IN-LIFE PHASE REPORT

Sponsor

URODELIA
Route de St Thomas
31470 St Lys, France

Study title

Vaccination against ectopic gliomas in rats using autologous HSPs. Results and tolerance

Study identifications

DETERCA Ref:

Author

Pr Jean Charles Le Huec

Study Completion Date

7 Avril 2008

Test site

DETERCA
University Bordeaux II

STUDY INFORMATION

SPONSOR	Urodelia Le Gaillard Route de St Thomas 31470 St Lys France
Sponsor Representative/Monitor	Patrick Frayssinet Tel.: +33 (0)5 34 47 86 10 Fax: +33 (0)5 34 47 71 45 E mail: patrick.frayssinet@wanadoo.fr

TEST FACILITY	DETERCA Université Victor Segalen Bordeaux II 146 rue Léo Saignat 33076 Bordeaux cedex, France
Study Director	Pr Jean Charles Le Huec Tel.: +33 (0)5 57 57 17 19 Fax: +33 (0)5 56 96 26 14 E mail: j-c.lehuec@u-bordeaux2.fr
Deputy Study Director	
Test Facility Management	Pr Jean Charles Le Huec Tel.: +33 (0)5 57 57 17 19 Fax: +33 (0)5 56 96 26 14 E mail: j-c.lehuec@u-bordeaux2.fr

TIME SCHEDULE	
Study initiation date	15 dec 2007
In-Life phase Start date	16 Jan 2008
In-Life phase End date	01 Apr 2008
Study completion date	15 Apr 2008

AUTHENTICATION

We, the undersigned, were responsible for the conduct of the work and reporting of the results; we agree with the views expressed in the Discussion.

STUDY DIRECTOR

Jean Charles Le Huec
DETERCA

Date

SPONSOR REPRESENTATIVE

Patrick Frayssinet
Urodelia

Date

PERSONNEL involved in the study

In-life Units

Jean Patrick Chesnus
Technician, In-life Units

Julie Petit
Technician, In-life Units

Histology Laboratory:

Patrick Frayssinet
MD, PhD

1. SUMMARY :

Heat Shock Proteins are used as therapeutic vaccine for some cancerous tumors. Their purification allows to get a fingerprint of the cancer cell synthesis in particular the abnormal peptides potentially recognized by the patient immune system. These proteins are purified from tumors using hydroxyapatite particles which are then injected subcutaneously into the patient. The particles are used for purification, as carrier and adjuvant. There are numerous publications reporting the safety of vaccine using HSPs in humans and there are some indications that the composite HA/HSPs could have an effect on some type of tumors.

In order to test the efficacy and tolerance of this kind of vaccine against high grade glioma, in particular regarding autoimmune myelo- encephalitis, we have injected in the left flank of CD344 Fischer rats syngenic 9L gliosarcoma. The vaccine were obtained from the cells grown in monolayer. The vaccine consisted in one shot (5mg) every week for one month before the injection of the glioma cells. Four groups were created, the control only receiving the cells, a group vaccinated with the HA particles associated to the HSPs, a group vaccinated with HA-particles loaded with the HSPs and cancerous cell membranes, another group injected with the HA-particles alone. The animals were euthanasied at 21, and 35 days after the cell injection.

The daily observation of the rats did not show any sign of secondary effects, and the activity of the vaccinated rats was not different from the control. At the autopsy, the brains and medullas of the vaccinated rats were not macroscopically different from the control and the histology did not evidence any inflammatory process.

The cancerous cells induced tumors in the flanks and the vaccination significantly slowed down the tumor growth.

2. CONTENTS

3. INTRODUCTION

Human subjects are able to mount a T cell mediated response to cancer based on their recognition of epitopes from mutated proteins, the products of overexpressed oncoproteins or ectopically expressed developmental genes.

The antigen presenting cells (APCs) originate from the monocyte cells. They are professional phagocytes and can be macrophages or dendritic cells. When recognized, the tumor antigens are internalized by the APCs and then presented at the cell surface to the cells of the immune system.

The heat shock proteins (HSPs) are a class of proteins synthesized when the cells are submitted to a stress and in particular to heat. Due to cell stress existing in the tumors for the reason of the poor vasculature and metabolism anomalies, the cancer cells generally oversynthesize HSPs. They stabilize other cell protein folding and thus are intimately associated to these late proteins. They also have other functions. In particular, some of them such as gp96 or HSP 70 are involved in the process of T-cell cross-priming by antigen presenting cells.

As the HSPs are associated to many peptides synthesized by the cell, the purification of these proteins produced by the cancer cells allows to get a good finger print of the cancer cell peptide synthesis. They are indeed specific of the patient, its tumor, and the time of purification during the tumor development due to the genetic instability of the cancer cells.

HSPs complex can interact specifically with receptors and are taken up by APCs. The peptides carried on HSPs are then processed and delivered to the cell surface in association with MHC class I antigens for recognition by antigen-specific $CD8^+$ T cells. Thus tumors HSPs can be used to make some of the associated peptides recognised by the T cells.

The aim of this study was to assess the tolerance and efficacy in animal of the vaccination against high grade gliomas using autologous Heat Shock Proteins extracted from the own animal tumor with HA-chromatography and carried by the same HA-particles.

The tumors were obtained ectopically by injection of 9L cells in the flanks of CD344 Fischer rats for which they are syngeneic. The vaccine was obtained from the 9L cells. It is admitted that the immune reaction against brain tumors can show characteristics different from that obtained in connective tissue but the risk for inducing an autoimmune reaction against the central nervous system is identical as the vaccine is obtained from intracellular proteins. Furthermore, this experiment allowed to check if the HSPs extracted from the tumors should be associated or not with membrane proteins acting as co-factors.

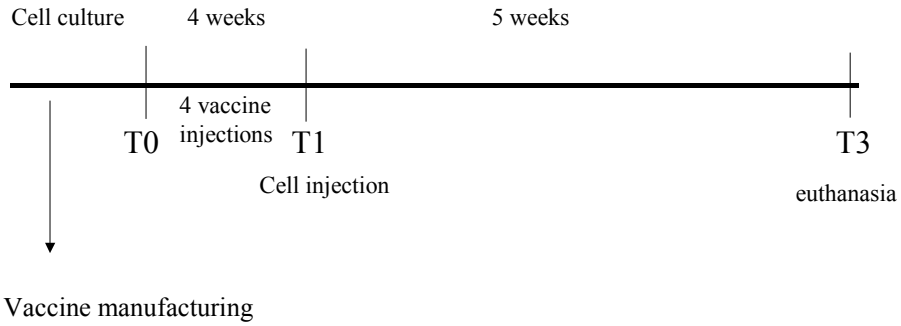
4. MATERIALS AND METHODS

4.1. GLIOMA CELL CULTURE:

Upon reception from the ATCC, the cells were defrost and cultured according to the ATCC instructions. The culture medium was DMEM supplemented in 10% fetal serum and glutamine. The cells were grown in triple Falcon flasks allowing to have a great culture surface. The culture medium was changed three times a week and the cells were subcultured when they reached confluence.
Before their use for the vaccine preparation, the cells were grown one hour at 42°C in order to increase the synthesis of different HSPs, the cells were then washed with the culture medium scraped and placed in PBS before to be frozen.

4.2. VACCINATION PROTOCOL:

The syringe containing the sterile frozen vaccine were supplied by Urodelia and contained 5 mg of the protein loaded HA-particles.

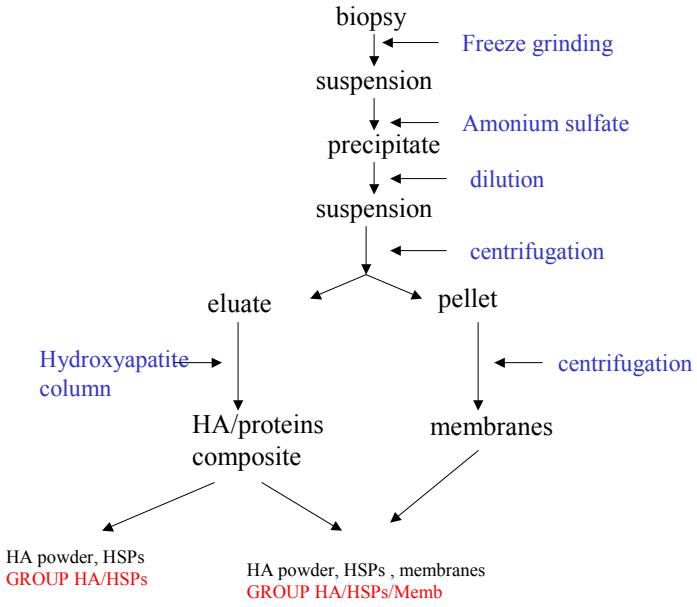


The vaccine was obtained from the 9L cells grown in monolayer. The rats received one shot every week for 4 weeks. Due to the difficulties linked to the novelty of the technology, a few days of variation in the injection time was tolerated. Four groups were made; 1; receiving the microparticles alone, 2; microparticles loaded with HSPs, 3; microparticles loaded with HSPs and cell membranes, 4; the control group without any injection. At the end of the vaccination period, the rats were injected in the left flank with 10^6 cells in suspension in 0.5 ml of DMEM medium without fetal calf serum.
It must be noted that very few information are available about the natural history of 9L tumor development in ectopic sites of CD344 rats. In case of doubt about the tumor development, animals would be sacrificed and autopsied. One animal of each group will be euthanasied. The rats injected with the tumor cells in which a tumor did not grow were not included in the statistics. All animals whose follow up was more that two weeks are included in the toxicity study.

4.3. VACCINE PREPARATION :

10^9 cells were obtained by scrapping or by trypsinizarion, suspended in demineralized water and submitted to several freezing- thawing cycles. The freezed cells were then pulverized in a mortar (kept at -20^0 C), and the powder transferred to a Khan tube on ice, adding 750 µl of $NaHCO_3$ (30 mM, pH 7). The tissue was then homogenized and the solution transferred to Eppendorff tubes and centrifuged at 10,000 rpm for 30 min at 4^o C. The supernatant was saved for posterior use. The pellet was re-suspended in 400 µl of phosphate buffer (30 mM, pH 7) and used for membrane preparation.
Membranes were separated using two Eppendorf tubes each containing a sucrose gradient (400 µl sucrose 40%, 400 µl sucrose 35% and 400 µl sucrose 30%). Then, 200 µl of the resuspended pellet were carefully layered onto the Eppendorf tubes, and the tubes were centrifuged in a microcentrifuge (11,000 rpm for 30 min at 4° C). The material present in the 40% and 35% sucrose interface was recovered with a Pasteur pipette and placed in 4 sterile vaccine glass containers.
The supernatant obtained in the first stage was used for HSPs purification by HA column chromatography with the following steps: 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). B) Prepare the column (chromatography columns, Poly-prep, Cat. 731-1550, Bio Rad) with HA de 40-80 µm, 2 cm high, equilibrating with 10 volume of phosphate buffer (20 mM pH7). The resuspended pellet was then added. C) The column was washed with 3 ml of a 100 mM NaCl solution. The resulting powder (1 mg) was used in 0.1 ml of isotonic NaCl as vaccine for the HA/HSPs group.
The association of this solution with 0.1 ml of the membrane solution constituted the vaccine of the HA/HSPs/memb group.



4.4. STATISTICS:

The different parameters (tumor weight and size) were evaluated following an analysis of variance. The rats in which tumor development had failed were not included in the statistics.

4.5. TEST SYSTEM

4.5.1. INCLUSION CRITERIA

- CD344 Fischer rats
- Weight: mean body weight 243 g (SD: 6.04 g) at treatment
- Number and sex: 24 males
- 7 animals were attributed to every group, three were kept as negative control.
- Selection: upon arrival, all animals were observed for external signs of sickness and healthy rats were included in the study.

4.5.2. IDENTIFICATION

Each animal group was clearly identified on his box:

-positive control group
-HA/HSPs group
-HA/HSPs/memb group.

4.5.3. SUPPLIER

Animals were obtained from Harlan France SARL, Z.I. Le Malcourlet-RN9 BP 98 03800 GANNAT France.

4.5.4. HOUSING

Animals were housed in the small animal facilities of DETERCA, in individual cages.
Room temperatures was maintained within a 18 – 23°C interval. Room humidity was maintained between 40 to 70 %. Lighting pattern was 12 hours light, 12 hours darkness. Air flow was 10-12 changes/hour without re-circulation.

4.5.5. ACCLIMATISATION

Acclimatisation was carried out over a 5 day-period.

4.5.6. FOOD AND WATER

Rodent controlled pelleted diet was offered to animals *ad libitum*.
The animals had free access to water distributed in bottles.

4.5.7. MANAGEMENT AND OBSERVATION OF ANIMALS

Each animal was observed once daily. Any abnormal findings were recorded as they were observed and reported to the Study Director and the Sponsor Representative. Serious adverse events characterized as significant hazards (immediately life-threatening, resulting in death or disability) should have been reported to the Study Director and the Sponsor Representative.
In particular, the clinical evaluation of a potential autoimmune myelo- encephalitis was based on the observance of comportmental anomalies and/or objective signs of neural suffering. They were monitored daily as follows: no neurological sign = grade 0, weak tail = grade 1.5, wobbly walk or limb paresis = grade 2, one leg paralysis = grade 2.5, placed on its back the animal is returned to its normal position = grade 3, hind leg paralysis, nasal bleeding or incontinence = grade 4, moribund = grade 5, death = grade 6.

4.5.8. CONCURRENT MEDICATION

No drug or other vaccine was administered during the trial.

4.5.9. WEIGHING AND MEASURMENTS

The animals will be weighed before euthanasia. The tumors are measured after removing and weighed after dehydration in alcohol, before embedment in polymer for histology .

2.3.10. TISSUE SAMPLING AND AUTOPSY:

At the time of euthanasia, the kidneys, spleen, liver, lungs and brain and lower part of the spinal cord were removed. Metastasis were searched in kidneys, spleen, liver and lungs. The brains were analysed for checking the possible lesions of autoimmune encephalitis.
After a dorsal incision of the skin, the subcutaneous tissue was searched for the occurrence of tumors which were removed, measured under a dissecting microscope and weighted.
The organs and tissues removed were fixed in ethanol to preserve the enzymatic activity of the cells. They were then embedded inside hydroxy methyl etacrylate blocks or methyl methaccrylate according to the manufacturer instructions. 7 µm sections were performed. They were stained with giemsa and for the galactosidase activity of the tumor cells with the sigma kit according to the instructions. Some organs and tissue sampling were embedded in wax in order to preserve galactosidase activity and to check the possible presence of tumor cells in these samples. The sections of the central nervous system were

4.6. IN-LIFE PHASE

4.6.1. APPLICATION OF TEST ITEM

4.6.1.1. Route of administration

Subcutaneous injection.

4.6.1.2. Time of vaccine administration

The first injection was done at T0 and one injection was done every week up to one month. A few days of variation in the injection time was tolerated as some difficulties occurred in the vaccine manufacturing due to the scarcity of available cells. The total number of injection was 4.

4.6.1.3. Dosing method

In each syringue, there was a sufficient quantity of vaccine for one shot.

4.6.1.4. Tumor induction in the subcutaneous tissue:

The cell monolayer was washed with PBS and then resuspended in DMEM using a trypsin EDTA solution. The cell suspension (0.5 ml) was injected in the subcutaneous tissue of the left flank at a 5. 10^6 cell/ml concentration. The cells were injected one week after the last shot.

4.6.2. SAMPLE LABELLING

The samples were removed after animal euthanasia.
Each sample was identified with study number, animal number, group number, specimen sampled, time of sampling.

5. RESULTS :

5.1. TOXICITY AND SECONDARY EFFECTS :

None of the rats showed sign of suffering and kept a normal behaviour and activity. In particular, they did show any motility anomalies of the tail and legs (table 1). They were all rated grade 0. None of the animals showed any activity, eating or drinking disturbance.
The histology of the rat central nervous system at the autopsy did not show any sign of inflammation. No inflammatory cells were evidenced in the brain parenchyma, vessels or cavities. None of the cells of the sections showed apoptotice cells.

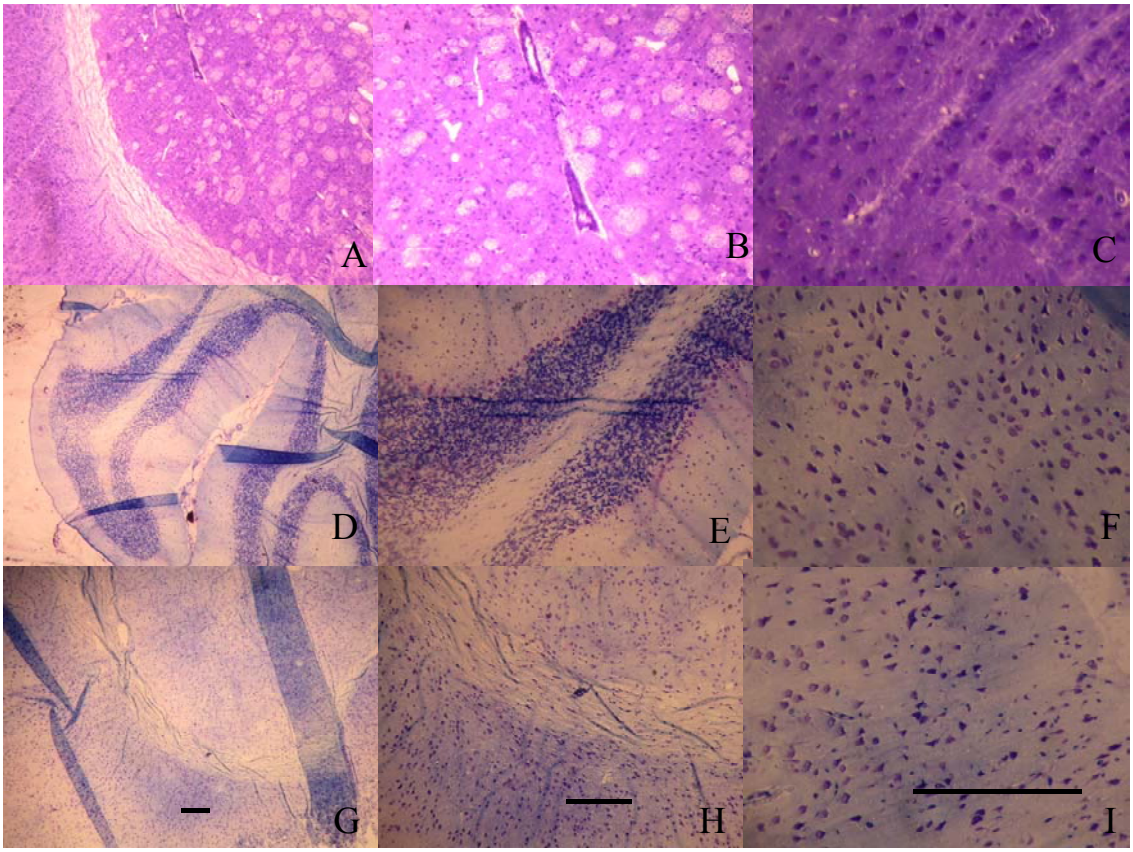


Figure 1: histological sections of rat forebrain after vaccination 21 days before animal euthanasia. The first line sections were obtained from HA/HSP/memb vaccinated rats, the second line from HA/HSPs and the third line from control groups. None inflammatory cells were evidenced infiltrated in the brain parenchyma. No apoptotice cells were found. Bar: 500 µm

Table 1: number of rats of each vaccinated group for each neurological grade

Number of animals	HSPs group	HSPs/memb group
Grade 0	7	7
Grade 1.5	0	0
Grade 2	0	0
Grade 2.5	0	0
Grade 3	0	0
Grade 4	0	0
Grade 5	0	0
Grade 6	0	0

5.2. TUMOR DEVELOPMENT:

5.2.1. 17 DAYS:

At 17 days after cell injection, a rat of each group was sacrificed to evaluate the tumor development. Tumors in the left flank can be found by palpation in some of the rats of every groups. At the autopsy, there are some clear mass at the surface of the muscle membranes (fig. 1). The histology revealed that they were constituted by adipocytes and did not contain tumor cells.
The control group showed anomalies of the vessels present at the inner surface of the derm (fig. 2). The tumor of the left flank of the control group have a soft consistence and show a heterogeneous structure. Tumors evidenced in the vaccinated groups were much smaller than in the previous group (fig. 3).
The tumor histology showed cell anomalies with a nucleus/cytoplasm ratio much higher than usual, a margination and condensation of the chromatin. In the HA/HSPs group, the tumor structure was quite homogeneous, with a lot of lymphocytes among the tumor cells (fig. 5, 6, 7). The percentage of the lymphocyte also high in the control group although tumors were much bigger (fig. 8, 9, 10). The tumor structure was heterogeneous but showed a very distinct delimitation.
We could not show any sign of metastasis in other organs in any of the groups.

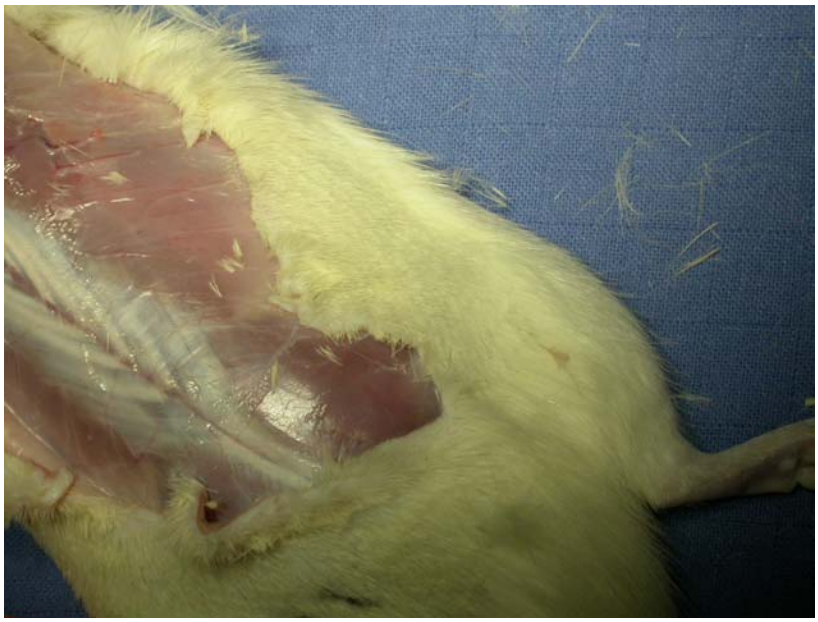


Figure 2: rats vaccinated with proteins and membranes (HA/HSPs/memb), 17 days after cell injection. There are some clear masses like bubbles at the aponevrose surface.

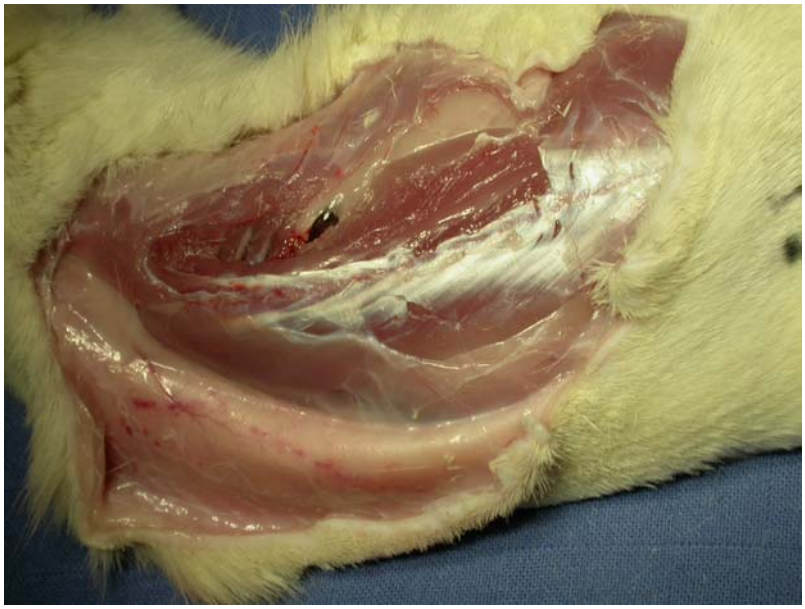


Figure 3: control rat 17 jours after cell injection. There are vasculature anomalies on the inner face of the derm.

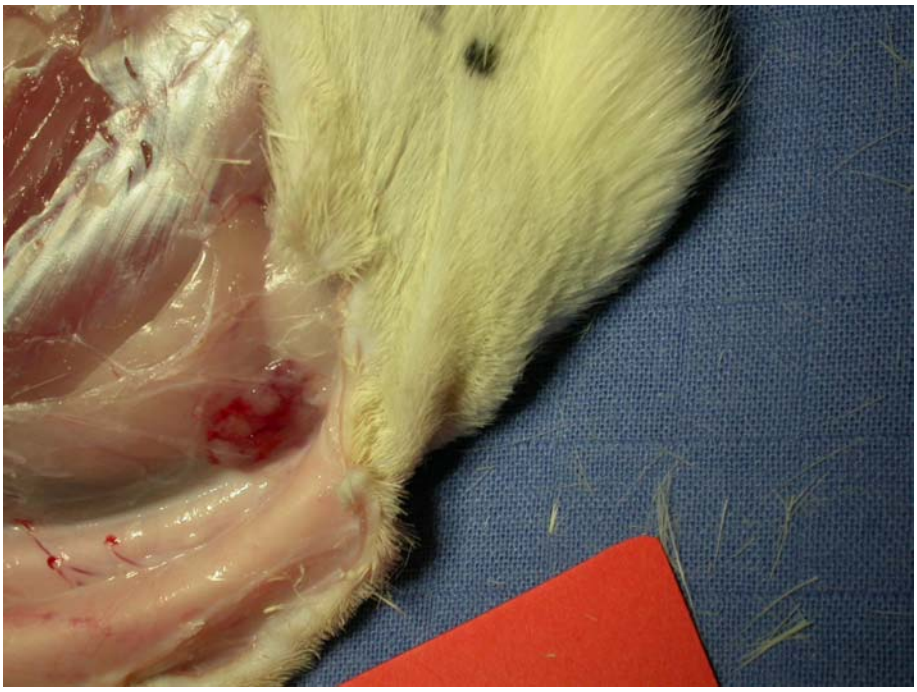


Figure 4 : control rat 17 jours after cell injection. There is a tumor in the left flank.

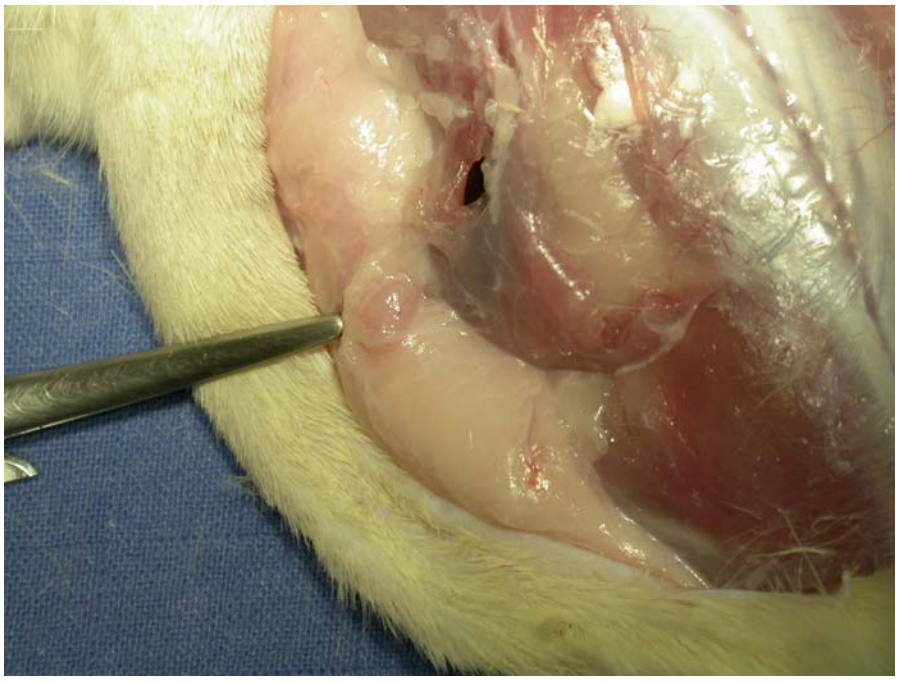


Figure 5 : rat vaccinated with only the HA particles associated to the HSPs. There is a small tumor in the subcutaneous tissue

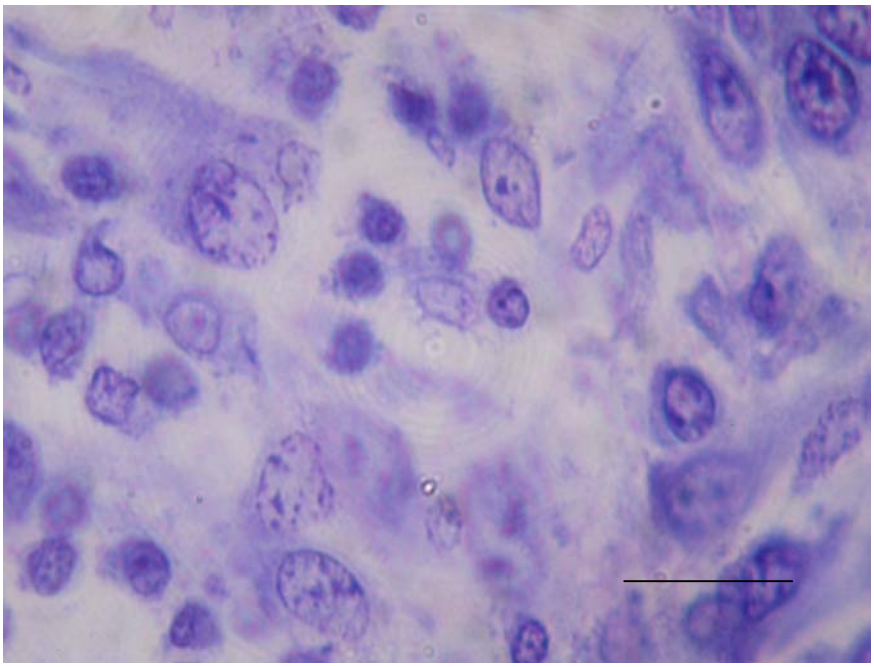


Figure 6: histology of the tumor shown in fig. 4. The tumor is ingrown by many lymphocytes. Bar: 5 µm

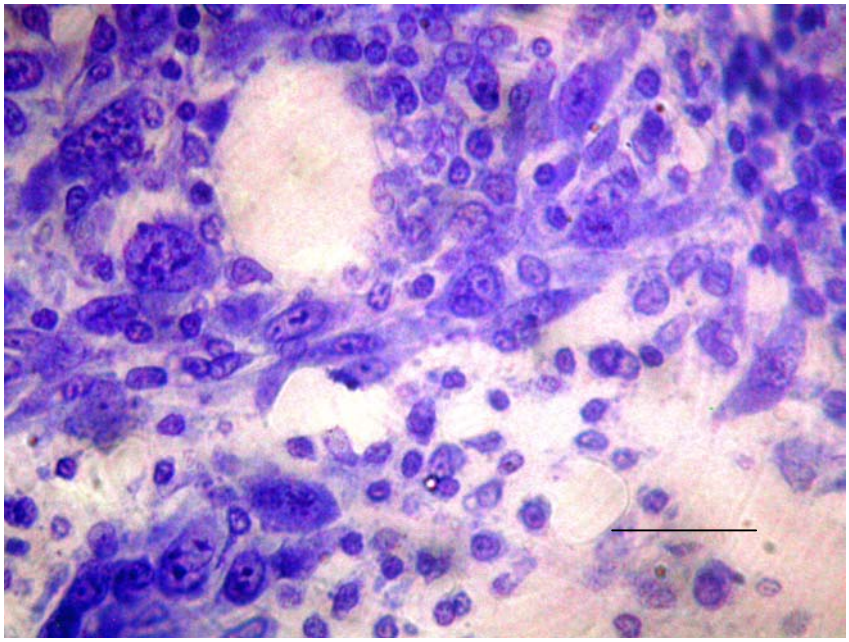


Figure 7: Section of the same samples showing the high percentage of monocytes. Bar: 15 µm.

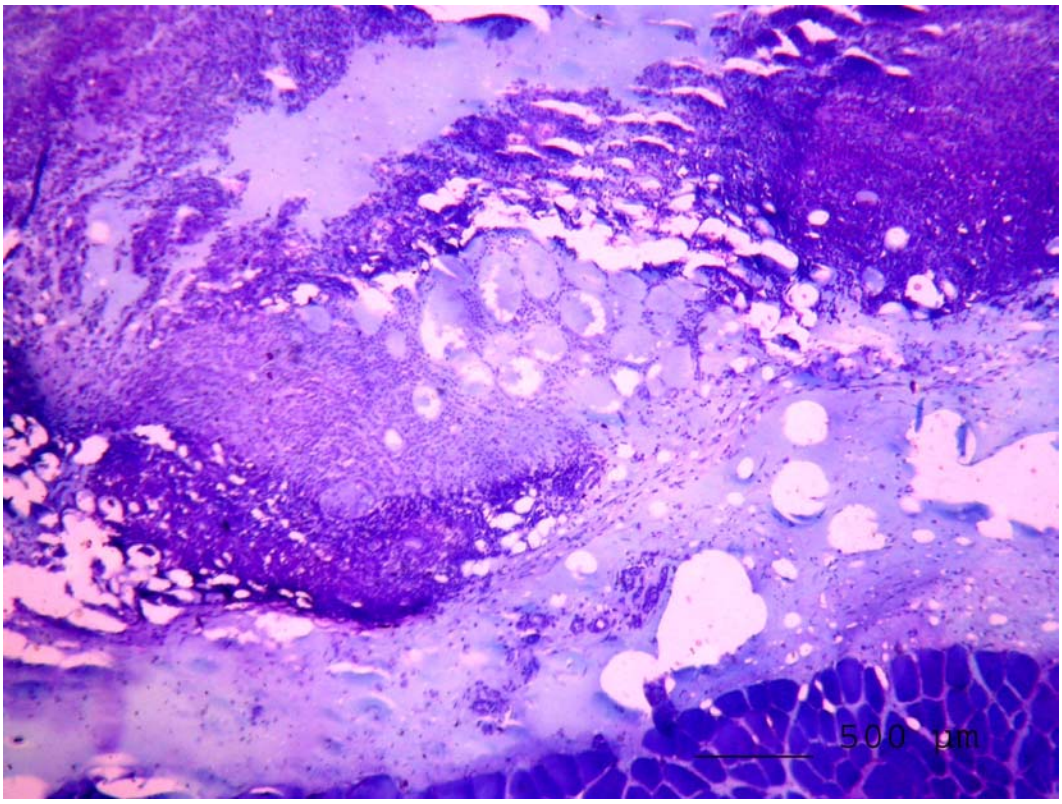


Figure 8: Histological section of a control rat tumor rat at 17 days. The tumor is heterogeneous, well separated from the muscle and its periphery is ingrown by lymphocytes.

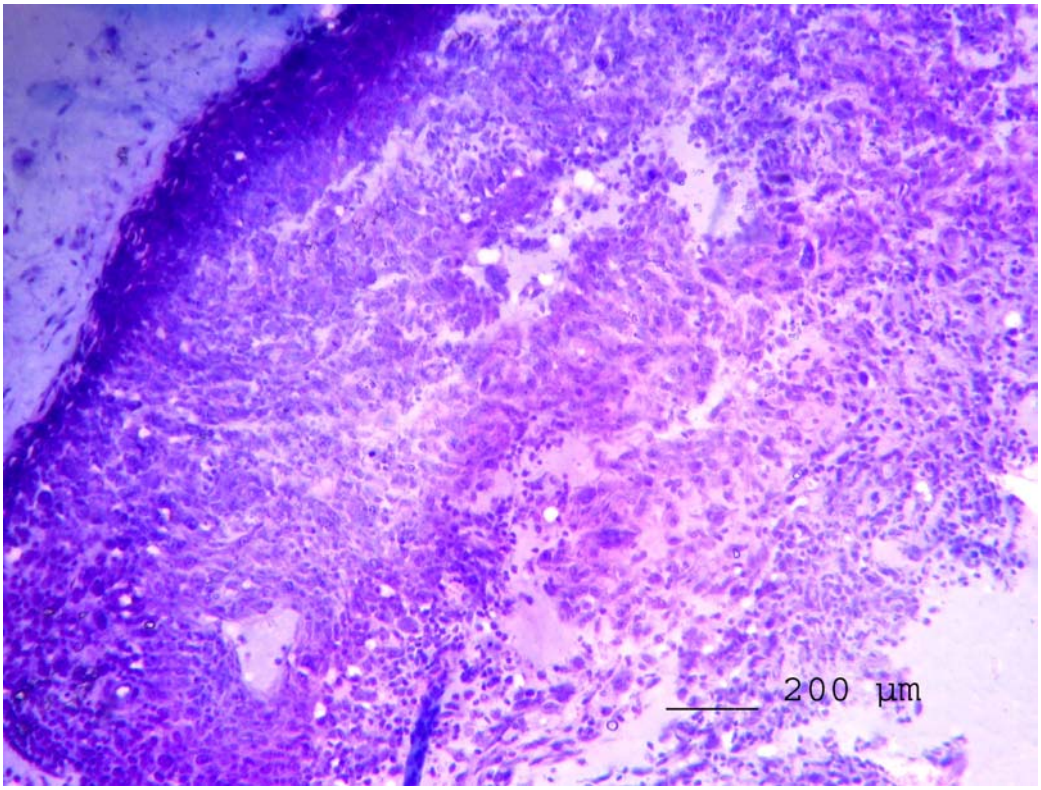


Figure 9: same histological section at higher magnification

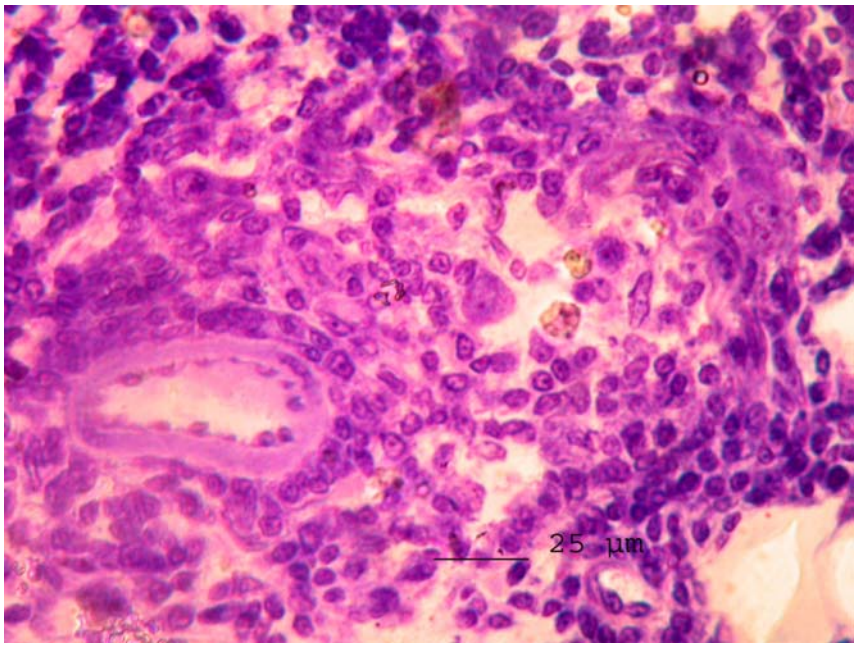


Figure 10: the tumor shows an important vascularization with many marginated cells

5.2.2. 29 DAYS :

The rats did not show neither behavior anomaly nor noticeable slimming. Tumors of the left flanks can be palpated in the control rats and in a rat of the HA/HSPs group. At the autopsy, the control rats had one or several tumors which did not invaded the muscles or the derma (fig. 10). The examination of the other organs (spleen, kidneys, liver, brain, lung) did not present any sign of macroscopic metastasis. In some of the vaccinated rats smaller tumors than the control group were also found. No sign of metastasis could be evidenced (fig. 11, 12, table 1).

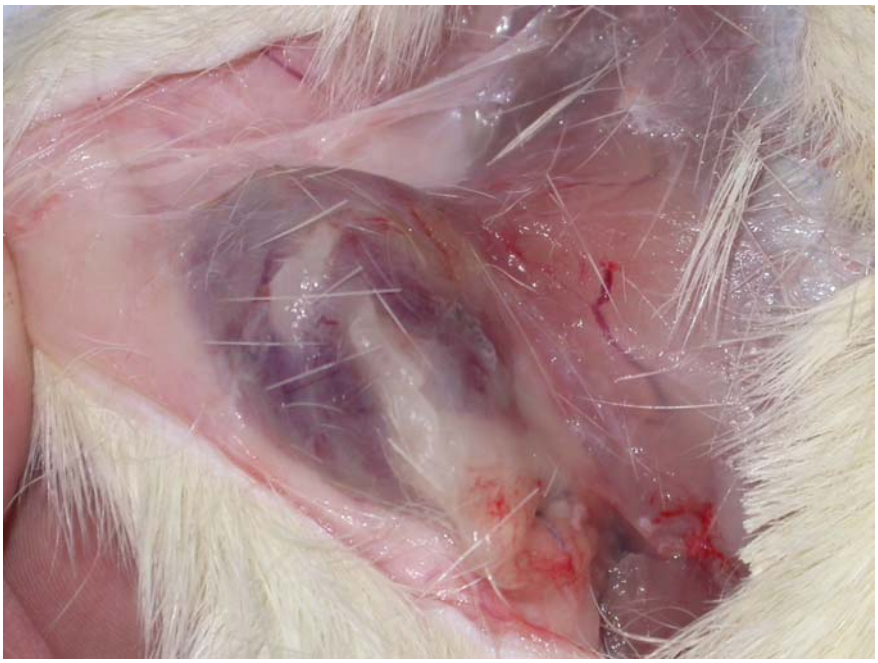


Figure 11: Control rat tumor 29 days after the cell injection

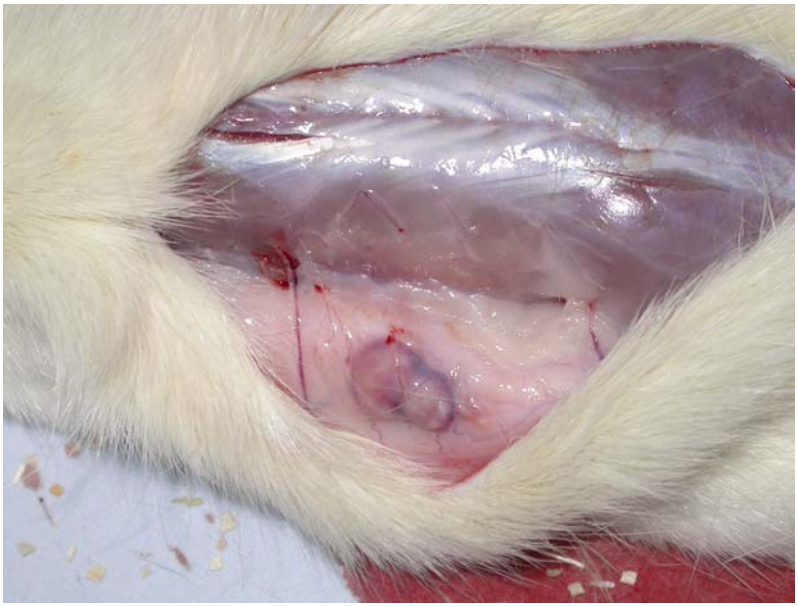


Figure 12 : tumor of a HA/HSPs/memb group rat 29 days after cell injection.

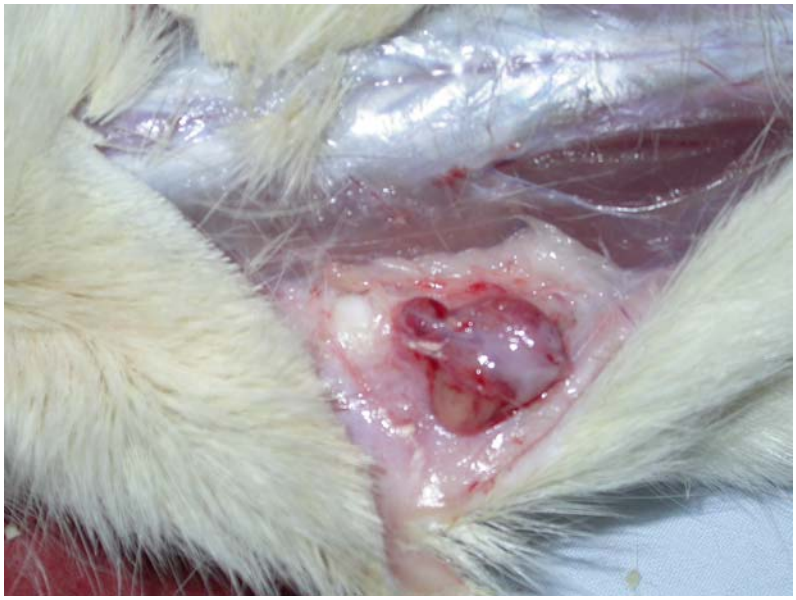


Figure 13: tumor of a HA/HSPs group rat 29 days after cell injection.

The histology of the tumor sections of the HA/HSP vaccinated rats showed that inside a connective coating, the largest volume of the tumor was necrotic. Only small parts of the sections were made by living tumoral cells in particular in the outside region of the tumor. In this region the tumor was invaded by a lot of immune cells. There were many tumor cells in an apoptotic process. The cells in apoptosis were in majority in the sections making the tumor almost free of living tumor cells.

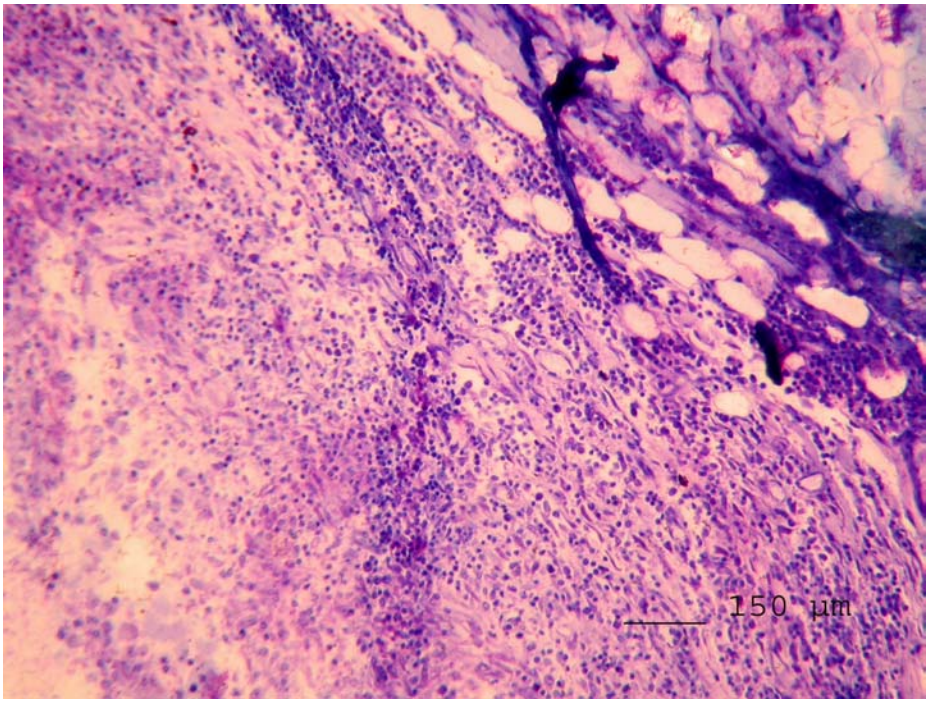


Figure 14 : Tumor of a HA/HSPs vaccinated rat 29 days after cell injection. The outside zone is infliltrated with lymphocytes and limited by connective tissue

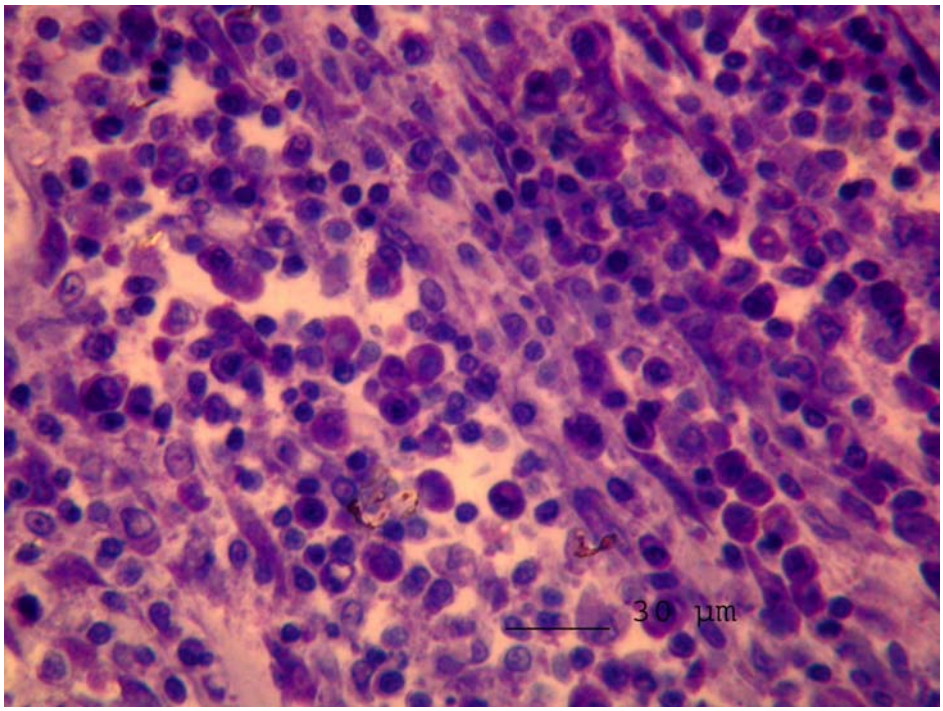


Figure 15 : in this zone the majorityof the cells is constituted by lymphocytes

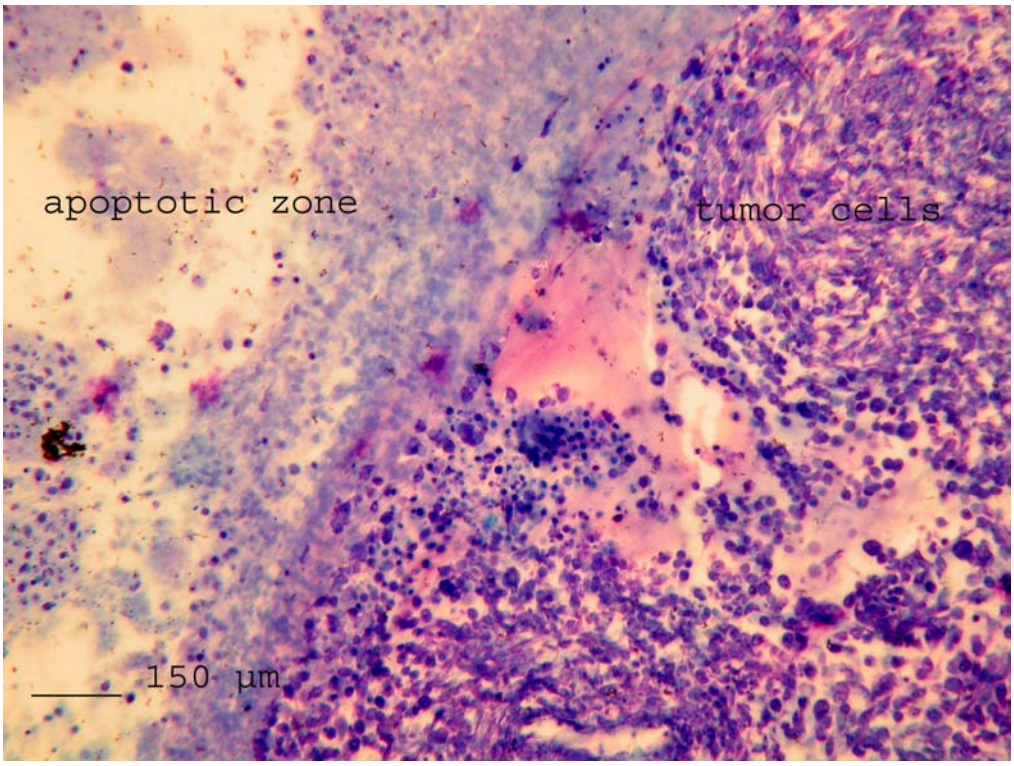


Figure 16 : the tumeur center of the HA/HSPs vaccinated rats is occupied by a few tumor cells and large zone of necrosis with apoptotic cells

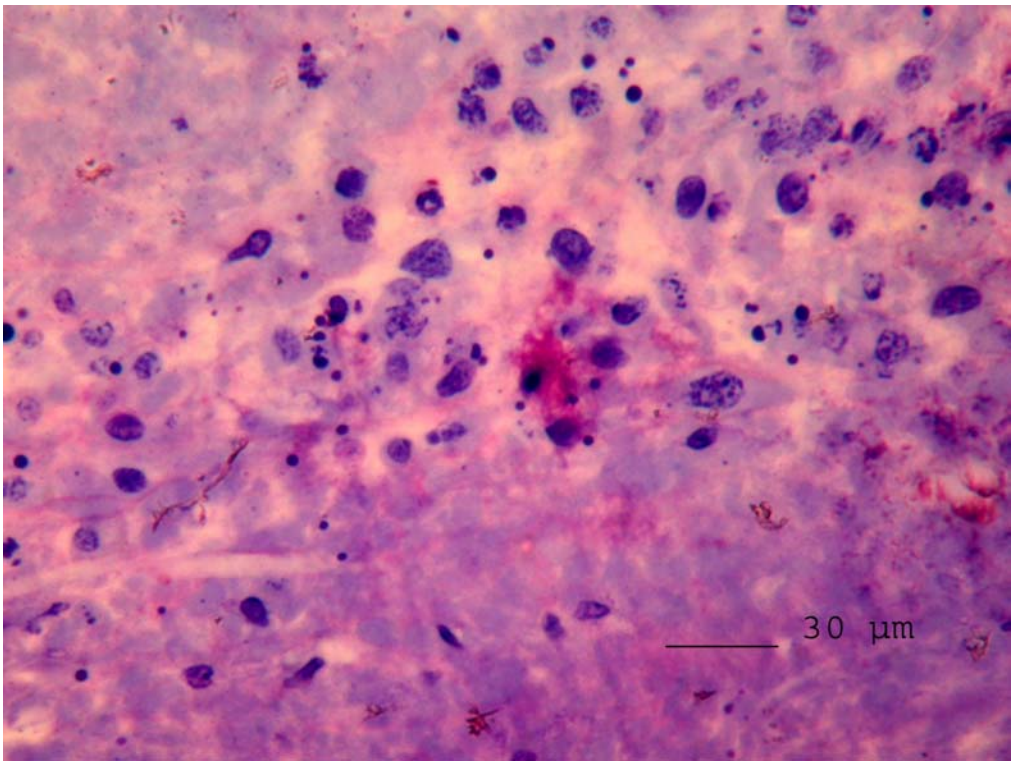


Figure 17 : most of the cells of the necrosis zone are apoptotic.

The big sized control tumors did not show many necrotic zones. Macroscopically the tumors were homogeneous. The microscopic examination showed that the number of lymphocytes among the tumor cells was much lower than in the vaccinated rats. However there were some zones containing dispersed apoptotic cells.

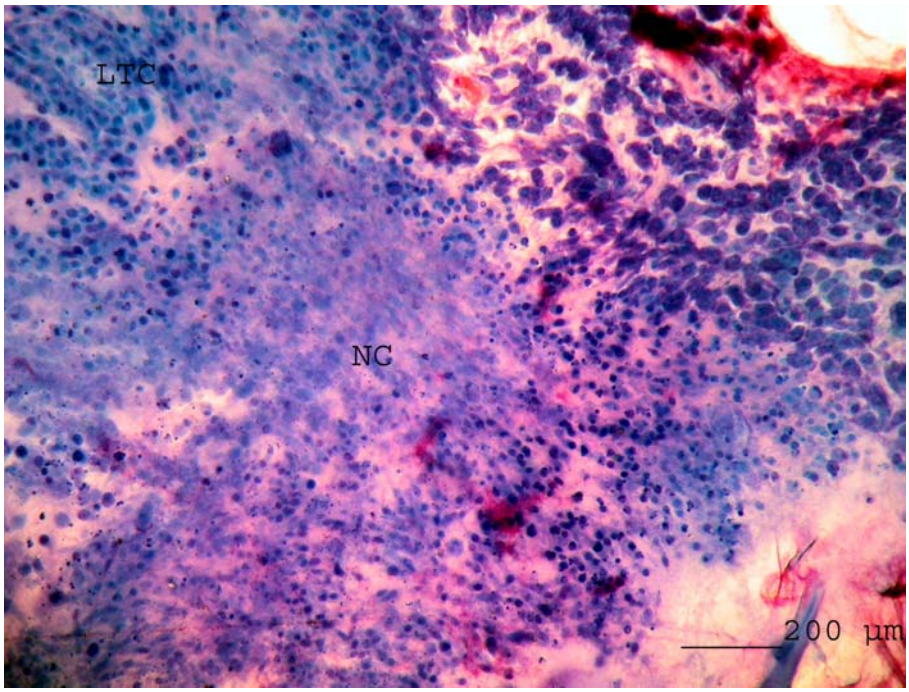


Figure 18: histological section of a control rat tumor. Among the tumor cells (LTC), some small zones of necrotic tissue with apoptotic cells (NC).

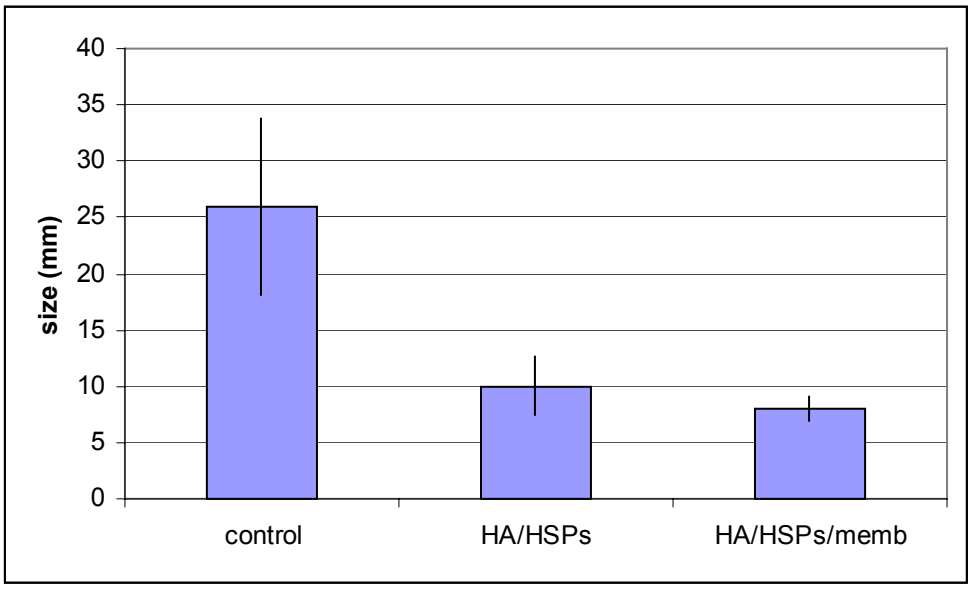


Table 2 : mean tumor size 29 days after the cell injection.

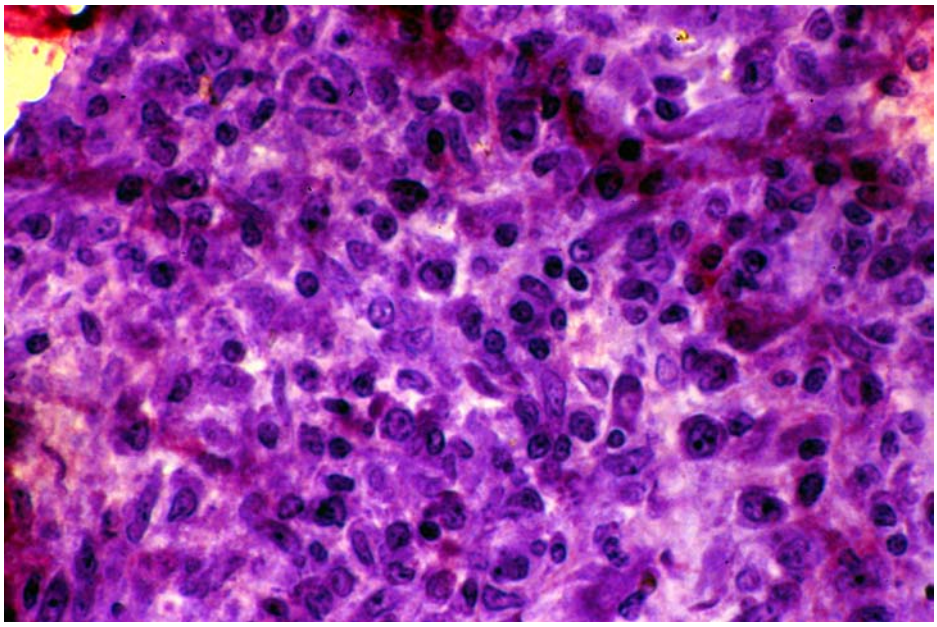


Figure 19: section of a HA/HSPs rat showing numerous lymphocytes infiltrated among the cancer cells

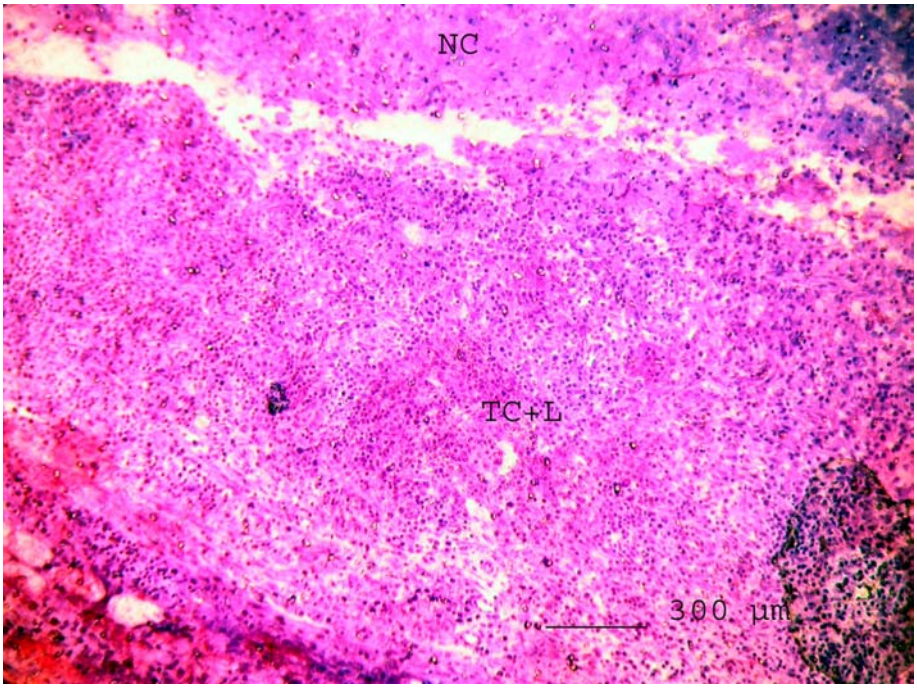


Figure 20: the oustside layer of the tumor of rat vaccinated byHA/HSPs/memb contains many Rats vaccinated using HA/HSPs/memb:

There is a strong infiltration by lymphocytes and very large necrotic zones were found. The apoptotic cell number was very high.
At 29 days, there was a statistical difference ($P < 0,005$) between the size of the vaccinated and non vaccinated rat tumors when compared to the control group.

Tableau 2: One-way ANOVA of the different vaccinated group 29 days after cell injection.

29 th days	source of variation	sum of squares	df	mean square	F
between the vaccinated groups	between groups	8	1	8	2,25
	within groups	21,3	6	3,55	
	total	30	7		
between HA/HSPsmemb and control	between groups	512	1	512	128
	within groups	24,83	6	4	
	total	648	7		
between HA/HSPs and control	between groups	792	1	792	132
	within groups	35,7	6	6	
	total	136	7		

6. DISCUSSION AND CONCLUSIONS:

This study showed that the vaccination using tumor extracts with HA adjuvants slowed down the growth of the tumors obtained by injection of the cancerous cells. The vaccination did not triggered any secondary effects and in particular no clinical signs of myelo- encephalitis were noted. A strong immunitary reaction leading to a cell death by apoptosis was induced by the vaccination.
The number of lymphocytes is always very high in the outside layers of the tumor. The apoptotic zones are located in the inner part. It seems that the tumor cells are in contact with the lymphocytes in the outside layer and then die by apoptosis in the tumor center.
The effect of the vaccine with the tumors HSPs did not seem to necessitate the presence of the membrane as co- factor.