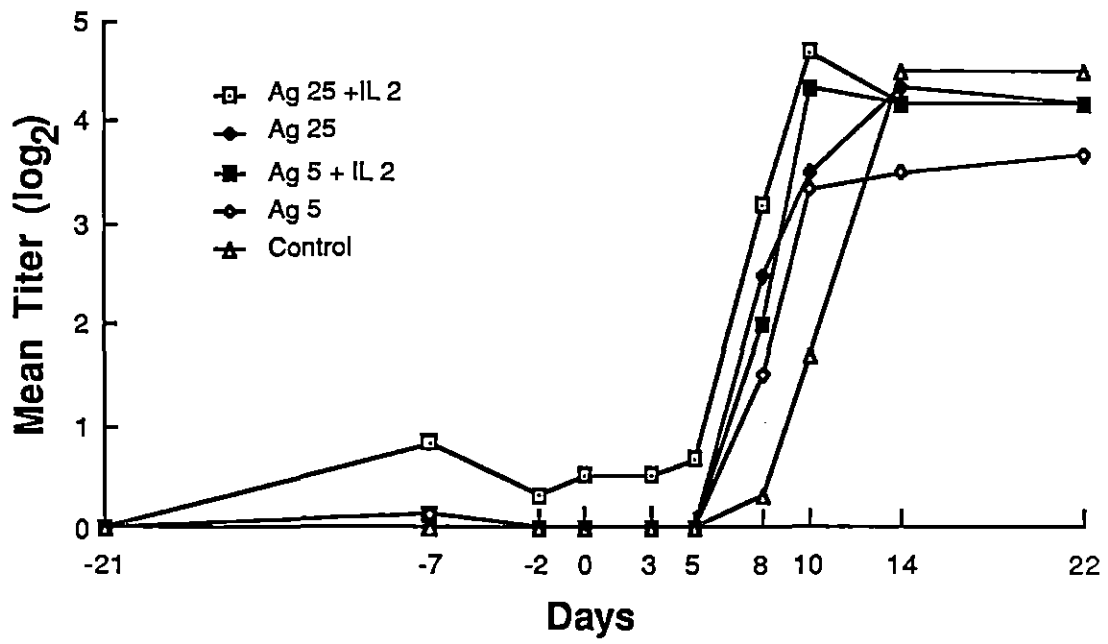


Table 4. Humoral antibody response of IL-2 treated and non-treated subunit-vaccinated pigs pre and post nasal challenge with virulent pseudorabies virus (PRV) as determined by the enzyme-linked immunosorbent assay

Treatments <sup>a</sup>	Mean corrected optical density (OD) <sup>b</sup> + SE									
	Day pre and post nasal challenge with 10 <sup>5.0</sup> PFU of virulent PRV strain Be									
	-21	-7	-2	0	3	5	8	10	14	22
25 ug/dose + IL-2	0.010 +0.003	0.215 +0.012	0.167 +0.006	0.202 +0.020	0.190 +0.021	0.207 +0.009	0.435 +0.001	0.493 +0.022	0.482 +0.032	0.477 +0.029
25 ug/dose	0.012 +0.005	0.138 +0.005	0.130 +0.017	0.131 +0.017	0.132 +0.018	0.184 +0.020	0.392 +0.028	0.388 +0.032	0.383 +0.050	0.422 +0.017
5 ug/dose + IL-2	0.021 +0.015	0.135 +0.041	0.112 +0.039	0.146 +0.030	0.125 +0.035	0.163 +0.030	0.383 +0.014	0.408 +0.018	0.430 +0.012	0.453 +0.021
5 ug/dose	0.015 +0.008	0.106 +0.041	0.100 +0.044	0.099 +0.044	0.098 +0.041	0.123 +0.042	0.279 +0.035	0.314 +0.029	0.343 +0.019	0.369 +0.008

<sup>a</sup>Pigs were vaccinated twice s.c. with lectin extracted PRV glycoproteins at 3 week intervals. Recombinant interleukin 2 (rIL-2) was given s.c. at a rate of 10<sup>5</sup> units/kg during two 5 day periods beginning on the day of the first and second vaccination. N=3 for all groups.

<sup>b</sup>Corrected optical density (OD) equals OD of serum plus test antigen minus OD of the same serum plus cell control antigen. Mean corrected OD + SE of 12 known negative serums was 0.001 + 0.0005.

Figure 5. Humoral antibody response of IL-2 treated and non-treated high dose subunit-vaccinated pigs following nasal challenge with  $10^{5.0}$  PFU of virulent pseudorabies virus as determined by the enzyme-linked immunosorbent assay. Day 0 is the virus challenge day. N=3 for all groups

Figure 6. Humoral antibody response of IL-2 treated and non-treated low dose subunit-vaccinated pigs following nasal challenge with  $10^{5.0}$  PFU of virulent pseudorabies virus as determined by the enzyme-linked immunosorbent assay. Day 0 is the virus challenge day. N=3 for all groups

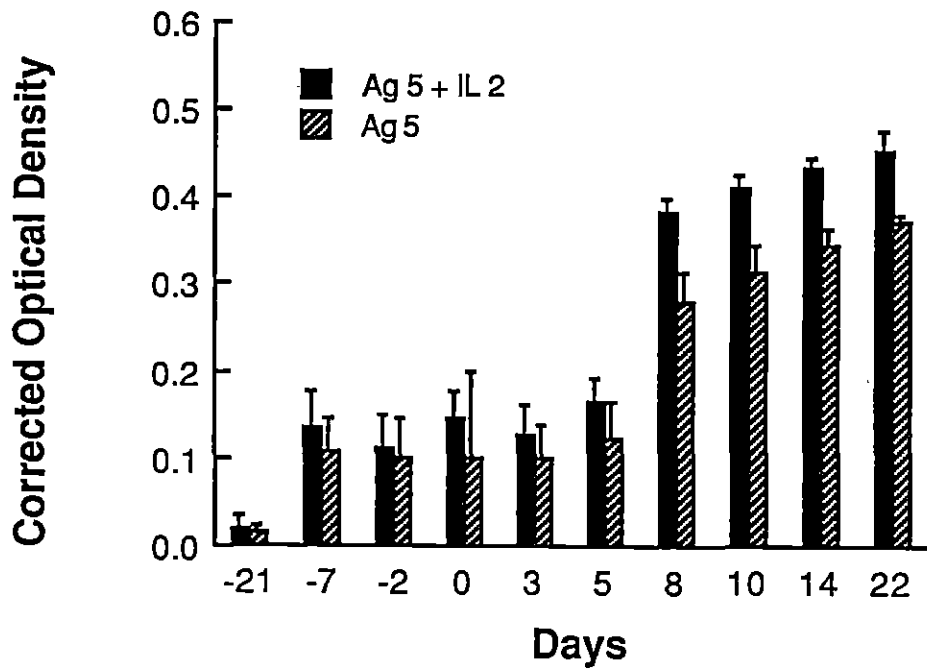
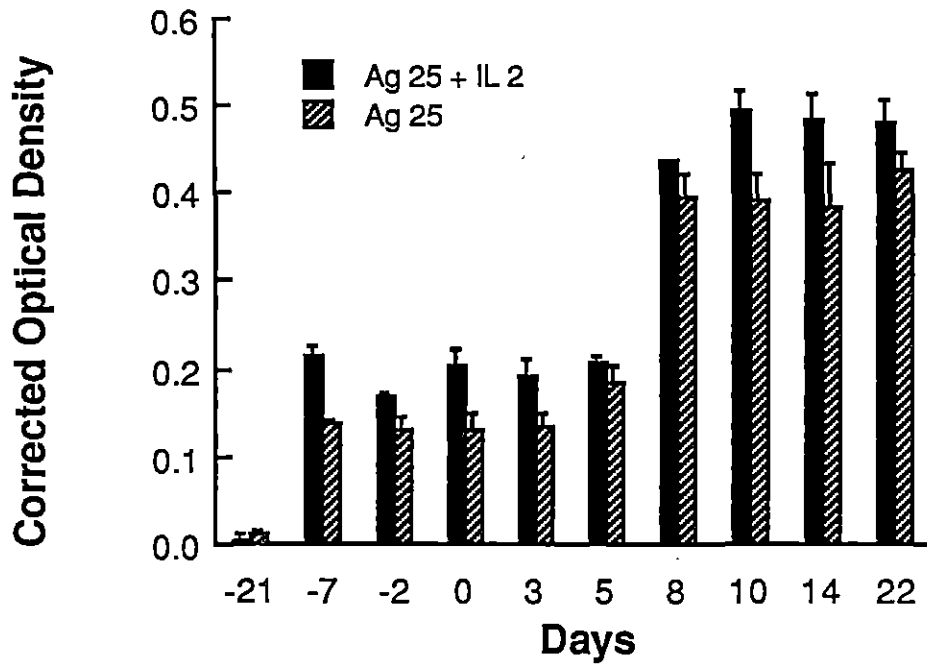


Table 5. Humoral antibody response of IL-2 treated and non-treated subunit-vaccinated pigs pre and post nasal challenge with virulent pseudorabies virus (PRV) as determined by a commercial enzyme-linked immunosorbent assay<sup>a,b</sup>

Treatments <sup>c</sup>	Mean corrected optical density (OD) <sup>d</sup> ± SE									
	Day pre and post nasal challenge <sup>e</sup>									
	-21	-7	-2	0	3	5	8	10	14	22
25 ug/dose + IL-2	0.244 ±0.038	0.997 ±0.021	0.957 ±0.029	0.906 ±0.050	0.857 ±0.044	0.818 ±0.014	1.240 ±0.002	1.280 ±0.071	1.230 ±0.035	1.340 ±0.074
25 ug/dose	0.165 ±0.063	0.569 ±0.022	0.638 ±0.050	0.527 ±0.045	0.408 ±0.029	0.539 ±0.055	1.170 ±0.035	1.070 ±0.037	1.140 ±0.062	1.190 ±0.030
5 ug/dose + IL-2	0.165 ±0.065	0.498 ±0.200	0.546 ±0.200	0.550 ±0.200	0.477 ±0.161	0.561 ±0.129	1.001 ±0.013	1.183 ±0.024	1.201 ±0.036	1.290 ±0.010
5 ug/dose	0.160 ±0.068	0.446 ±0.204	0.477 ±0.206	0.423 ±0.193	0.400 ±0.178	0.446 ±0.168	1.110 ±0.061	1.066 ±0.041	1.157 ±0.030	1.206 ±0.021
Control	0.017 ±0.004	0.011 ±0.006	0.008 ±0.004	0.019 ±0.011	0.026 ±0.006	0.024 ±0.065	0.334 ±0.065	0.789 ±0.052	1.023 ±0.068	1.161 ±0.140

<sup>a</sup>Agritech Inc., Portland, ME.

<sup>b</sup>ELISA assay was performed by Veterinary Diagnostic Laboratories, Iowa State University, Ames, IA.

<sup>c</sup>Pigs were vaccinated twice s.c. with lectin extracted PRV glycoproteins at 3 week intervals. Recombinant interleukin 2 (rIL-2) was given s.c. at a rate of  $10^5$  units/kg during two 5 day periods beginning on the day of the first and second vaccination. N=3 for all vaccinated groups. N=3 for control group through day 10, thereafter N=2.

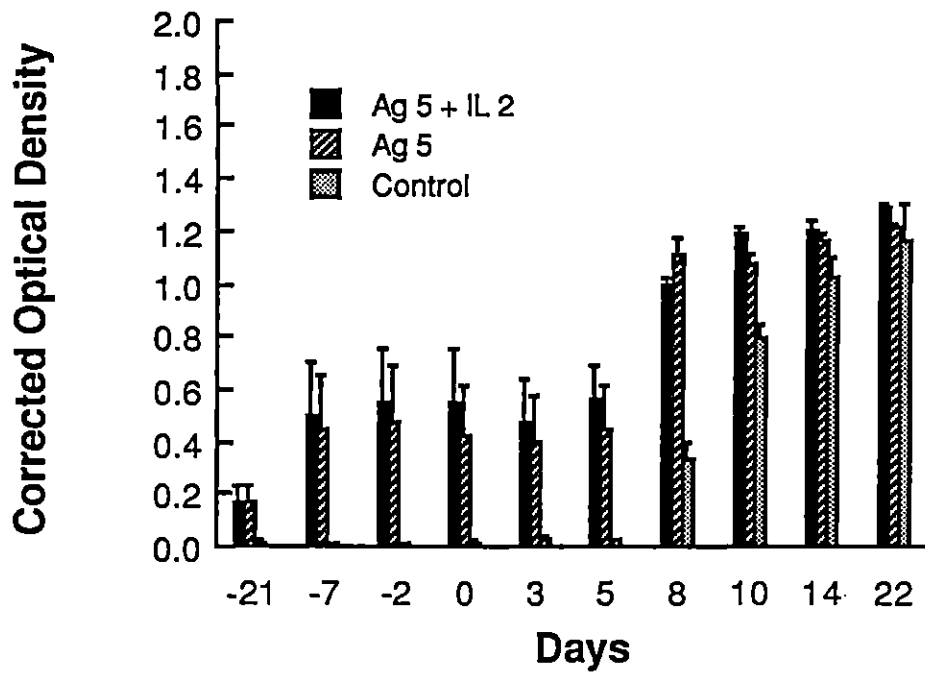
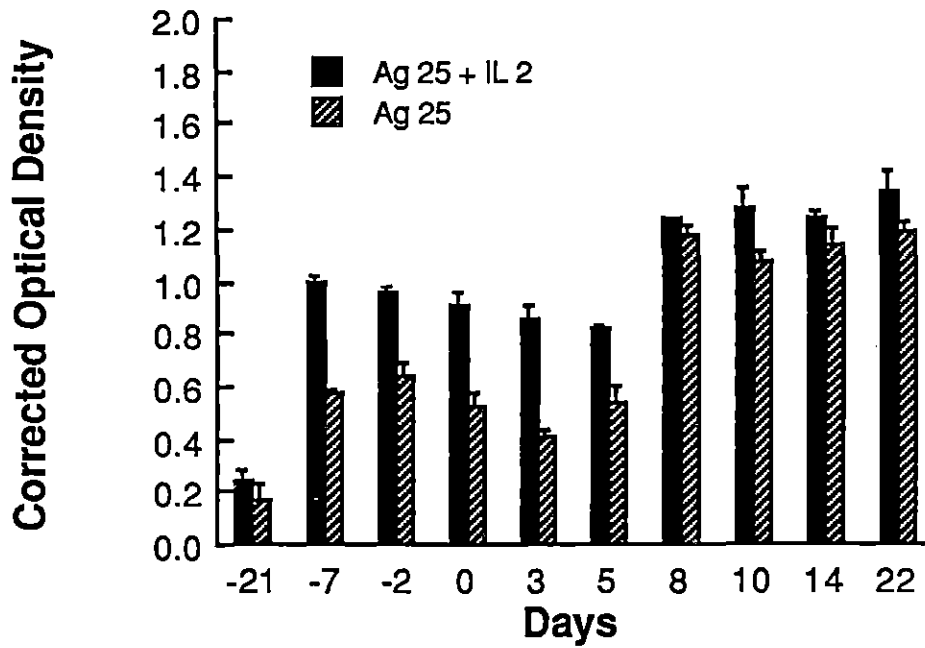
<sup>d</sup>Corrected optical density (OD) equals OD of serum plus test antigen minus OD of the same serum plus cell control antigen. Mean corrected OD  $\pm$  SE of 21 known negative serums was  $0.008 \pm 0.004$ .

<sup>e</sup>Pigs were nasally challenged with  $10^{5.0}$  PFU of virulent PRV strain Be.



Figure 7. Humoral antibody response of IL-2 treated and non-treated high dose subunit-vaccinated pigs following nasal challenge with  $10^{5.0}$  PFU of virulent pseudorabies virus as determined by a commercial enzyme-linked immunosorbent assay (Agritech Inc, Portland, ME). Day 0 is the virus challenge day. N=3 for all group

Figure 8. Humoral antibody response of IL-2 treated and non-treated low dose subunit-vaccinated pigs following nasal challenge with  $10^{5.0}$  PFU of virulent pseudorabies virus as determined by a commercial enzyme-linked immunosorbent assay (Agritech Inc, Portland, ME). Day 0 is the virus challenge day. N=3 for all group



## DISCUSSION

The preceding study was conducted to determine if human recombinant interleukin 2 (IL-2) could be used to enhance the immune response of pigs to a pseudorabies subunit vaccine. Results of this study showed that while the administration of IL-2 with vaccine antigen did not enhance the immune response of pigs to pseudorabies as determined by clinical signs and the cell-mediated immune (CMI) response (Tables 1 and 2), it did induce consistently higher SN and ELISA antibody titers (Tables 3, 4 and 5). These differences were highly significant ( $P < 0.01$ ) during the pre-challenge and anamnestic periods among high dose vaccinates when measured by the commercial ELISA (Table 5 and Figure 7). The ability of the commercial ELISA to detect statistically significant differences that were not revealed by the non-commercial ELISA may have been due to quantitative differences in the composition of the test antigen. The inability of the SN test to reveal differences as significant as those which were revealed by the ELISA may be due to the specificity of the test for neutralizing and not non-neutralizing antibody.

The higher antibody titers induced by IL-2 may be an indication that a stronger immune response was generated in IL-2 treated pigs. However, this suggestion is not supported by the clinical and CMI responses of treated and non-treated pigs. The inability to detect potential differences between groups by these parameters may have been due to the size of the vaccine dose used in the study. Earlier work in this

laboratory suggested that 10 ug of vaccine antigen approached the minimum effective immunizing dose.<sup>48</sup> Based on this finding, 5 ug of vaccine antigen was selected as a dose that would be small enough to demonstrate potentiation by IL-2. The use of smaller antigen doses might be required to demonstrate potential immuno-enhancing effects of IL-2. Higher virus challenge doses may also be more effective in revealing differences in the clinical response of treated pigs.

The increased antibody production observed in the current study was expected based on the work of Kawamura et al.<sup>31</sup> These workers were able to demonstrate increased levels of antibodies in mice that were inoculated with a mixture of IL-2 and antigen in Freund's complete adjuvant. The increased antibody production observed in the current study may have been due to clonal expansion of antigen-specific  $T_h$ , which has been shown to be induced by IL-2.<sup>29,47,60,61</sup> An expanded population of  $T_h$  can indirectly cause an increase in the size of antigen-specific  $T_c$  and B lymphocyte populations through secreted lymphokines.<sup>29,39,40,43</sup>

However, an expanded antigen-specific lymphocyte population was not reflected by the lymphocyte blastogenesis assay (Table 2) which measures the relative activity of  $T_h$ ,  $T_c$ , and B cells. The absence of differences in the stimulation indexes between IL-2 treated and non-treated vaccinates might be explained by the high degree of variability which is normally associated with the lymphocyte blastogenesis assay and the relatively small number of animals tested. It is also possible that the number of antigen-specific lymphocytes present in peripheral blood was too small to be detected. Activated lymphocytes may have been localized

in lymphoid organs. This hypothesis is supported by the work of Wittmann et al.<sup>73</sup> These investigators demonstrated in a PRV challenge experiment that lymphocytes derived from spleen or lymph nodes are more blastogenic to virus antigen than peripheral lymphocytes.

Although the preceding study suggests that human recombinant IL-2 may be a useful immuno-enhancing agent in the pig, additional experimentation will have to be conducted in order to assess its effectiveness for this purpose. This experimentation will have to utilize: larger number of animals; a broader dose range of IL-2 and immunizing antigen; and more sensitive assays to evaluate the CMI response.

## SUMMARY

The effect of interleukin 2 (IL-2) as an immune enhancing agent in pigs was evaluated by comparing the clinical and immune response of IL-2 treated and non-treated pseudorabies virus (PRV) subunit vaccinated pigs. Weanling, cross-bred pigs were randomly divided into 5 groups of 3 principles each. Two groups received 2 high vaccine doses of 25 ug each given s.c. 3 weeks apart. The other two groups received 2 low vaccine doses of 5 ug each given in the same manner. A fifth group served as unvaccinated controls. One high and one low vaccine dose group were treated two times with IL-2 given s.c. at the rate of  $10^5$  units/kg for five consecutive days beginning on the day of each antigen inoculation. All pigs were housed together and challenged with  $10^{5.0}$  PFU of virus three weeks after the second vaccine inoculation. Clinical responses were evaluated by determining the survival rates, weight responses, and virus shedding patterns. Humoral immune responses were measured by the ELISA, and microtiter serum neutralization (SN) assay. Cell-mediated immune (CMI) responses were measured by the lymphocyte blastogenesis assay. No differences were detected in the clinical responses and CMI responses among treatment groups. However, the ELISA and SN antibody titers were consistently higher in IL-2 treated vaccinates. Differences determined by the ELISA among high dose vaccinates were highly significant ( $P < 0.01$ ). These results suggested that human recombinant IL-2 may be a useful immune enhancing agent to PRV subunit vaccine.

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## ACKNOWLEDGEMENTS

I would like to thank first to my major professor, Dr. Kenneth B. Platt for his generosity and encouragement throughout my study program. Without his enthusiastic encouragement, this work would never be accomplished. I also wish to thank my committee members, Drs. George W. Beran and Wayne A. Hagemoser for their invaluable guidance in my graduate study.

Special acknowledgement is extended to Dr. David R. Kinker, Iowa Veterinary Diagnostic Laboratory, who kindly supplied many field swine serums and allowed me to utilize a ELISA microplate reader. All faculty and staff members of the Department of Veterinary Microbiology and Preventive Medicine are also greatly appreciated, especially Drs. James A. Roth and Merlin L. Kaeberle for allowing me to utilize facilities in the Immunology laboratory. I also extend my appreciation to my fellow graduate students who helped with handling pigs and provided opportunity for invaluable discussion throughout my studies. Nancy E. Kelso is also appreciated for her technical help in the Virology laboratory.

I also want to extend my gratitude to Dr. Tony Matsubara, professor in the Azabu University (Japan), who encouraged me to attend Iowa State University and to Dr. Lauren L. Christian, professor in the Department of Animal Science, Iowa State University for his encouragement throughout my studies.

Finally, I am profoundly grateful to my parents, Mr. Kiyoshi Kawashima and Mrs. Ikuko Kawashima, for their generosity in providing me with an educational opportunity in the United States of America.

This work was supported in part by USDA-ARS cooperative agreement # 58-6125-6-4, and the Iowa Livestock Health Advisory Council.