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Análisis de la potencia de vacunas inactivadas del virus de la enfermedad de Aujeszky (ADV), cepa RC/79: estimulación de linfocitos en cerdos inmunizados

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ORIGINAL ARTICLE

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Potency analysis of inactivated vaccines for Aujeszky's disease virus (ADV), strain RC/79: Lymphocyte stimulation in immunized pigs

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ABSTRACT. Mitogenic and antigenic lymphocyte stimulation was examined in pigs that had been immunized with 2 inactivated vaccines which had been prepared with Aujesky's disease virus (ADV), strain RC/79. One vaccine was partially purified by ultracentrifugation (Vaccine A) the other one was not (Vaccine B). A second dose of vaccine had no influence over the blastogenic response when the lymphocytes were stimulated with phytohemoagglutinin (PHA). Lymphocyte response to the ADV antigen in the immunized pigs was significantly higher at day 30 post inoculation than at day 0 indicating that it was highly specific. Cellular antigens contained in the viral cultures produced a slight non-specific response as shown by a low increase in the levels of lymphocyte blastic transformation (LBT) in the control group at day 30 p.i., this group only received a non infected Vero cell suspension. This was the case in pigs that received vaccine A as well as in those that were vaccinated with vaccine B. Vaccine B contains a greater quantity of contaminating cellular antigens, since it is an impure vaccine. Such antigens could act as non-specific immunomodulators, potentiating cell-mediated immunity (CMI). This assay demonstrated that inactivated vaccines produced with VPR-RC/79, partially purified and unpurified are capable of inducing a humoral immune response. The blastogenic reaction of the peripheral blood lymphocytes to antigens of ADV strain RC/79, indicated that the employed immunogens also induced the CMI. Results indicate that the analyzed immunogens could be considered for the possible implementation of epidemiological measures, which imply the use of vaccines to prevent pseudo-rabies in Argentina.

Key words: Aujesky's disease virus, strain RC/79, lymphocyte stimulation, inactivated vaccines, cell mediated immunity (CMI).

RESUMEN. La estimulación linfocitaria mitogénica y antigénica fue examinada en cerdos inmunizados con 2 vacunas inactivadas preparadas con el virus de la enfermedad de Aujeszky (VEA), cepa RC/79; una parcialmente purificada por ultracentrifugación (vacuna A) y otra sin purificar (Vacuna B). Una segunda dosis de vacuna no tuvo influencia sobre la respuesta blastogénica cuando se estimuló a los linfocitos con la fitohemoaglutinina (PHA). La respuesta de los linfocitos al antígeno del virus de la EA de los cerdos inmunizados con ambas vacunas, fue significativamente más alta al día 30 post inoculación (p.i.), respecto del día 0 de iniciada la experiencia; indicando que fue altamente específica. Los antígenos celulares contenidos en los cultivos virales produjeron una ligera respuesta inespecífica evidenciada por el pequeño incremento que mostraron los índices de transformación blástica linfocitaria (TTBL) de los cerdos controles al día 30 p.i.; cerdos que sólo recibieron una suspensión de células Vero sin infectar. Esto aconteció tanto en los cerdos que recibieron la vacuna A como en aquéllos vacunados con la vacuna B. La vacuna B contiene mayor cantidad de antígenos celulares contaminantes por tratarse de una vacuna impura, los que podrían actuar como inmunomoduladores inespecíficos potenciando la inmunidad mediada por células (IMC). En este ensayo se demostró que las vacunas inactivadas producidas con el VPR-RC/79 parcialmente purificadas y sin purificar son capaces de inducir la respuesta inmune humoral. La reacción blastogénica de los linfocitos de sangre periférica a los antígenos del virus Aujeszky, cepa RC/79 indicó que los inmunógenos empleados indujeron también la IMC. Los resultados indican que los inmunógenos analizados aquí podrían ser considerados para la posible implementación de medidas epidemiológicas que impliquen el uso de vacunas para prevenir la pseudorrabia en la Argentina.

Palabras clave: Virus de la enfermedad de Aujeszky, cepa RC/79, estimulación linfocitaria, vacunas inactivadas, inmunidad mediada por células (IMC).

INTRODUCTION

The vertebrate immune system comprehends a complex and interrelated set of defenses against the invasion of foreign material, mostly of microbial nature. Viral infections stimulate a cellular as well as humoral specific immune response inducing B and T lymphocytes, natural killer cells, and neutralizing antibodies.[24] It is known that cell-mediated immunity (CMI) plays an important role in the recovery from various infections by Herpes virus and in the prevention of recurring illness.[6,15] In vitro stimulation of lymphocytes has been used as an indicator of cellular immunity and has been applied to a large number of infections by Herpes virus. *Steele & col.*[20] proved that humans with recurring labial herpes had a lymphocyte blastogenic response to cells infected with Herpes simplex type 1. In addition, *Rouse & Babiuk*[14] when working with the virus of bovine infectious rhinotracheitis (BIR) demonstrated a similar temporary response, in peripheral blood lymphocytes of cows that had suffered primary and recurring infections.

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Regarding the ADV the CMI's role in infected pigs has been studied and demonstrated by several techniques. *Thawley & col.*[21] confirmed ADV infection in pigs recovering the viral agent after a treatment with dexamethosone and measuring lymphocyte blastogenesis in vitro. *Wittmann & col.*[27] proved a blastogenic response *in vitro* to the viral antigen in pigs that were infected by the pseudorabies virus, such response was not different in lymphocytes from the spleen than in lymphocytes from the lymphatic nodules. This author also achieved measuring macrophage migration inhibition (MMI).[26] On the other hand, this same technique was developed by *Gutekunst*,[8] whose results permitted determining CMI in vaccinated pigs and pigs infected with pseudorabies. With these backgrounds the purpose of the present work was to demonstrate if ADV, strain RC/79 inactivated vaccines (found in Argentina) awakened the CMI. To this end, lymphocyte sensitization in pigs, immunized with these immunogens, was measured using the lymphocyte blastic transformation technique in vitro (LBTT).

MATERIAL AND METHODS

Virus and cell culture. Field isolated Aujeszky's disease virus, strain RC/79 was used.[1] Characterized by its virulence,[4,17] and by the induction of neutralizing antibodies (NAb) synthesis in convalescent pigs,[28] this strain was kept by serial passage in Vero cells: the latter were grown with MEM-Eagle-Eagle, supplemented with 8% of inactivated bovine fetal serum and 30 µg/ml of gentamicin. The viral lot showed a titer of 1 x 10^8 $DICC_{50}$/ml and was used for the preparation of the vaccines and of the antigen used in the LBTT.

Vaccine preparation. The supernatant fluids of the cellular monolayers, infected at an infection multiplicity of 1, were collected when the cytopathic effect (CPE) affected more than 90% of the cellular monolayer at 48 h to 72 h p.i. This was done through two freeze-unfreeze cycles and clarification by centrifugation at 3,000 x *g* during 15 min in a Bolco centrifuge (AMC-2500). The viral suspension was divided in to fractions:

Fraction 1 or Vaccine A: A new clarification was done by successive centrifugations at 5,000 rpm and at 10,000 rpm for 20 min each time. The supernatant was centrifuged at 33.000 rpm during 2.5 h in a Spinco ultracentrifuge rotor SW-27. The precipitate was resuspended in PBS, until it reached a concentration of 10X with respect to the original volume. This immunogen was considered partially purified.

Fraction 2 or Vaccine B: The viral fluid was partially purified at 10,000 rpm but it was not ultracentrifuged and it was considered unpurified.

Both viral fractions were inactivated with Bromo ethylen imine (BEI) 0.1 M at 1% through the night. stopping the reaction by adding sodium thiosulphate 1 M at 1%. Inocuity controls were done by three successive passes in Vero cell monolayers. Sterility control was done by inoculation of thioglycolate medium.

For each viral fraction protein concentration was determined by the Bradford method.[2]

Animals and immunization scheme. 26 hybrid pigs, both sexes, were used. All pigs came from a single establishment, were sero-negative to the ADV -as determined by a microseroneutralization test (MSN) described by *Hill & col.*[9] modified by *Zanon y col.*[28] were 40 days old and weighed 8 kg in average at the beginning of the experiment. The following animal groups were used:

Group I: 10 animals numbered from A1 to A10, immunized with vaccine A.

Group II: 10 animals numbered from B1 to B10, immunized with vaccine B.

Group III: 6 animals numbered from C1 a C6, controls.

Group I and II received the vaccine with aluminum hydroxide as an adjuvant, through deep inoculation to the neck.. Each animal received 2 doses of 2 ml of the vaccine that contained 1 x 10^8 $DICC_{50}$/ml, with an interval of 15 days between each application.

Group III did not receive the vaccine, but a non-infected Vero cell suspension was given. These Vero cells had been treated the same way as the monolayers infected with the virus and served as antigen control. This antigen was also coadjuvated with aluminum hydroxide.

All animals were tried 53 days p.i. with 2 x 10^8 $DICC_{50}$/ml of the active virus. The animals were kept in separate cages and their food intake and hygiene were strictly controlled during the whole experiment.

Antigens used for the LBTT. The following were used as antigens for the test of lymphocyte stimulation of the immunized animals and controls:

a) Suspension of Aujeszky's disease virus inactivated through heat exposure for 1 h at 56ºC, as described by Sabini & col. [18]. The virus thus treated was not infectious as determined by three successive passes in Vero cells. One ml of the antigen was equivalent to 1 x 10^8 $DICC_{50}$/ml of the virus. This antigen was stored at -20ºC until its use.
b) The mitogen PHA (phytohemoagglutinin M-Difco) at a final concentration of 0.5% per culture.
c) Suspension of uninfected Vero cells treated in the same manner as the infected monolayers.

Obtainment and preparation of the lymphocytes for the culture. All animals from the three groups were bled

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by jugular puncture with a heparinized syringe on day 0 of the experiment before vaccination and at day 30 post vaccination (p.a.). The syringe containing the blood sample was kept in a vertical position to allow red cell decantation. The supernatant plasma containing the leukocytes was moved into a sterile flask covered with a rubber stopper. In an aliquot, a leukocyte count and a lymphocyte differential count as well as their viability was determined by exclusion with trypan blue.[13] In all cases, viability was greater then 87%. Lymphocytes were then resuspended in TC-199 DIFCO medium supplemented with 10% of bovine fetal serum, 30 mg/ml of gentamicin and 2 mM/ml of glutamine, until reaching a final concentration between 5 x 10^5 and 10^6 leukocytes/ml.

Lymphocyte blastic transformation (LBTT). For blastogenesis determination the methodology described by Brarda & col.,[3] was performed, doing the assays three times. Each cell culture consisted of 2 ml of TC-199 DIFCO medium supplemented with serum and glutamine as described above, with or without stimulants and 0.1 ml of the lymphocyte suspension. In the lymphocyte antigen stimulation assays 0.1 ml of ADV was used prepared as described above or with 0.1 ml of PHA added to the described solution or 0.1 ml of free extracts of uninfected Vero cells. Cultures were incubated at 37ºC during 4 days, and then 0.01 mL of H^3 thymidine were added to each of them (6,6 Ci/nmol New England Nuclear, 1/10 dilution in sterile physiological solution). They were incubated again for 3 h.

Determination of H^3 thymidine capture. After the incubation period the cultures were agitated, to separate the lymphocyte layer from the flask's bottom and 10 to 12 ml of sterile physiological solution were added. The resulting cellular suspensions were centrifuged at 3.500 rpm during 15 min. After discarding the supernatant, the cultures were washed again as described. The sediment in each tube was resuspended in 1 ml of distilled water and individually transferred to scintillation vials.

Each tube was washed with 1 ml of distilled water and the washing fluid was transferred to its corresponding vial. The uncovered vials were set in the stove at 180ºC until dry. 10 ml of scintillating fluid were added to each vial: 5 g de 2,5-Diphenyloxazole (PPO) and 50 mg de (1,4-bis[5-Phenyl-2-oxazolyl]-benzene, 2,2´-p-Phenylene-bis[5-Phenyloxazole) (POPOP), per liter of toluene). Radioactivity was determined in a Beckman LS-6000IC liquid scintillation counter

The thymidine capture index and the stimulation index (SI) were calculated by using the following formula:

$$IE = \frac{CpmI - CpmE}{CpmI}$$

Where Cpml is the count average per minute taken from cultures stimulated with PHA or with the viral antigens and CpmE is the count average of cultures without stimuli, or spontaneous count. When a difference greater than 20% was observed among the triplicates the assay was discarded.

Data were statistically analyzed by analysis of variance and non-parametric tests.[25]

Microseroneutralization test. In all three groups of animals, the detection and titration of neutralizing antibodies (NAb) against ADV, followed the methodology described by *Hill and col*.,[9] modified by *Zanon and col.* [28].

RESULTS

Antibody production. Pigs from groups A and B immunized with both vaccines responded synthesizing NAb detected for the first time at a value of 7. Initial values were of 4 to 8, except two animals: A9 and B6, which showed a titer of 2. After two doses of vaccines, at day 47 p.i., titers were between 128 and 256, except pig B5, which had a titer of 64. After inoculation with the active trial strain, all animals responded by increasing their levels of NAb and the majority reached a maximum value of 512. Regarding pigs in group C-non-vaccinated controls-it was observed that they stayed sero-negative until day 63 of the experiment, that is, 10 days post-trial (p.t.). During this time Nab were detected in titers of 8 and did not exceed 32 at the end of the assay.

Clinical observations. The vaccinated pigs from groups A and B remained healthy during the entire study and did not present symptoms of illness after the trial, except for a light anorexia. Animals from group C after being confronted with the virus, showed a biphasic febrile state between days 2 and 8 p.i. with an average temperature higher than 40ºC, light coughing, anorexia and general decay. These signs disappeared on day 11 p.i.

Mitogenic stimulation. Lymphocyte response to PHA by the vaccinated and control pigs was followed simultaneously with the viral and cellular antigen stimulation tests. All three groups of pigs showed a similar response to mitogen PHA, having H^3 thymidine capture values between 11,000 and 25,000 c.p.m. This yielded stimulation indexes between 0.5 and 0.78 with the exception of 1 control pig, which had a SI of 0.89 after 30 days from the first day of the experiment.

A representative result of the average lymphocyte proliferation indexes obtained as a response to PHA is shown in Fig. 1

To analyze the SI an analysis of variance was applied[25] after corroboration that the SI met the criteria necessary to apply such analysis.

No difference among the medians of all three groups was found, $p = 0.78$ for day 0 and $p = 0.77$ for day 30 p.i. (Table 1).

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However, to analyze the obtained data in each group of pigs on day 30, it was necessary to transform the results through square root to meet the normality criterion, which was proven by applying the Shapiro-Wilks test.[25]

When applying the t test for paired data, values of p = 0.02 were obtained for group C, p = 0.0032 for group A and 0.0233 for group B (Table 1).

The differences found were not statistically significant, confirming that revaccination did not significantly affect the mitogenic lymphocyte stimulation in any of the three groups of animals.

Viral antigenic stimulation. There were considerable variations between the SI measured at day 0 and day 30. A variation among the pigs individually was also noted. Oscillation of SI values in the vaccinated pigs had a range of 0.41 to 0.58, in contrast to the controls whose SI was between 0.07 and 0.30.

The pigs immunized with vaccine A as well as those immunized with vaccine B developed a marked lymphoproliferative response when confronted in vitro with the inactivated antigens of ADV at day 30. At that time, the median of incorporation of H^3 thymidine reached 15,000 cpm in the majority of the animals. In contrast, the control pigs did not exceed 7,000 cpm.

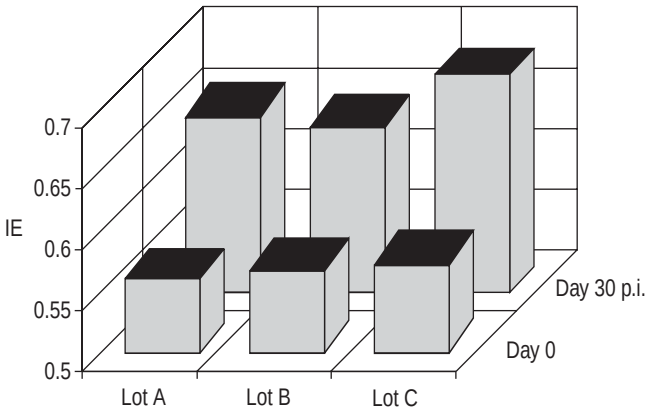

Figure 1. *Median values of lymphocyte stimulation indexes of vaccinated and non-vaccinated Pigs induced* in vitro *with PHA.*

Lymphocyte SI values in response to the antigens are represented in Fig. 2.

Table 2 shows the median values and the standard deviation of the SI before the homologous viral antigens.

When the observed data were statistically analyzed to compare the results between animal groups, it was noted that they did not meet the criteria for an analysis of variance. For this reason, the data were transformed, and even then, they did not meet the Anova's requirements. Therefore, non-parametric tests were applied.[11]

1) The *Kruskall-Wallis* test was applied, showing that there was a significant difference between the control group, group 1 (vaccine A) and group 2 (vaccine B), with a $p < 0.001$.
2) *Dunn's* test was applied to compare the vaccinated groups against the control group. A significant difference was detected between group 1 (vaccine A) and the control group with a probability value of $0.01 < p < 0.05$.

In the same manner, the re was a significant difference between group 2 (vaccine B) and the control group with a probability value of $p < 0.01$.

The data obtained on day 30 with respect to those obtained on day 0 were also analyzed, verifying a significant difference in the SIs of animals in group A and B between 0 and 30 days, with a value of $p < 0.0001$ for both groups. Animals in group C, in contrast, had a difference with $p = 0.04$.

Antigenic cellular stimulation. The average stimulation indexes of the lymphocytes in all pigs by the cellular antigens, measured at day 0 and day 30 of the experience are represented in Fig. 3.

Table 1. Median values and standard deviations of the lymphocyte stimulation indexes before PHA.

PHA	Control Pigs	Pigs immunized with Vaccine A	Pigs immunized with Vaccine B	Observed F	p
Day 0	X = 0.583 SD = 0.065	X = 0.564 SD = 0.048	X = 0.576 SD = 0.056	0.25	0.78
Day 30 p.i.	X = 0.688 SD = 0.122	X = 0.644 SD = 0.093	X = 0.630 SD = 0.096	0.27	0.77
t test day 0 *vs.* day 30 p.i.	t = - 3.36 p = 0.0200	t = - 3.99 p = 0.0032	t = - 2.73 p = 0.0233		

Observed F. = Statistical value of the ANOVA (analysis of variance).
p = level of significance of the test.

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The animals from group C, inoculated with uninfected Vero Cells, developed a marked lymphocyte response to the cellular antigens at day 30 of the experiment, in contrast to groups A and B.

In the majority of the animals in all groups, the H^3 thymidine incorporation median in lymphocyte cultures stimulated with cellular antigens was between 5,000 and 12,000 cpm with the exception of pig A10.

The animals in group A, immunized with the partially purified vaccine did not exceed 9,700 cpm.

Table 3 shows the median values and standard deviations of the lymphocyte stimulation indexes obtained before the cellular antigens.

The comparison of data through the analysis of variance, between the three groups of animals at day 0 showed that there were no significant differences between the obtained results and that the value of p was of 0.11.

In contrast, there were significant differences in the results obtained at day 30 p.i. between the three groups of animals, finding also significant differences between pairs of groups: group A *vs.* group C, group B *vs.* group C with a $p < 0.001$.

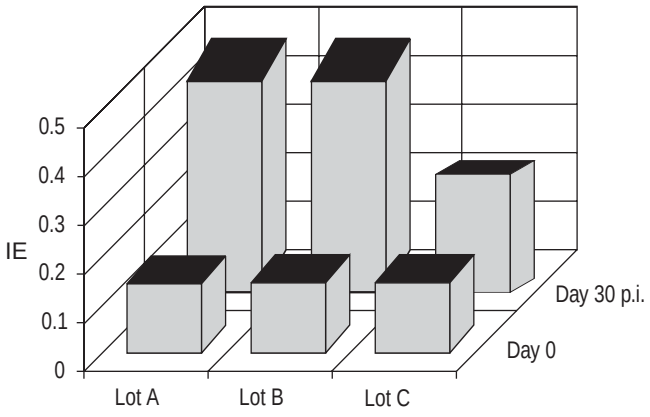

Figure 2. *Lymphocytic transformation index induced by viral antigens in pigs from three lots before and after immunization.*

DISCUSSION

The values of the LBTT indexes with PHA showed that the animals responded in a normal way to this mitogen. A second dose of the vaccine did not exert any influence on the subsequent stimulation of the lymphocytes, since the values at day 0 and day 30 were practically the same, without any significant differences bet (Table 1). The normal response to the mitogen indicated that the animals were acceptable for this study.

Results in table 2 show that animals immunized with vaccine A as well as those immunized with vaccine B developed a lymphocyte response to the virus. This latter turned out to be highly significant since only the immunized pigs increased their LBTT values at day 30 p.i., when the viral antigens were added, with respect to day 0. The cellular antigens contained in the viral cultures produced a light non-specific response since the control pigs had a slight increase of LBTT indexes at day 30 in the blastogenesis induced with the viral antigens. Similar non-specific data have been reported by other authors. *Koszinowski y Bandlow*[12] described a lymphocyte response to the host cells used for propagation of the vaccine virus in guinea pigs. Nevertheless, *J.T. Van Oirschot*[23] demonstrated that, neither vaccinated pigs, nor ADV infected pigs developed a lymphocyte response to the antigens of PK-15 cells, used for viral propagation. Control pigs, inoculated with a suspension of uninfected PK15 cells did not react to the ADV antigens either, indicating the lymphocyte response described by this author turned out to be highly specific to ADV antigens.

Table 2. Median values and standard deviation of the stimulation indexes before the homologous viral antigens.

Viral Ag	Control pigs	Pigs immunized with Vaccine A	Vaccine B	K-W	p
Day 0	X = 0.160	X = 0.148	X = 0.150		
	SD = 0.050	SD = 0.056	SD = 0.050	9.28	0.001
Day 30	X = 0.203	X = 0.440	X = 0.453		
p.i.	SD = 0.078	SD = 0.114	SD = 0.14		
t test					
day 0 *vs.* day 30 p.i.	t = 2.81	t = - 10.34	t = 9.74		
	p = 0.0375	p = 0.0001	p = 0.0001		

K-W = Statistical value of the *Kruskal-Wallis* test
p = level of significance of the test.

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Although our data generate a degree of non-specificity in the immune response, this latter turned out to be non-significant, and it did not impede the specific immune response to develop.

It should be pointed out that a cruda fresh preparation contains other antigens besides the viral suspension, for example cellular surface antigens induced by the virus. These play an important role in the production of CMI, (*Thurman & col*.),[22] (*Koszinowski & Bandlow*).[12] Just as there was a non specific lymphocyte response in the control pigs when confronted with the viral antigens, the same took place in the animals from group B with respect to groups A. Vaccine B, considered impure, has a higher quantity of contaminating cellular antigens. *Chaher & col*.[5] propose that vaccines to inactivated viruses that are available to fight the aphtose fever virus (AFV), give protection only for a few months, and they suggest including immunomodulators (IM) in the vaccine's formulations. Immunomodulators are substances that increase the immune response to the vaccines through specific and non-specific mechanisms. In their work, they evaluate the humoral and cellular immune response generated by inactivated vaccines against VFA that included natural and synthetic IM. These authors conclude that including IM in the vaccines, on the one side, increases significantly the virus' immunogenicity, and on the other, sensitizes the memory cells-which would translate into a fast and efficient secondary immune response. For our study, we suggest that the contaminating cellular antigens could possibly act as non-specific IM.

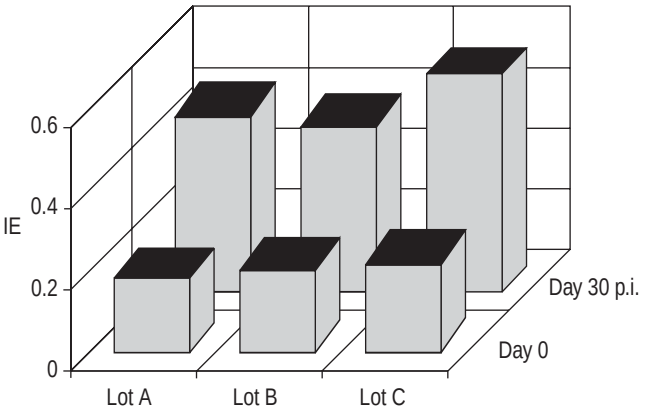

Figure 3. *Lymphocytic transformation index induced by viral antigens in pigs from three lots before and after immunization.*

Generally, the pigs that were immunized with both vaccines, developed a blastogenesis that was similar to the ADV virus, and there was a correlation with the production of neutralizing antibodies. These data agree with *Van Oirschot's*,[23] who showed a simultaneous production of humoral and cellular immune responses. The existence of a continuous lymphocyte migration between peripheral blood and the lymphoid organs, is known (*Ford, W.)*.[7] (*Holt y col*.)[10] suggested that the movement of human lymphocytes that are reactive to specific antigens between peripheral blood and the extra-circulation lymphoid compartments, could be periodical and, at least partially synchronized. A similar migration of lymphocytes that are reactive to ADV could explain the fluctuation in the lymphocyte response observed from certain pigs (A10, B4 B7).

In this assay, the blastogenic reaction of the peripheral blood lymphocytes to the ADV's antigens, indicated that the used immunogens induced the CMI. This was also proven by using the same pigs, which were subjected to the subcutaneous delayed hypersensitivity tests.[16] Since the CMI plays a critical role in the protection mechanisms against infectious agents, we inferred that the immunogens used in this assay could be applied the prevent pseudorabies in Argentina.

Table 3. Median values and standard deviation of the lymphocyte stimulation indexes before the cellular antigens.

Cellular Ag	Control pigs	Pigs immunized with Vaccine A	Vaccine B	F	p
Day 0	X = 0.128 SD = 0.017	X = 0.117 SD = 0.2359	X = 0.142 SD = 0.027	2.68	0.11
Day 30 p.i.	X = 0.525 SD = 0.040	X = 0.173 SD = 0.0320	X = 0.243 SD = 0.034	199.34	0.00001
t test day 0 *vs.* day 30 p.i	t = 18.96 p = 0.0001	t = - 7.34 p = 0.0001	t = 6.23 p = 0.0002		

Observed F. = Statistical value of the ANOVA (analysis of variance).
p = Level of significance of the test.

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