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Stimulation of Phagocytosis and Production of Antibodies against Canine Herpesvirus Type 1 by Pidotimod (Adimod™)

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Abstract

Neutrophils are the most important circulating phagocytes. Circulating monocytes and precursors of tissue macrophages also have the ability to phagocytize. Pidotimod (ADIMOD™) exerts immunostimulatory and immunoregulatory effects through the stimulation and regulation of cellular immune responses by lymphocytes Canine herpesvirus (CHV) mainly affect puppies between the first and second weeks of age, causing high morbidity in the litter. To date, there is only one commercial vaccine in Europe to prevent disease. In this work, inactivated CHV cultures were inoculated in rabbits, adsorbed and not adsorbed to chitosan nanoparticles. Phagocytosis in the presence or absence of specific antibodies was measured. Response of virus neutralizing antibodies was also evaluated. Adimod™ enhanced the nonspecific and specific phagocytotic response. The association of the virus to the nanoparticles increased the phagocytic ability of blood cells; however, Adimod™ alone had a greater effect on phagocytic activity and generated a stronger immune response that corresponded to the increased phagocytosis ($p < 0.05$). Moreover, the level of neutralizing antibodies was higher and increased more rapidly when Adimod™ was used.

Keywords

Phagocytosis, Canine Herpesvirus, Pidotimod Stimulation

1. Introduction

Antibodies are potent determinants of the humoral immune response. Although they are generated through interactions between B and T cells, antibodies trigger their cytotoxic effects by interacting with complement and innate effector cells; thus, they provide a functional link between the adaptive and innate immune systems [1].

Neutrophils are the most important circulating phagocytes as they provide the first line of defense against invading particles, especially bacteria [2]; however, circulating monocytes and precursors of tissue macrophages also have the ability to phagocytize [3].

Pidotimod [(R)-3-(S)-(5-oxo-2-pyrrolidinyl)-carbonyl-thiazolidine-4-carboxylic acid] [4] (ADIMOD™) exerts immunostimulatory and immunoregulatory effects on T lymphocytes, enhances and stimulates the immunological mechanisms involved in the humoral and cellular immunity mediated by T lymphocytes and simultaneously stimulates macrophage migration, which is an essential aspect of the cellular immune response that enables adequate phagocytosis and clearance of infectious microbes [4]. Pidotimod is almost completely absorbed when administered orally, and urine recovery reaches nearly 93% of the unchanged product. Furthermore, it is widely distributed throughout the body and mainly eliminated in the urine and feces, and its bioavailability reaches approximately 45% and is dose-dependent. Pidotimod undergoes only slight hepatic metabolism; therefore, it is excreted unchanged in the urine and no metabolites are known. Its plasma protein binding is very poor, which is advantageous to prevent interactions with other medications. Pidotimod has a half-life of approximately 4 h [5].

The mechanism of action of Pidotimod remains unknown but is considered to occur through the stimulation and regulation of cellular immune responses by lymphocytes, mainly T lymphocytes, with a concurrent stimulatory effect on macrophage migration. It should be noted that macrophages are involved in the cellular immune response; therefore, the display of Pidotimod is important for the mobilization of macrophages to allow them phagocytize and obtain the proper clearance of infectious microbes.

Pidotimod also presents a protective effect; although he did not have direct bactericidal or bacteriostatic activity, it globally stimulates the immune system, primarily macrophages.

To date, the role of Pidotimod in veterinary medicine, especially in small species, is unknown; therefore, the aim of this study was to evaluate its use as a vaccine adjuvant against Canine Herpesvirus (CHV).

2. Methods

Were used sixty rabbits of the New Zealand breed with an approximate weight of 1700 g. The rabbits were obtained from the Animal Isolation Unit (Bioterio) of the Faculty of Higher Studies Cuautitlán, UNAM. Clinical and hematological evaluations were performed to ensure the health of the animals and set the initial parameters. Every procedure was conducted according to the use and care of

experimental animals protocols mentioned in the Official Mexican Norm 062 (NOM062).

The virus was previously isolated from a 3-day-old dead puppy [6]; Canine Herpesvirus (CHV) was replicated in MDCK cells at a concentration of 10^5 viral particles per ml and was inactivated by UV light exposure, as briefly described; the virus culture was placed in a 54 mm diameter glass Petri dish, capless at a distance of 20 cm from a mercury germicidal lamp (Osram HNS 10W), emitting mainly at 254 nm wavelength light. After 30 min an absorbed dose of approximately of 104 J/m^2 (3.6 quantum/nucleotide) occurred, this UV dose has been described to cause inactivation of alphaviruses and herpesvirus.

Chitosan nanoparticles (NANO) were prepared at the UNAM-FESC Biotechnology Laboratory, and the particles presented an approximate molecular size of 1000 μm with a weight of approximately 1.5×10^6 Da [7]. A 1:10 dilution of 10^5/ml inactivated CHV was prepared using a chitosan solution. All preparations were photographed using transmission electron microscopy (TEM).

The experimental design was established as shown in Table 1.

All animals were bled from the jugular vein at 0, 7, 15, 30, 45 and 60 days. A complete blood count, CHV antibody detection in the serum and an *in vitro* phagocytosis test were performed.

An *in vitro* test to evaluate specific and nonspecific phagocytic activity was modified from a previously described protocol [1]. Blood samples were collected in plastic syringes containing 30 U of heparin per ml of blood. A 5% lysing solution was added to the sample, followed by centrifugation at 150 g, at 6˚C, for 8 min to separate the leukocyte-rich plasma. The leukocytes were washed by adding Hank's balanced salt solution (HBSS) and centrifuging at 1118 g for 8 min. The cell pellet was re-suspended in Medium 199 (*In Vitro*™). The cell concentration was adjusted to 2.0×10^6 leukocytes per ml.

The phagocytosis assay was performed as follows: a suspension of peripheral blood leukocytes at a concentration between 10^3 and 10^4 per ml was prepared, and 0.5 ml of the suspension was added into 12-well Nunc™ plates. The chitosan-virus suspension (0.5 ml) was added to six wells in each plate, and 0.5 ml of the chitosan suspension was placed in the other six wells. Every well was filled with 0.5 ml of MEM medium containing 5% fetal bovine serum (*In Vitro*™) and 0.5

Table 1. Experimental design to determine the effect of Pidotimod (Adimod™) on phagocytosis in rabbits.

Treatment	Group 1	Group 2	Group 3	Group 4	Group 5
CHVi 1 ml sc	Yes				Yes
CHVi + NANO 1 ml sc		yes			
NANO 1 ml sc			Yes		
Adimod® oral seven days 2 ml				Yes	yes
Inactivated MDCK cells 1 ml sc			Yes	Yes	

Twelve animals in each group (n = 60) CHVi, canine herpesvirus inactivated; NANO, chitosan nanoparticles; sc, subcutaneous.

ml of serum from each rabbit. The plates were incubated overnight at 37˚C. The content of each well was transferred to an Eppendorf tube and centrifuged at 1118 g for 8 min. at 6˚C, the pellet was washed three times with PBS, and the pellet was re-suspended in 0.5 ml of MEM medium. For leukocyte counting, four smears from each well were fixed with cold acetone for 10 min. Then, 15 μL of fluorescein conjugated anti-CHV (VMRD™) were added. Finally, 100 cells were counted and the number of labeled cells per ml was calculated.

Total amount of serum antibodies against CHV was determined with a previously described seroneutralization test using 10^3 virus particles per well [8] [9]. The sera were inactivated at 57˚C for 30 min, followed by a centrifugation step at 27,000 g for 10 min in a microcentrifuge. Because the virus used has a very low cytolytic effect, we employ a combination of routine test SN and observe cytopathic effect of the virus by the detection of cell infection by an immunoperoxidase technique, using antibodies against canine herpes virus of dog and anti canine IgG subsequently antibodies conjugated to peroxidase (Sigma™) developed by diaminobenzidine (Sigma™) on wells with cells [9]. Neutralizing antibodies were tested in all serum samples. A plaque formation test was used for viral titration: ten-fold dilutions of the virus culture (1:10, 1:100, 1:1000, 1:10,000) were made, and 0.5 ml of each was added to each well of a 12-well NUNC™ plate containing a monolayer of Madin-Darby canine kidney MDCK cells (*In vitro*™, p117) at 90% confluence in minimal essential medium (MEM) (*In vitro*™) with 5% newborn calf serum (NCS) (*In vitro*™). The cells were incubated for 1 h at 35˚C to allow for viral absorption, with mixing every 15 min. Finally, MEM was added slowly with 1% newborn calf serum and 0.6% agarose (Bioline™) at 45˚C to allow for solidification, and then incubated at 35˚C for 3 days. A drop of formaldehyde (JTBaker™) was then added to each well so that the agarose could be subsequently removed. The wells were washed carefully with phosphate buffered saline (PBS) and fixed with acetone/methanol (ICR™) for 10 min. Subsequently, 0.5 ml of a 1:80 dilution of positive anti-CHV-1 control rabbit serum was added to each well and incubated for 30 min at 37˚C. The wells were washed three times with PBS, and anti-rabbit IgG peroxidase (Sigma Aldrich™) was added and incubated for a further 30 min at 37˚C. The plate was washed three times with PBS, and a 0.01% diaminobenzidine solution (Sigma Aldrich™) with hydrogen peroxide (ICR™) was added and held at 15 min at 37˚C The plate was washed again with PBS and stained with Harris hematoxylin (Sigma Aldrich ™) for 15 s, and the plates were examined microscopically for the presence of plaque forming units, termed peroxidase-positive spotlights (PPS), which were counted only in wells containing 20 - 100 plaques.

The neutralization assay was performed by incubating equal amounts of virus (50 PPS) with rabbit sera at different dilutions (1:4, 1:8, 1:16, 1:32, and 1:64; pretreated for 30 min at 56˚C) for 30 min at 37˚C. The mixture (0.5 ml) was inoculated to NUNC™ plates containing a monolayer of MDCK cells at 90% confluence. Viral adsorption was allowed for 1 h at 35˚C, while redistributing the inoculum every 15 min, and MEM supplemented with 1% calf neonate serum and

0.6% agarose at 45˚C was added slowly, allowing for solidification. The plate was incubated at 35˚C for three days, and then a drop of 40% formaldehyde was added to each well and the agarose layer was removed the next day. The titer was calculated as the inverse of the highest dilution in which a reduction of at least 50% of the number of plaques was observed. A serum sample was considered negative if the 1:8 dilution of serum did not show a decrease of 50% or more in plaque formation.

The results were analysed by the Mann-Whitney U-test used Statistic© software The numbers of samples are stated in the figure legends. The level of significance was set at $p < 0.05$. These is nonparametric test for the null hypothesis that there is no difference in phagocytosis between the two experiments. Assuming that we do not know if the experiments behave in a normal distribution, both experiments are independent and determine the difference between the two.

3. Results and Discussion

The average counts of blood leukocytes in the 12 animals were not significantly different ($p > 0.05$) over time (results not shown). Therefore, the doses used for the immunization of the rabbits were low because there were not showed inflammatory effects.

Abundant viral particles were adsorbed to the prepared chitosan particles, as observed on transmission electron microscopy (Figure 1).

In Figure 2, phagocytosis of viral particles in the absence (a) or presence (b) of specific antibodies was observed. Adimod™ enhanced the nonspecific and specific phagocytotic response ($p < 0.05$). In addition to their role in neutralization, antibodies mediate additional functions including the recruitment of innate

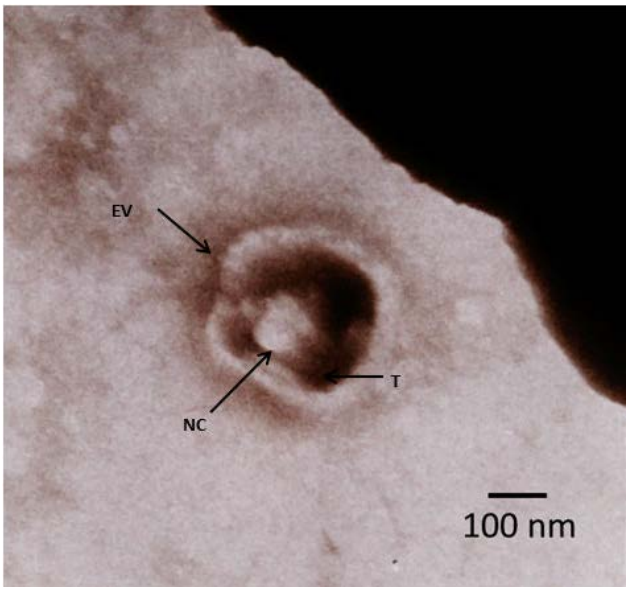
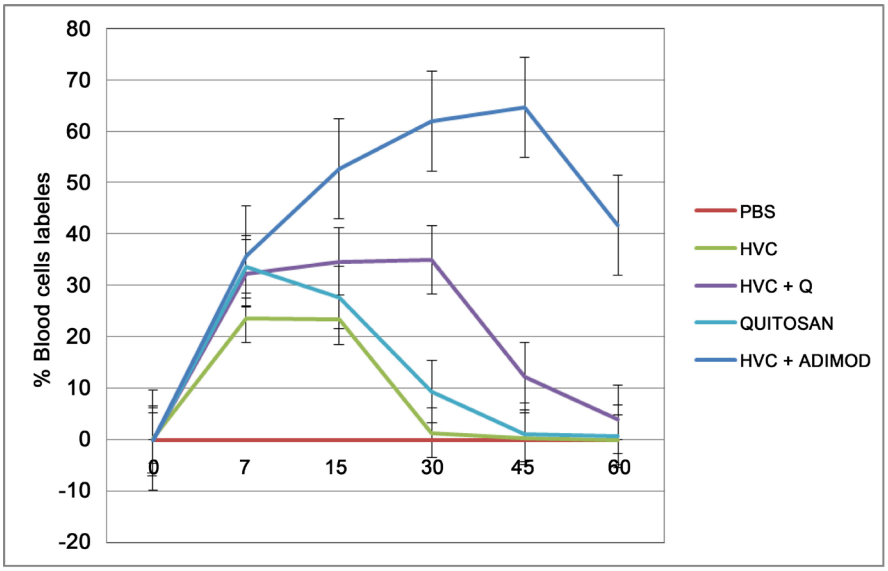
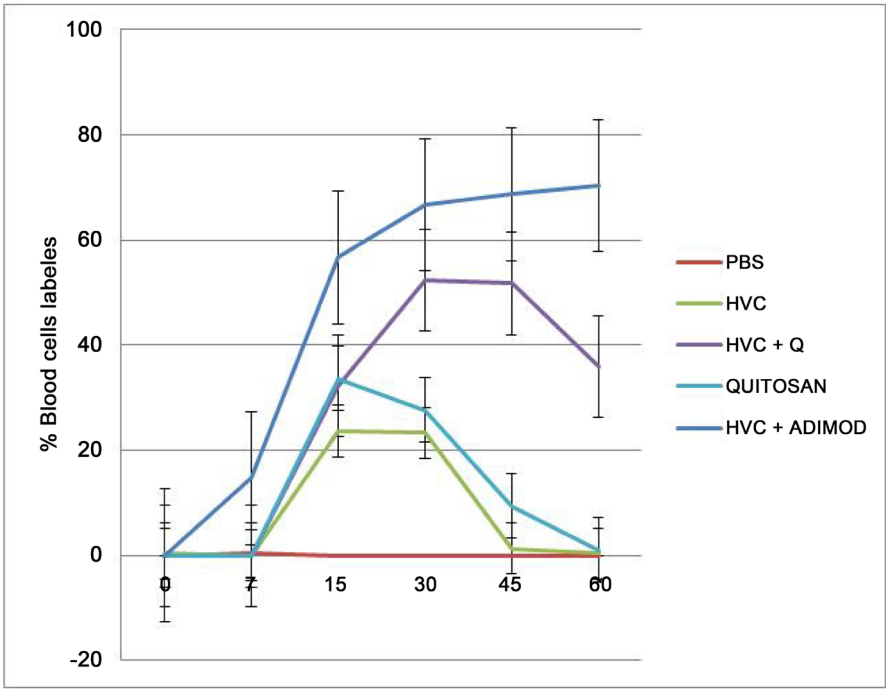


Figure 1. Micrograph taken by transmission electron microscopy showing adsorbed particles of herpesvirus (CHV) with an average diameter of 100 nm for the capsid and an average diameter of 240 nm for the sheath. Nucleocapsid (NC), the tegument (T) and the viral envelope (EV). Magnification: 20,000×.



(a)



(b)

Figure 2. (a) Results indicate the average value for 12 rabbits per treatment. Chitosan particles adsorbed with inactivated canine herpesvirus (CHV) were placed in contact with ((a) blood leukocytes and evaluated by fluorescent antibodies against CHV. (b) the serum of rabbits and with leukocyte blood, and after incubation, they were evaluated using fluorescent antibodies against CHV).

immune responses to eliminate antibody-opsonized material. Furthermore, antibodies promote phagocytosis, which may play an important role in the rapid containment and clearance of a pathogen following infection. However, robust assays that are able to capture differences in the quality of antibody-mediated phagocytosis are lacking [1]. Thus, in this study we developed a novel high-throughput assay by using monocytic cell blood to provide a platform to eva-

luate antigen-specific antibody-mediated phagocytosis. Briefly, the antibodies of interest are captured on the surface of chitosan beads (NANO) coated with highly fluorescent spiked viral particles, and the beads are then incubated overnight with monocytes prior to analysis by immunofluorescence. Because the beads used may be coated with an antigen of choice, this assay allows the evaluation of antigen-specific phagocytosis without requiring purification of the respective antibodies.

We observed variation in the phagocytosis tests; however, comparison of data from microbiological *in vitro* studies on the phagocytosis and killing of *C. albicans* blastoconidia by phagocytes is hampered by divergent methodologies. The results of some studies are equivocal, and the reported differences may result from the various assay conditions and methods that have been used to determine phagocyte candidacidal activity [10]. However, to date, minor variations have been observed in other studies of the phagocytic ability of leukocytes.

We were not able to determine whether phagocytosis was performed by blood neutrophils or monocytes because both of these cell types have blood phagocytosis capability after opsonizing [11]; however, all rabbits in this study showed an average cell content of 73% neutrophils and 4.5% monocytes; therefore, most of the fluorescent cells observed corresponded to neutrophils.

In the present experiments, the association of the virus to the nanoparticles increased the phagocytic ability of blood cells; however, Adimod™ alone had a high effect on phagocytic activity. Pidotimod was also observed to generate a stronger immune response that corresponded to the increased phagocytosis ($p < 0.05$).

In Figure 3, the results of seroneutralization tests are shown for each group, and the mean of the inverse of the highest dilution that provided 50% protection is plotted. Our results confirm that Adimod™ enables a faster immune response ($p < 0.05$) than chitosan's response after 45 days, which has been observed to be

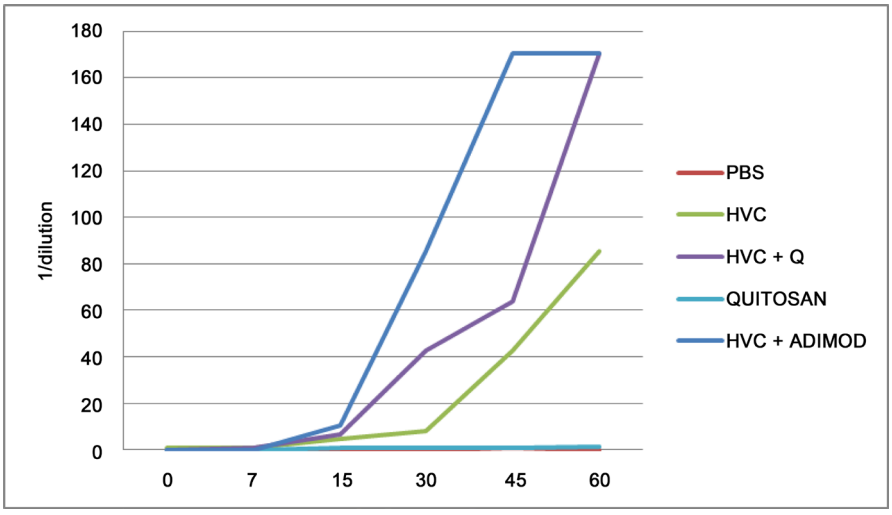


Figure 3. The results refer to 12 rabbits per treatment; the seroneutralization test was performed in triplicate using 103 viral particles per well, and the average of the inverse of the dilution that caused 50% lysis was plotted.

as immunogenic as a vaccine adjuvant [12] [13].

Pidotimod [(R)-3-(S)-(5-oxo-2-pyrrolidinyl)-carbonyl-thiazolidine-4-carboxylic acid] has been shown to significantly increase the survival time after challenge with low doses of herpes simplex virus and influenza virus [4] Pidotimod was also shown to induce dendritic cell (DC) maturation and up-regulate MHC class II cell surface receptor (HLA-DR) and the co-stimulatory molecules CD83 and CD86, which are essential for communication with adaptive immune cells, and it was demonstrated that Pidotimod promotes strong and specific humoral and cellular immune responses *in vivo* when co-administered intranasally with an antigen model; it was also shown that administration by oral route increases resistance to viral infections [5] [14] [15] [16].

In this experimental design, we did not determine the combined effect of chitosan and Adimod™ because the purpose was to demonstrate Adimod's effect, but it is inferred that the immune response, using Adimod and nanoparticles combined, would possibly be higher and faster.

Studies developed to compare the intestinal absorption of various drugs in humans and experimental animals (e.g., rats and rabbits) have concluded that these animals are a good alternative model for preclinical studies for absorption in humans [17].

Vaccination of pregnant bitches has been effectively used in other countries to lessen the impacts of canine herpes virus infection in puppies; however, to date, no data is available for the use of vaccination against canine herpesvirus to protect an open population. Although it is true that protection against viral diseases is more efficient when mediated by cellular immunity, the development of higher amounts of serum antibodies in animals can help to eliminate viral reactivation after chronic infections or latency observed in herpes viruses; thereby decreasing the possibility of childbirth transmittance [18].

Evaluation of chemotaxis, phagocytosis and microbicidal killing may enable the differential diagnosis of immunodeficiency-related diseases as herpes virus infections observed in dogs and other species [11]. The methodology developed in our study may be useful to evaluate the effects of immunosuppressive diseases such as herpes virus and also to evaluate the effectiveness of immunomodulators and adjuvants.

The level of neutralizing antibodies was higher and increased more rapidly when Adimod™ was used (**Figure 2**).

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