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Hydroxylapatite (HA) Powder for Autovaccination Against Canine Non Hodgkin's Lymphoma

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1. Introduction

Lymphoma is the most common neoplasm of the canine hemolymphatic system. It represents about 15% of all malignant neoplasms. It has a very poor prognosis, the mean survival with high grade non Hodgkin's lymphomas being two months without treatment [1]. Incidence is increasing.

Lymphomas in dogs, as in humans, can be divided into numerous types depending on the cell line involved and their immunophenotype [2]. T lymphomas have a worse prognosis than B lymphomas and late clinical stages obviously have a very short survival period.

Lymphomas are known in human medicine to respond to chemotherapy, and some of them can even be cured by complex chemotherapy protocols, although severe side effects are noted.

Chemotherapy protocols have also been developed for dogs in the last 40 years. They have proved to be effective for the overall survival of the treated animal. Although the order of drug administration and duration of the maintenance part of the protocol vary considerably, most oncologists agree that a doxorubicin-based (eg CHOP) combination chemotherapy protocol provides the longest period of disease control and overall survival [3].

Even with chemotherapy, survival is relatively short and adjuvant therapies have been developed to improve prognosis. Immunotherapy protocols are of particular interest for this purpose as they may arm immune system cells against the abnormal proteins synthesized by cancer cells [4]. It is a selective way of destroying cancer cells and a treatment with much fewer side effects than chemo- or radiotherapy.

The immune system can destroy cancer cells by different methods:

- Synthesis of TNF- α (tumor necrosis factor alpha) and an oxygen intermediate such as nitric oxide by macrophages activated by IFN- γ (interferon gamma)
- Activation of NK (natural killer) cells by IL-2 (interleukin 2)
- Adsorption of an antibody against tumor cell antigens targeting macrophages and cytotoxic T lymphocytes.
- Activation of CD8+ T lymphocytes by an MHC(major histocompatibility complex) - mediated cell contact mechanism between antigen-presenting cells (APCs) and CD8 cells

Several kinds of immunotherapy protocol are available both in human and veterinary medicine [4, 5, 6, 7]. Heat shock proteins (HSPs) such as gp96 or HSPs70 which are synthesized by the cells submitted to stress are advantageous vaccination adjuvants due to their chaperone properties and their role in antigen presentation [8]. As chaperone molecules, almost all the cell peptides are associated with these proteins and HSP purification provides a fingerprint of the cell's protein synthesis [9]. This is particularly useful for cancer cells which synthesize numerous abnormal proteins during their natural progress [10]. These cells being genetically unstable, their abnormal protein synthesis differs from patient to patient and during the course of the disease. The HSPs and their associated peptides (AAPs) have special receptors (CD91) on dendritic cells which allow the internalization of the AAPs and their modification in order to be expressed at the surface of class I HLA proteins on the cell membrane, triggering activation of the CD8 T cells if they are abnormal [11].

Cancer cells are stressed by the mechanical and metabolic characteristics of the tumour. They synthesize many HSPs [12]. We thus isolated these proteins in order to make an autologous vaccine against the tumour. The HSPs were purified using a hydroxylapatite powder (HA) column. The powder carrying the HSPs was then injected subcutaneously to stimulate the immune system's response to the tumour.

Purification of HSPs using classical way is long and tedious. The use of hydroxylapatite powder allows a much faster purification process. Hydroxylapatite chromatography has been described by Tiselius in the early seventies. Hydroxylapatite chromatography is an adsorption chromatography. The adsorption mechanism is very poorly understood. The surface of the material is occupied by Ca^{++} and PO_4^- . These ions are supposed to interact with the chemical groups of the proteins. However, post synthesis treatment such as sintering or spray-drying process modify the physico-chemical properties of the material surface. Furthermore, the interaction of the material surface with biological fluids triggers epitaxial growth of carbonated apatite at the surface of the material [13].

It was decided that the proteins purified will be injected carried by the particles for several reasons: the hydroxylapatite particles have been described as vaccine adjuvant, they are phagocytosed by the APCs and can deliver the proteins directly in the APCs, they trigger an afflux of APCs at the injection site [14]. Most of the adjuvants used in anti-infectious vaccines are nano or microparticulate.

The aim of this study was to check the feasibility of this protocol using HA-particles with dogs suffering of high grade lymphoma and to know if secondary effects were detected.

2. Materials and methods

2.1. Dogs to be treated

Two dogs (Poodle, 6 years, Jagdterrier, 8 years) suffered from polyadenopathy without any sign of immune deficiency. One dog (mixed breed, 10 years) had a liver metastasis. The last one had a cutaneous form. Their general condition remained reasonable, with no real weight loss or fever. One dog (Poodle) had a splenomegaly. The blood numeration revealed that the white cells and calcaemia for both dogs were in the normal range. Node biopsies were performed. One was sent to the pathologist and the other was frozen (-20°C) and used to manufacture the vaccine. Disease staging was performed using the WHO-staging criteria for canine lymphoma.

A chemotherapy treatment was proposed by the physician but rejected by the dog's owners due to side effects and an informed consent was signed.

After vaccination, the dogs were subjected to a clinical examination whenever they came for the following injection and side effects noted. The volume of a control node was externally measured.

2.2. Toxicity and response assessment

The side effects were graded every week for the first month and every month for the next five months according to the National Cancer Institute's common Toxicity Criteria (version 2.0).

The dogs were also submitted to a physical examination at the same frequency to assess their clinical response. A complete response (CR) was defined as the disappearance of all the nodes and metastasis for at least 4 weeks. A partial response (PR) was defined as a decrease by at least 50% of the product of the 2 longest lengths of all the nodes without the appearance of new lesions for at least 4 weeks. A minor response (MR) was defined as a decrease by less than 50% using the same criteria. Stable disease (SD) was defined as the same criteria unchanged for at least 4 weeks. Progressive disease (PD) was defined as an increase by >25% of the product of the 2 longest lengths of all the nodes for at least 4 weeks or when new lesions appeared.

2.3. Hydroxylapatite powder characteristics

Hydroxylapatite powders (Fig. 1) can be used for adsorption chromatography under atmospheric or high pressure. In order that the protein solution did not fill in the column and to avoid the compaction, the powder was spray-dried then sintered at 1000°C.

The HA was transformed into a ceramic powder according to the following protocol. The synthesized calcium phosphate was suspended in a slurry which is liquid, spray-dried, then sintered. The spray-dried material was heated almost to fusion temperature which favors the migration of matter between the grains and the formation of bridges. As the surface energy was smaller for large than for small grains, their size increased and the distance between the grain centers and the particle surface area decreased.

Powder characteristics	
Nature of the charged groups	PO_4^{3-} , OH^- , Ca^{2+}
Electrocinetical potential (mV)	-35
Hydrophobicity	+
Surface pH	7,8
Granulometry (μm)	0-25
Surface area (m^2/gr)	4
Shape	spherical

Table 1. Characteristics of the powder

The sintering considerably reduced the surface area making the amount of proteins adsorbed on the powder lower. It also stabilized the powder structure. The powder was submitted to dissolution/ precipitation processes when soaked inside a saline solution [13]. The modification occurring at the powder surface affected the adsorption properties of the powder. The reduced surface area decreased the interactions with the saline solution containing the proteins. There was a microporosity between the ceramic grains inside the same particle allowing the protein solution to diffuse inside the particles. The characteristics of the powder used in this experiment was given in table 1

As the chromatography was carried out under atmosphere, the granulometry of the powder was an important factor in order to avoid any plugging of the column. The HSPs could be eluted from the powder by 200-300 mM NaCl solutions. The powder solution in NaCl was not stable enough to be injected as it decanted too fast in the syringe. Thus to improve the injectability the powder was put in suspension in a 2% solution of carboxymethylcellulose in 20 mM NaCl.

2.4. Vaccine manufacturing protocol

The tumor tissue and all the materials used to prepare the vaccine were handled in sterile conditions under a laminar flow. The frozen tumor (200 mgr) tissue was homogenized using a bead tissue homogenizer. 1 ml of NaHCO_3 (30 mM, pH 7) was added for 1 ml of homogenate. The resulting homogenate was then centrifuged at 1000 g for 15 mn at 4°C to remove all cell fragments.

The supernatant containing the cytoplasmic proteins was used for protein purification by HA column chromatography as follows: a) two precipitations with ammonium sulphate (first at 50% and then at 70%) recovering the pellets. The last pellet was resuspended in 1 ml phosphate buffer (20 mM, pH 7). The column was filled (chromatography columns, Poly-prep, Cat. 731-1550, Bio Rad) with 0.2 gr of HA (0-25 μm), equilibrated with 10 volumes of phosphate buffer (20 mM pH7). The resuspended pellet was then added. The column was then washed with 3 ml of a 100 mM NaCl solution (fig.2).

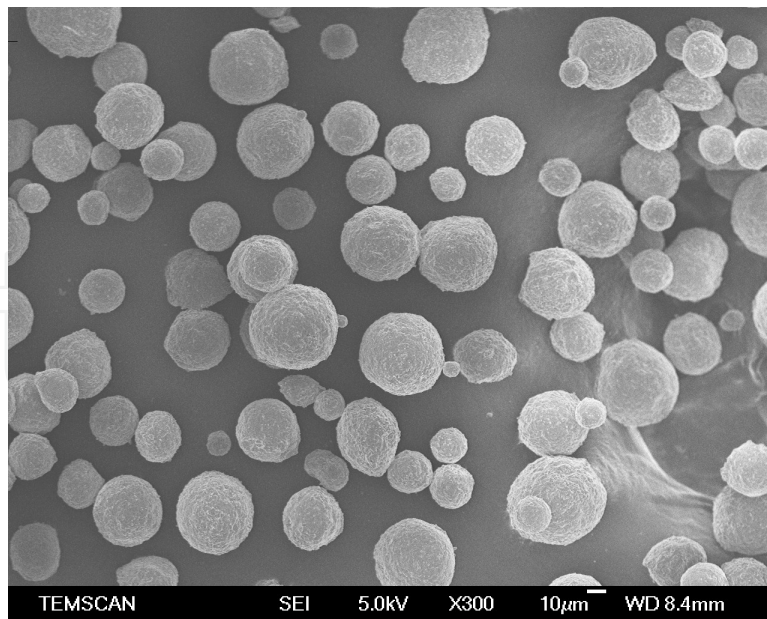


Figure 1. SEM of the HA-powder used for the vaccine.

The powder was then suspended in 5 ml carboxymethylcellulose (CMC) solution (2% in 20mM NaCl). 0.5 ml of this solution was used for each vaccine shot.

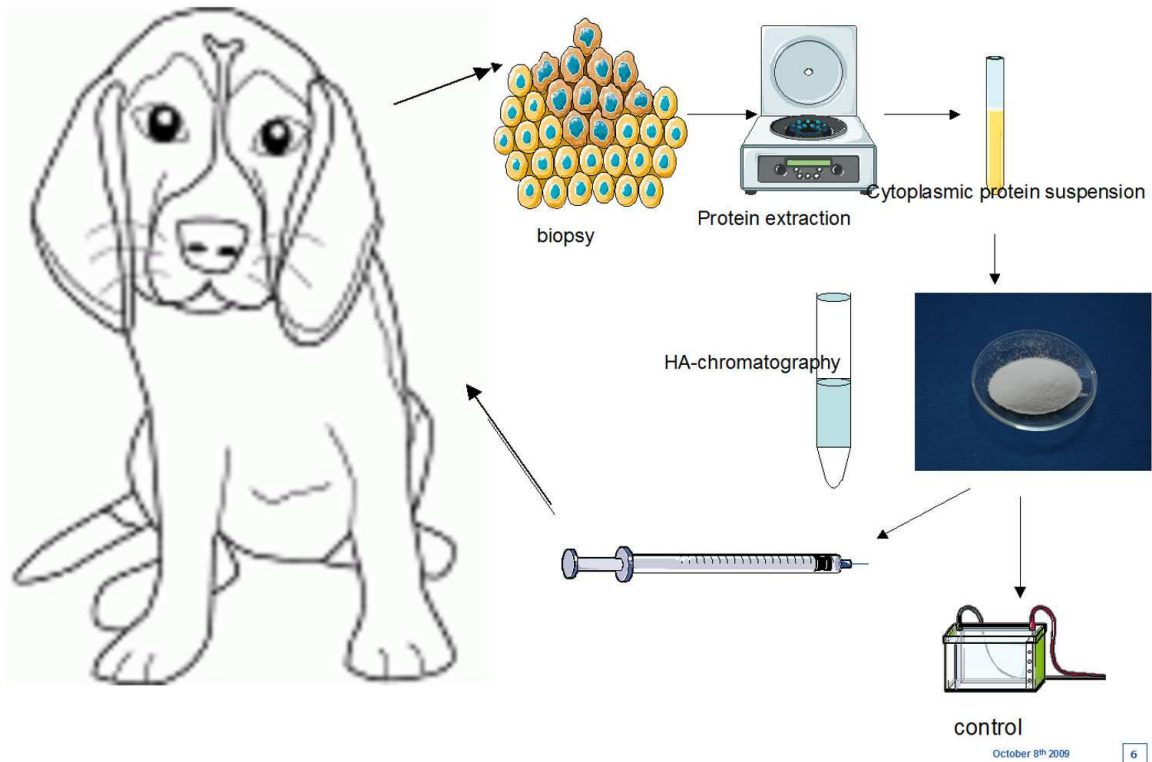


Figure 2. Autovaccine manufacturing scheme

0.2 ml of the previous solution was used to make electrophoretic control. The solution was centrifuged at 1000g 30 for seconds. The supernatant was discarded and the powder in the pellet washed with 0.1 ml of a 0.5 M NaCl solution. The solution was again centrifuged and the supernatant was used for a SDS-Page and for protein quantification using UV spectrometer. 10 µl of the solution was also used for dot blot with anti HSP70 and anti gp90 antibodies on a nitrocellulose membrane. The antibody labelling was evidenced using a westernbreeze™ (invitrogen) kit according to the manufacturer instructions.

2.5. Vaccination protocol

The dogs were injected with 0.5 ml (about 40 µg of proteins) of the vaccine subcutaneously on day 0. The dose schema consisted of 0.5 ml/dose every week for one month, followed by one dose every month for four months.

2.6. activation of TLR2 and 4

Some HSPs are TLR (Toll like receptor) agonist and the TLR activation was checked using two cell lines secreting an embryonic phosphatase alkaline when the TLR 2 or 4 are stimulated.

Two cell lines were used for TLR receptor stimulation. HEK-Blue™-hTLR2 and HEK-Blue™-hTLR4 cells (invivogen –France) were grown in DMEM supplemented with 10% fetal calf serum and 0,5% normocin (invivogen-France) to avoid mycoplasma contamination. 20000 cells were introduced in wells of 96-well plate, 4 hours later 20 µl of different concentrations of the vaccine was added and incubated at 37°C for 20 hours. Then the medium was removed and replaced by 180 µl of resuspended QUANTI- blue™ (Invivogen) and incubated for 1 hour and the positivity evaluated. The negative control was the HA- powder without any protein immobilized on its surface. The minimal concentration turning blue the medium was noted. It was estimated that the vaccine was functional when the minimal active concentration was less than the vaccine concentration.

3. Results

All the vaccine dilutions stimulated the TLR 4 but not the TLR2. The negative control (HA powder) did not stimulate any TLR. The SDS PAGE revealed two bands in the 95 kDa region which were previously demonstrated to be HSPs rich bands (fig. 3) The dot blots revealed that all the vaccine contained gp96 and HSP70 but the amount was different (fig.3 and 4).

Neither dog showed side effects after the injections, whether systemic or local. Two showed a decrease in nodule volume of less than 50% following the first month of injections and were rated MR. The other were rated SD. All dogs were rated PD about one month before they died.

No sign of infection such as fever was observed during the first month of vaccination. The Jagdterrier was diagnosed with an abscess of the collar by the fourth month, which was cured surgically. The lung radiographs did not reveal any lung metastases. The blood count did not show any anomaly in the white cell count up to the last month of survival.

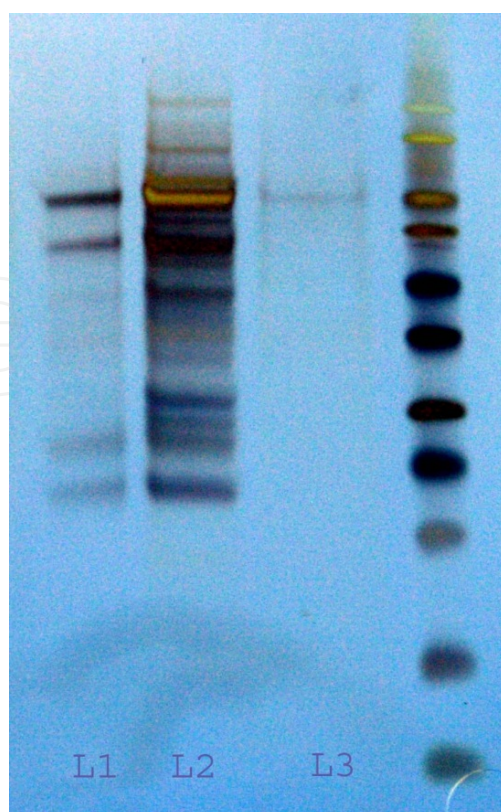


Figure 3. L1 was obtained after washing the powder with 0.3M NaCl, L2 was obtained after washing at 0.02M, L3 was obtained with 0.5M. At 0.02M almost all the contaminant proteins are eluted from the powder. At 0.3 M, the remaining proteins after the 0.02M elution are located in the 95 and 70 kDa zone. The 0.5 M column shows that after after 0.3 M there is almost no proteins remaining on the powder.

The poodle was euthanized 11 months, and the Jagdterrier 6 months, after the disease was discovered. During their survival period, both dogs had a normal activity. The Jagdterrier was still hunting two weeks before to be euthanized. The two other dogs were euthanized at 155 and 173 days (table 2). The mean overall survival time is 210 days.

Dog number	Breed	Histology	Stage	OS	Immunophenotype
1	Jagd terrier	Centroblastic polymorphic	IIIb	330	B cell
2	Poodle	Centroblastic polymorphic	IIIb	180	B cell
3	mixed	Centroblastic polymorphic	IVb	173	B cell
4	cocker	cutaneous	IVb	155	T cell

Table 2. Characteristics of the dogs treated by the vaccination protocol and overall survival time (OS) in days.

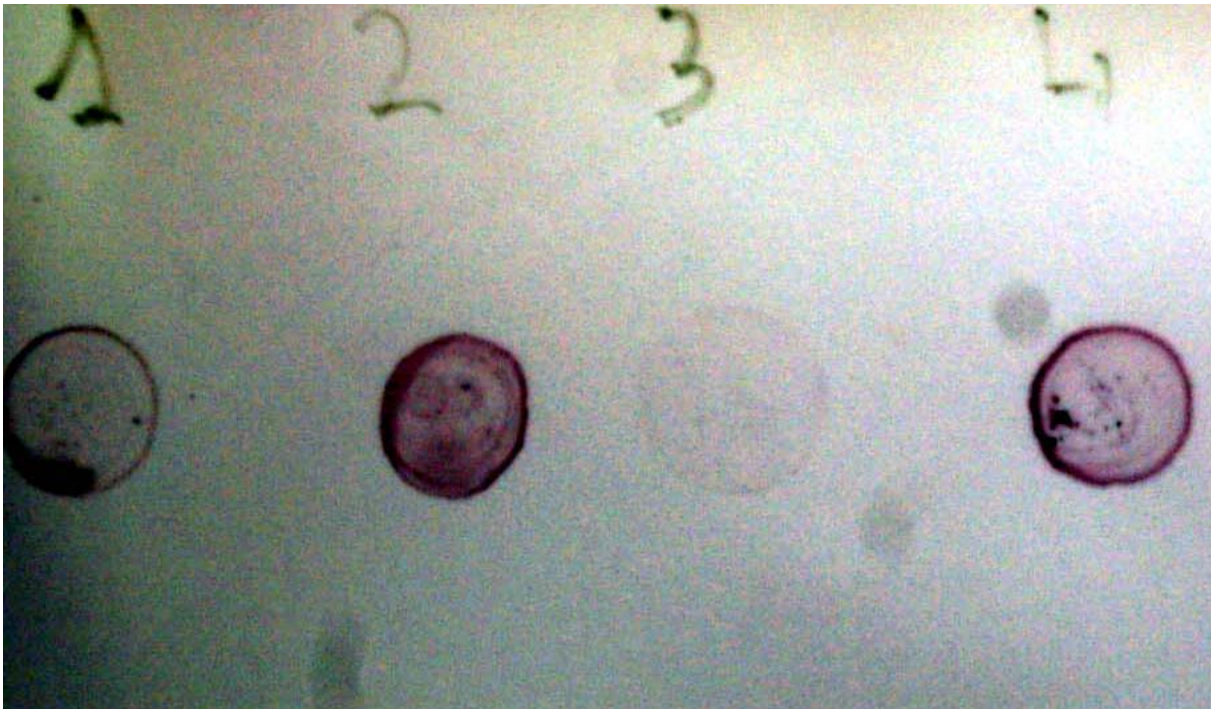


Figure 4. Dot blot after anti gp90 labelling

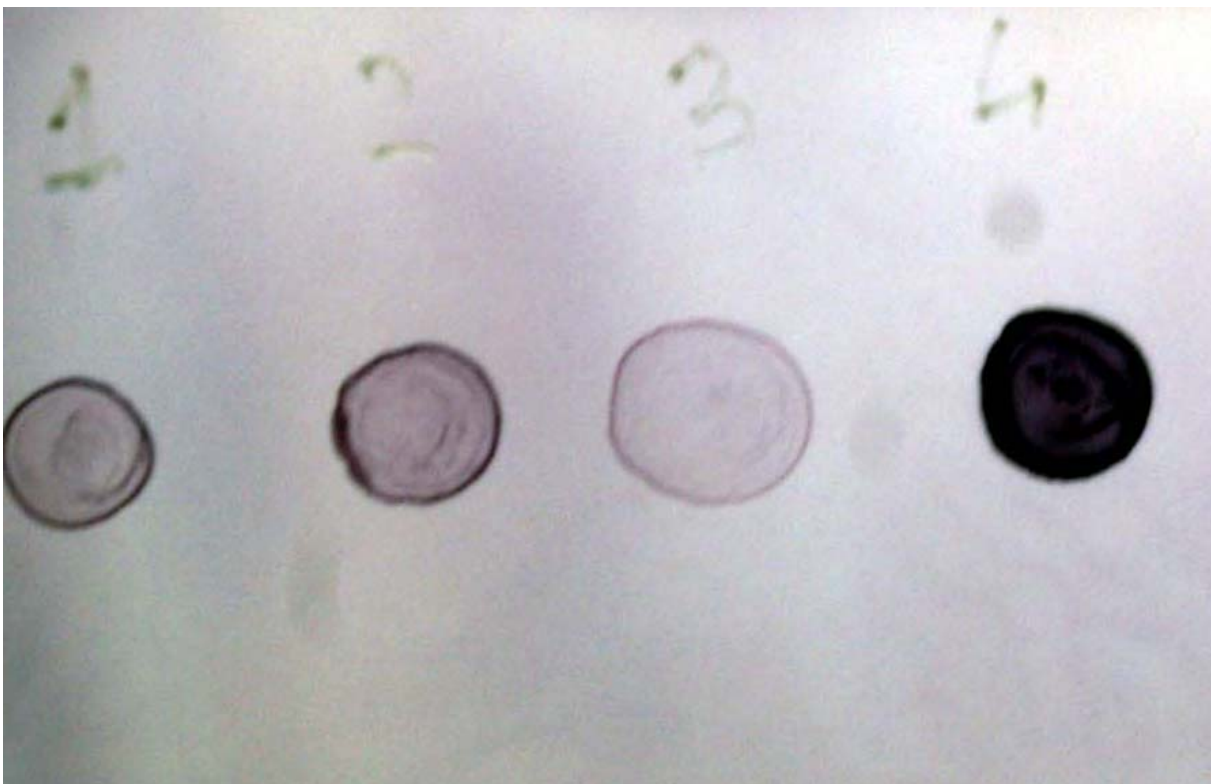


Figure 5. Dot blot after anti HSP70 labelling

4. Discussion

This experiment showed the feasibility of this protocol as an autologous vaccine for cancer in dogs in veterinary practice. We have also developed an *in vitro* test to assess the functionality of purified heat shock proteins. This test checks in a few minutes the vaccine's ability to stimulate antigen-presenting cells (APCs) through toll-like receptor (TLR) activation. Even if the sample size is small, the dogs' overall survival (210 days) was much higher than expected, as the average OS for this type of pathology is two months. In larger series of stage Vb lymphomas associating chemotherapy and the same vaccine protocol, we could demonstrate that after vaccination the dogs showed a delayed cutaneous hypersensitivity after their own tumor antigen injection in the derm (Marconato, unpublished results).

High grade lymphomas in dogs are an interesting model for cancer vaccines because survival is very short, so the effect on OS is very easy to measure. The clinical efficiency of vaccine is not always related to the vaccine's effect on biological parameters. Models with a short life expectancy are thus of interest. Furthermore vaccination without the use of other drugs is of particular interest.

The amount of proteins used in this vaccine was justified by the different experiments published about autovaccines using HSPs in animals and humans. Furthermore we checked that the amount of proteins could activate 20000 APCs *in vitro*. The presence of the tumor proteins at the surface of the HA-particles allows the activation of the TLRs. It indicates that the proteins are immobilized at the surface of the particles and their adsorption does not denaturize these proteins.

Cancer vaccines are the focus of great interest. Although cancer cells synthesise abnormal proteins, they are not recognised by the immune system. Different immunosuppression mechanisms by cancerous tumours have been described [15]. The use of multiantigen vaccines could reduce the cancer cell's "invisibility" to the immune system compared to monoproteic vaccines. Different kinds of immune therapy are under investigation. Cell engineering immunotherapy protocols have been tested, including activation of dendritic cells *in vitro* by tumour antigens before being reinjected into the patient [16]. Other trials concern amplification of the intratumoural lymphocytes (TILs) *in vitro* [17] before being reinjected into the donor. Recently, different types of antibodies were approved for the oncology field, in particular antibodies against VEGF (Vascular endothelial growth factor) to inhibit tumour vascularization [18, 19].

Heat Shock Proteins (HSPs) have proved to be of therapeutic interest in human medicine for some applications. In order to be useful as a cancer vaccination, these HSPs must be made available to APCs. HA-powder is a good material for use as a vector of HSPs to APCs. It has been shown that when injected in the dermis or subcutaneous tissue, it triggers a foreign body reaction and that the cells of this foreign body reaction could be transfected with a DNA molecule carried by the particles [20]. It suggests that, further to DNA vectorisation, the particles could help in the transfection of HSPs and their associated peptides in APCs.

Although gp96 has been described as able to stimulate TLR4, it is not sure that these proteins are the only proteins responsible for TLRs stimulation in this case. There are contaminating proteins in various bands of the SDS which could interfere with the TLRs. The non stimulation of the TLR2 indicates that the TLR4 is not activated by contaminating endotoxins.

Hydroxyapatite has been used as an adjuvant for various infectious vaccines such as diphtheria and tetanus [21]. The Hydroxyapatite lattice is a hexagonal structure which allows numerous substitutions. Ions and small amino acids can thus be trapped in the HA lattice. Consequently, HA powder has been used to purify DNA, proteins or even viruses from biological solutions [22]. In this case, the surface properties of the particles allowed both the purification of HSPs and their use as a vector to APCs and as an adjuvant.

The HA-powder characteristics seems well suited for its role of cancer vaccine adjuvant. The granulometry range allows the phagocytosis by the APCs as it was demonstrated previously [14]. Furthermore, the grain boundaries in each particle is degraded by the cells making the particles fragmented and thus decreasing the real granulometry a few days after injection. It was also demonstrated that the phagocytosis of these particles by the APCs induced the synthesis of various cytokines and lymphokines necessary for the cross-priming of the CD8. The HA-adjuvant effect does not seem to be due to TLR activation as HA-particles alone do not trigger TLR2 and 4 activation. Other mineral adjuvants such as aluminum oxide have been demonstrated to activate the inflammasome. The inflammasome is a multi-protein complex involved in the production of mature IL-1 β . The alumina-induced release of IL-1 β in macrophages is done under activation of the NLRP3 [23]. It is suggested from unpublished results that the adjuvancy effect of the HA-particles is also due to the inflammasome activation.

Tumor associated antigens show a very poor antigenicity. Thus the presence of an adjuvant like calcium phosphate particles is essential in order to increase the visibility of these antigens by the patient immune system.

This method was used in a short pilot study in humans and proved to be very safe, as only local effects (erythema) were reported in some patients (24). Although it was not the goal of the pilot study, some remission in the extent of the tumour was observed and constituted a good proof of concept. Gp96 has already been used in human medicine to treat a series of patients with indolent non Hodgkin's lymphoma [23]. The results are difficult to compare, as the patients could have been treated by chemo- or radiotherapy more than six weeks before the vaccination protocol. However, at three months most of the patients were rated SD, including those who were resistant to previous therapy. No patients suffered side effects.

5. Conclusions

The HA-particles are essential in this protocol both for their adsorption properties for proteins of interest and their adjuvant properties. They constitute a tool which allows to purify tumor associated antigens in a fast and reproducible way.

The overall survival of the four dogs was much better than expected. These preliminary results nonetheless show that the technique is feasible in private veterinary medicine. They are consistent with other results obtained with canine osteosarcomas or with a series of stage Vb lymphomas treated by chemotherapy and this protocol.

The association of the HA-particles to tumor associated antigens and HSPs did not trigger any adverse effects, confirming the safe results obtained for other applications in human medicine.

This technique could also be combined with conventional chemo- or radiotherapy to increase the animal's overall survival.

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