Statement of Work

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BORDEAUX-MOSCOW PROJECT: CONFIRMATION STUDIES OF THE RUSSIAN DATA ON IMMUNOLOGICAL EFFECTS OF MICROWAVES

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1 Summary

The work presented in a series of Russian and Ukrainian articles dealt mainly with the effects on the immune system of rats exposed to a CW RF EMF at around 2375 MHz (0.1 to 10 W/m^2).

The results of these studies have served in part as the basis for the setting of maximum exposure levels in the USSR.

Most of the 9 papers came from the same research group of G.I. Vinogradov at al. The work was published mainly between 1975 and 1986. None of these results have been published in major international journals, but a summary was published (Grigoriev et al. 2002).

The current intentional harmonization activity worldwide has motivated Russian scientists to conduct a retrospective analysis of the last results. Taking into account the fact that these bioeffects were observed after long-term low-intensity microwave exposure, it was deemed necessary to confirm the Russian findings with the help of independent laboratories. The results of these studies would be very important for RF EMF standards.

The main results of the immune system studies can be summarized as follows:

- The authors had previously found that microwave irradiation of rats disrupted the antigenic structure of brain tissue. They suggested that this action could provoke an autoimmune response. They evaluated the ability of brain extracts from microwave-exposed animals to induce an autoimmune response. The autoimmune response was assessed from basophile degranulation, plaque-forming activity, and content of complement-binding antibodies in blood serum. Experimental results revealed that semi-chronic exposure at 5 W/m^2 evoked a pronounced autoimmune response in comparison with sham-exposed animals.

- In a second phase of the experiments, intact rats were immunized by an intra peritoneal injection of saline brain extracts from control and exposed animals. The autoimmune response was assessed 2 and 3 weeks later by cytological and morphological alterations in brain, spleen, bone marrow, and blood cells. Brain extract from rats exposed at 5 W/m^2 evoked pronounced autoimmune response both 2 and 3 weeks after immunization: the basophile degranulation index

rose (21% vs 2% in controls) , antibody levels 2–2.5 times, and the percentage of plaque-forming cells rose (10% vs 1.5% in control). In addition, this treatment increased the content of plasmocytes in the spleen (0.6 vs 0.16 in controls) and decreased the number of small lymphocytes in bone marrow (9 vs 13 in controls).

The blood serum from the exposed donors (semi-chronic 30-day RF exposure at 5 W/m^2) was injected in intact (non exposed) female rats on the 10th day of pregnancy.
Embryo mortality was high in this group (28%) and absent in control groups. Significantly higher offspring mortality was noticed at the end of the first month of life (31% vs. 17% in controls).

The critical effect (as defined by ICNIRP) was thus found at 5 W/m^2. This incident power corresponds to a SAR of approximately 0.6 W/kg. However, the SAR levels were not given in the papers and the orientation of the rats with respect to the polarization of the incident wave is not known.

All the reviewed papers concerned effects in rats. Since these effects were found at rather low incident power, replication and extension of this work in independent laboratories should be done using the same exposure systems, modern and routine biological methods.

At the present time such results, awaiting replication and interpretation, cannot be used in setting exposure international guidelines. However, further research will help understand the validity and the importance of these results for health assessment.

2 Objectives

The present project is a one-year collaborative study between laboratories in France and Russia to confirm the results of the previous work:

2.1. Immune processes in the brains of rats exposed to low-level microwaves

The objective is two-fold:

- Evaluation of the effects of long-term low-level exposure to microwaves on immunological parameters in the brain of exposed rats.
- Determination of the mechanisms of the effects on cerebral tissues.

2.2. Influence of injection of blood serum from exposed rats on the teratology of female rats

The objective is to test the blood serum from exposed rats (semi-chronic RF exposure at 5 W/m^2) on the number of offspring and miscarriages in intact females rats.

3. Protocol

Identical protocols will be used in the two laboratories. External advisers will conduct site visits and report to a scientific committee (see the list of advisers in **Annex 1**).

Laboratory training might be provided by one laboratory to the other in the techniques unfamiliar or not frequently used. It should be considered for the technician or scientist that will perform or supervise the test/assay.

Experiment will be carried out according to:

- the WHO recommendations on EMF biological research and
- the methodological requirements of the original (early) experiments conducted in the USSR.

3.1. General scheme of the experiments

3.1.1. General requirements for carrying out the studies

Rats 120-130 g (4-5 weeks) at the time of purchase. One week quarantine and adaptation to the exposure system. Before each experiment rats are in quarantine during 14 days, and then adapted to the exposure system. Exposure will last for 7 hours daily (from 10:00 to 17:00 with lights on at 08:00 and off at 20:00) for 5 days per week, with a total of 30 days of exposure, at a power density of 5 W/m^2.
It would be optimal if the two laboratories use the same source of animals. However, in view of the major difficulties in exporting and transporting the animals, independent sources will be used
(in France: Charles River, BP 109, 69592 L'arbresle Cedex, France,
in Russia:-GO SCBMT RAMS, Solnechnogosk, Andreevka.
All details concerning animals' characteristics will be given: sex, age, weight, all together in this paragraph of the protocol.

Experiments in the laboratories in Russia and France will be carried out with full knowledge of each other schedules in order to maintain optimum coordination of effort.
The quality of the animal housing facilities is important, and the type of bedding to be used, the type of diet. Get name of supplier and formula of local standard pellets:
In France: Rats maintenance diet: Barley, Wheat, Maize, Extraction Soybean oil meal, Wheat bran, Soluble fish protein concentrate, additive premix, bicalcium phosphate, calcium carbonate, vit A, D3, E and Copper), Safe Route de Saint Bris 89290 Augy, France,
In Russia: Wheat, Barley, Maize, Soybean, Wheat bran, Soluble fish protein concentrate, Dried milk, Sprout mail ,calcium carbonate, vit A, B1, B12, B2-6, D3, E, H, K3, endoks, Co, Cu, Fe, J, Mn, Se, Zn.
No food nor water during exposure or sham exposure. Food and water as libitum in the animal room, including cage-controls.

and the protocol of animal cage and room cleaning, the air flow in the animal rooms and the temperature of animal rooms will be as similar as possible in the Russian and French facilities.
(provide data to external advisers about general conditions in the animal facility (for example, the temperature range, humidity, light/dark cycle) Standardized housing conditions (animal per cage, saw dust change, etc.)
In France: Temperature range 21°C-23°C, humidity 40-60%, Light 12 hours, dark 12 hours, Air flow 100 m^3/hour, 4 rats/cage, 28 x 42 cm, Sawdust litter change: twice/week.
In Russia: Temperature range 21°C-23°C, humidity 40-60%, Light 12 hours, dark 12 hours, Air flow 100 m^3/hour, 4 rats/cage, 28 x 42 cm, Sawdust litter change: twice/week.

Biological samples will be collected and kept from exposed animals for future analyses (for brain histology, further ELISA of sera, etc.).

Statistical power (*beta* error) should be selected based on similar type of research, and proposed prior to conducting any studies. Add to protocol

A suggested schedule is presented in **Annex 2.**

3.1.2. Brain antibodies

- Pre-exposure procedure for laboratory animals (quarantine, adaptation to anechoic chamber conditions);
- RF exposure (see 3.1.1);
- Preparation of the homogeneous antigens from brain; (annex 3)
- Immunological analysis;
- Processing and analysis of results.

3.1.3. Teratology

- Pre-exposure procedure for laboratory animals (quarantine, adaptation to vivarium conditions);
- RF exposure the rats-donors, preparation of blood serum for subsequent injection in intact pregnant animals (see **Annex 4**). Preparation of the blood serum will be made at 14th day after the last exposure session (after 30 days of exposure).
- Intra-peritoneal injection the blood serum from exposed rats at intact pregnant animals at the 10th day after copulation.
- Supervision (pregnant rats and offspring);
- Processing and analysis of results.

3.2 Exposure

Conditions of microwave exposure will be made as similar as possible as those in the earlier Russian experiments. Exposure conditions in previous studies:
EMF CW exposure took place in anechoic chambers with power density 0.1, 0.5 and 5 W/m^2; 7 hours/day during 1 month (30 days) using "Luch-2" or "Luch-58" magnetron generators. The frequency of exposure was 2375±50 MHz. The conditions of exposure were simulated as an EM plane wave.

3.2.1. Exposure system

The RF EMF source will be a magnetron generator SMV-150-1 "Luch-11" provided by the Russian group along with the antenna and a spare magnetron. The working frequency is 2450 MHz (around 150 W). The antenna is a cylindrical horn antenna (diameter of 90 mm). The polarization is elliptic. The direction of exposure is from the top of the anechoic chamber where the antenna is placed. A schematic of the installation is presented in figure 1.

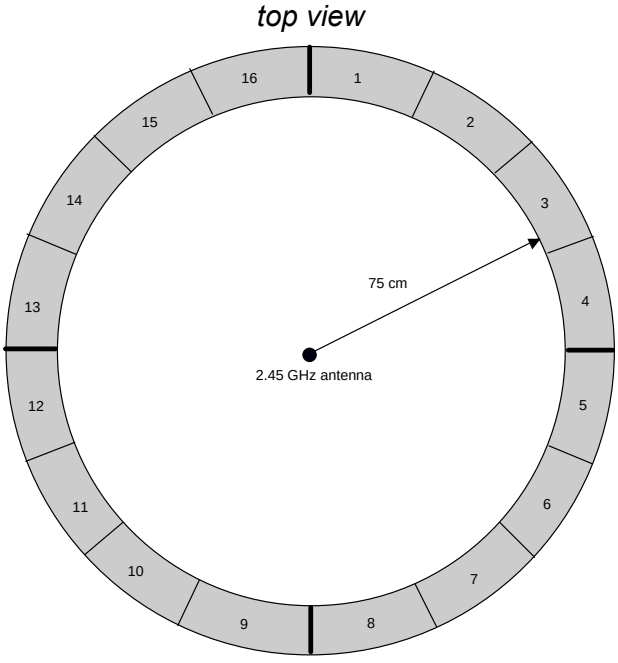

Figure 1 –Schematic of the exposure system

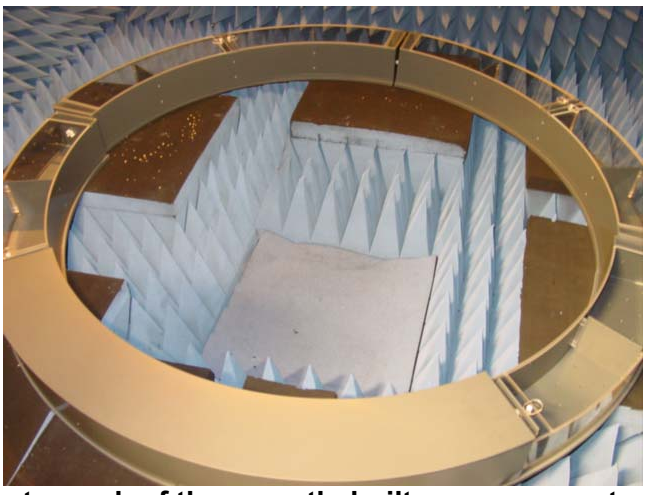

Figure 2 – Photograph of the recently-built exposure system at ENSCPB

Sixteen animals are placed in an annular fashion, in plastic cages (ca. 220×180×140 mm), closed from above by plastic covers with vents, with one animal per cage. Animals are free to move.

Cages are placed on top of a plastic stand above floor level in the far-field region of the antenna, at a distance of at least 150 cm from the antenna (distance set in Bordeaux and then adjusted in Moscow). During exposure, food and water are not provided to the animals (pending approval from the Ethics committee in Bordeaux). The duration of exposure is up to 7 hours per day (to be decided during the first site visit) Random rotatation of rat position within the ring each day.

The exposure system is placed in a shielded anechoic chamber. The absorbing material will be made of ferrite elements (5 cm thick) in Moscow and loaded foam with a pyramidal shape (thickness of 30 cm) in Bordeaux. The internal size of the anechoic chambers is ca. 3×3×3 m. Outer walls are covered with a metal screen.
Temperature, humidity, air flow change, controlled/monitored/known during exposure:
In France: 21-23°C, 40-60% humidity, air flow 160 m3/ h, min and max temperature and humidity will be measured during exposure
In Russia: 21-23 C, 40-60% humidity, air flow 160 m3/h,min and max temperature and humidity will be measured during exposure.

3.2.2. Conditions of exposure

- whole-body exposure at 2450 MHz (continuous wave) in anechoic chambers
- single exposure level: 5 W/m^2,
- 30-day exposure (excluding weekends), up to 7 hours per day, for 6 weeks.
- the room temperature of air is from 20 to 24°C, at relative humidity 40-60%. Air renewal is carried out using a ventilation system.
- Light level: using high-efficiency bulbs light flux measured using a lux meter at the cage level. Try to get same levels over cages in both labs according to animal room regulations.

In case of failure of exposure equipement: 1 hour (replacing the magnetron): no change in exposure protocol; 1 day failure: add one day at end of the 30 day exposure.

3.2.3. Dosimetry

The incident power density will be measured at the location of the cages in the absence of the animals with the help of a broadband meter (EMR-20/-30, Narda STS, Germany, or equivalent). (points of measurements to be agreed by both labs with help from P. Lévêque, e.g. probe on top of lids) Such measurements will be carried out at regular intervals and the power of the generator

monitored in real time. Overall dosimetry will be determined initially by an independent expert identified by WHO and monitored regularly by local experts.

4.2 Biology

NB: the text is binding but not the diagrams in the annexes

4.2.1 Assay of antibodies (Ab) against various antigens (diagram —Annex 4, tested antigens — Annex 5).

This approach is based on previous experience with investigations of autoimmune processes. Most of the antigens in the table are markers of antigenic modifications induced either by processes involving free radicals or by exposing antigens that are hindered in the CNS. Some of these are markers of neurodegeneration and inflammation (Geffard et al., 1998, 2002).

Assaying these circulating antibodies is completed on blood samples using ELISA with plates that are readily available in Bordeaux. Coated plates will be provided to the Moscow laboratory. This can help predict the time evolution of the effects of microwaves. Any notable increase in Ab titres is related to damage of the nervous tissues. Immunoglobulin isotypes of A, E, M, D and G will be evaluated.
These experiments will be performed on 16 exposed, 16 sham-exposed and 16 cage-control rats, in a blind manner. Blood will be collected on all animals on days 7 (from tail) and 14 after exposure (by killing the rats) Positive controls will also be included (sera of rats immunized with chosen antigens described in annex 5)
Detection method for ELISA results should be part of the detailed protocol with listing of the controls (positive and negative) and plate reader

4.2.2 Development of tissue specific circulating antibodies in reaction to complement fixation and ELISA (this part of the experiment will be performed in Russia and replicated in France if positive).

Experimental animals: male Wistar rats (weight 120–130 g). Sixteen animals in each group ("exposure", "sham-exposure", cage-control).
Antigens will be obtained from a water-saline brain extract from all animals.

At various time points (7^{th} and 14^{th} days after exposure), blood serum samples from sham-exposed and exposed animals will tested for their ability to react with the brain extracts in a reaction of complement fixation. Antibodies against myelin basic proteins will also be evaluated using ELISA.

Brain preparation and storage used by the French scientists

5. Teratology (Annex 6)

1. Mating of 90 female + 30 male Wister rats during 10–14 days.
2. Selection of 45 pregnant rats.
3. Distribution of these rats into 3 groups (exposure, sham-exposure, cage-control).
4. Preparation of a blood serum on day 14 after termination of exposure.
5. Blood serum (1 ml) from exposed and sham-exposed animals is injected I.P. in pregnant rats on day 10 after copulation.
6. Study of pre-natal embryo mortality on 5 rats from each group (to analyze intra-uterine death).
7. Observation of birth for 30 pregnant rats.

8. Observation of about 300 offspring during the following 30 days (weight, mortality, physical development).

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Annex 1

Oversight committee

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Annex 2

Experimental Schedule

1st trimester	2nd trimester	3rd trimester	4th trimester
Building exposure system and tests	Immune tests on brain	Teratology experiments	Final experiments and analysis

Annex 3

Method of preparation of the blood serum

French scientists will use the standard, modern technique for preparation of blood serum and for preparation of homogenous antigens from brain. The Russian scientists will use the technique, described below, that was used in the original studies. If there are any differences in the study results, the French scientists will replicate using the original technique.

Collection of the serum samples in the French laboratory

Rats are anesthetized with isoflurane (*5%*),the thorax is opened and blood is collected with a seringe through the left ventricle. Sera are prepared as described below. Samples are aliquoted in 200 μl and frozen at –80°C in cryotubes for several weeks.

Russian technique for blood serum preparation

Each rat is injected intra-peritoneally with 0.75 ml of 5 % hexenal solution. 1 - 2 minutes after injection the rat becomes anaesthetized. The rats are held on an operating table and the chest surface sterilized with 5 % a solution of iodine in spirit. The thorax is opened and the lungs and heart gently cut. The blood in the chest cavity is then collected with a sterile Pasteur pipette and placed in sterile test tubes and sealed with a cork. At least 1ml of blood is collected from each rat. Collected blood is placed in an incubator and maintained at 37°C for 30 minutes. Then, using a Pasteur pipette, remove the clots of fibrin from the walls of the test tube. For the best formation of serum, place the test tube for 30 - 60 minutes in a refrigerator (+ 4°C). Then collect the serum using a sterile Pasteur pipette and centrifuge at 3000 rpm for 5 minutes. Transparent serum is used in serological reactions (1 ml). The remaining serum is place in a sterile hermetically sealed tube and kept frozen at - 20°C for three weeks.

Method of preparation homogeneous antigens from brain (the original method)

In Bordeaux

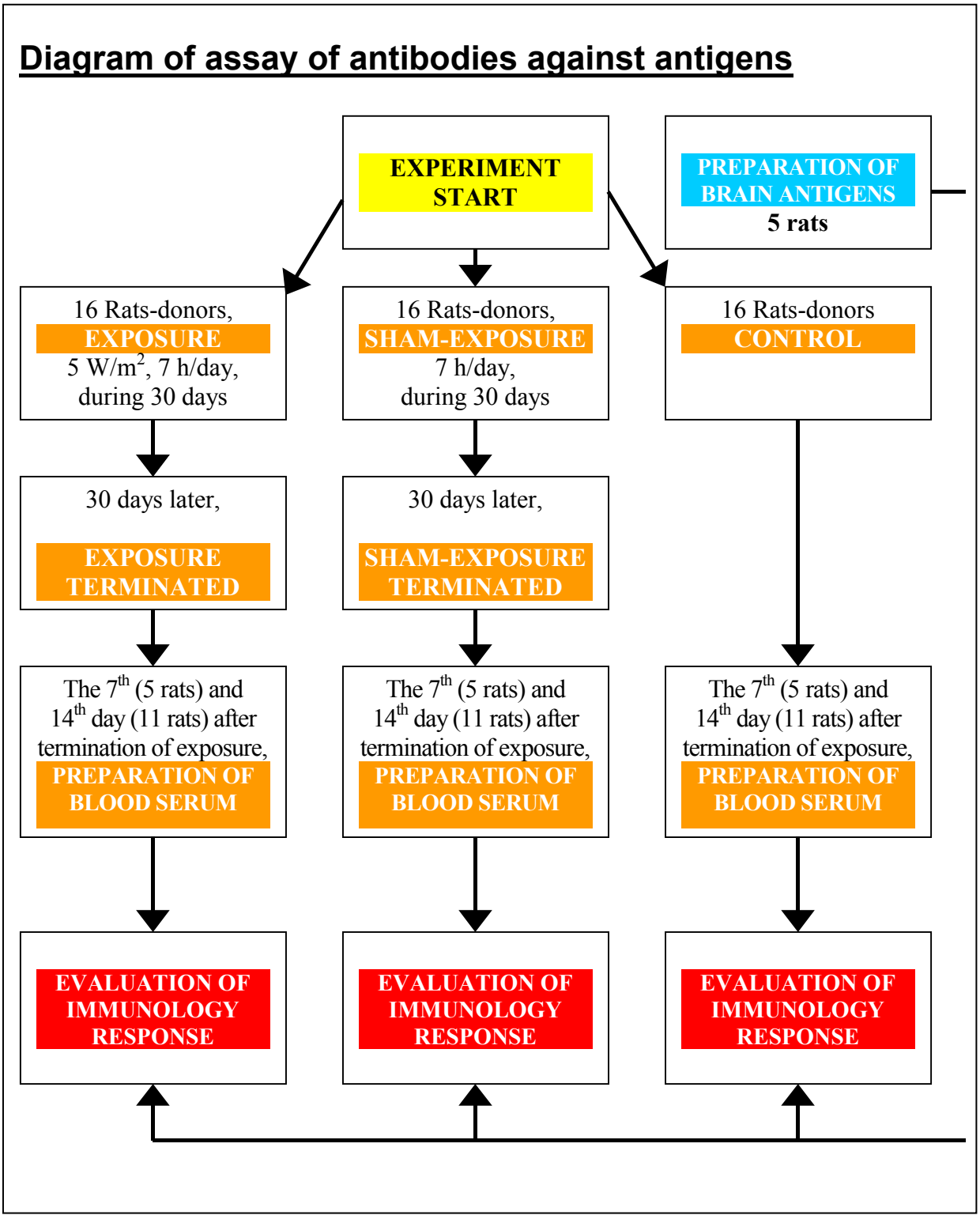
Rats are anesthetized with isoflurane (*5%*), perfused intracardially with PBS and brains are rapidly removed. Brains are dissociated with a tissu press and antigens are prepared as described below.

In Moscow (the original method)

Brain tissue is separated from connecting and fatty tissue and washed in physiological solution so that it is free of blood. Brain crush scissors are used on the clean brain. Then add one part, by weight, of crushed brain to four parts of sodium chloride physiological solution. Then make homogeneous using crushed glass and centrifuge the mix at 1000G for 20 minutes. The fluid above the brain mix is placed in ampoules, sealed and kept frozen at – 20° C for 60 days. Ampoules with antigen are use one at a time, and not repeatedly frozen and unfrozen. When used, the antigen is diluted 15 times in physiological solution.

The above method was reconstructed by Prof. A. Ivanov using (1) the publications by G. Vinogradov and (2) his own experience during the years 1970-1980.

Diagram of assay of antibodies against antigens

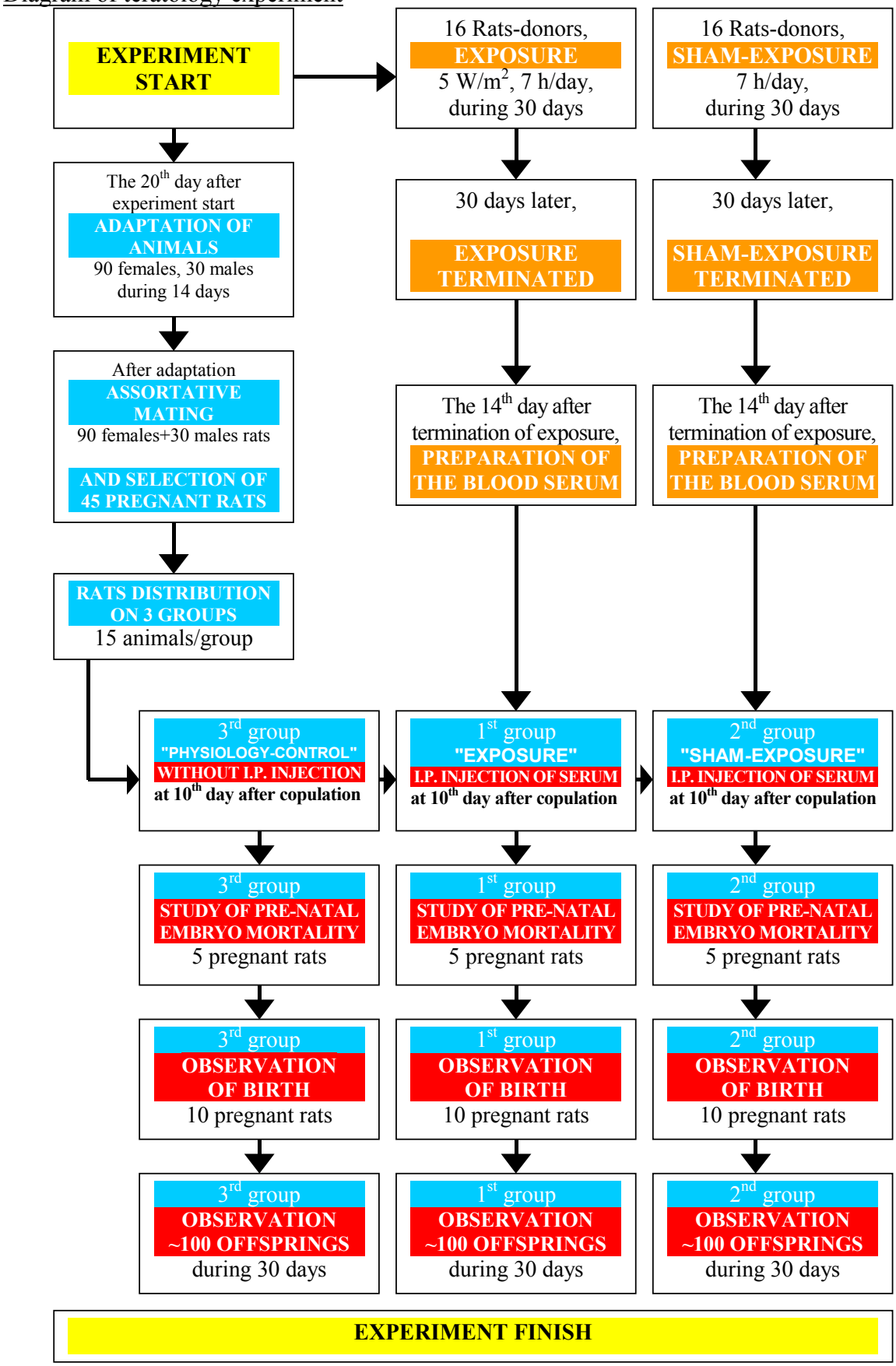


Annex 5

Tested antigens

Conjugated Ag	Origin	Pathological significance
Fatty acids: (C14-C18: palmitic, oleic, myristic, other fatty acids with long and short tail (C6-C12) and hydroxytated fatty acids (C6OH-C12OH)) Farnesyl Cystein	Endogenous compounds linked by amid bond	Non accessible to the immune system: recognized following membrane alteration or after abnormal interaction with other constituents of the self.
Azelaic acid Phosphatidyl inositol	Modified endogenous compounds (amid bond)	Products of oxidative processes and hydrolysis of unsaturated fatty acids
Malondialdehyde residue 4-hydroxynonenal	Compound highly reactive to endogenous species	Final products of lipoperoxydation
NO-Cysteine NO-Tyrosine NO-Trytophane NO-Histidine NO2-Tyrosine NO-Methionine NO-Asparagine NO-Arginine NO-Phenylalanine NO-Creatine NO-Citrulline	Endogenous compounds modified by NO and EOR	neoantigenes resulting from an hyperproduction of NO and peroxynitrite
Kynurenine Hydroxykynurénine Tryptophan Kynurénic acid Quinaldic acid Quinolinic acid Xanthurénic acid Anthranilic acid Hydroxy-anthranilic acid Picolinic acid	Tryptophan	Tryptophan metabolites
Cysteinyl catecholamines	L-DOPA, dopamine, noradrenaline, adrenaline, HVA, ...	Oxidation of catecholamines and nucleophilic attack by SH of thiol groups
Acetate, propionate, lactate, pyruvate, alanine, formiate, succinate	Products of bacterial metabolism	Role of bacteria

Diagram of teratology experiment



Elisa protocol in Bordeaux

	Tryptophan metabolites	Fatty acids	NO-conjugated	Bacterial metabolism
Coating 1 night 4°C	50µg/mL	50µg/mL	10µg/mL	10µg/mL
Saturation 1h, 37°C	PBS+BSA 2,5g/l	/	PBSTween+BSA 2,5g/l	PBSTween+BSA 2,5g/l
Washes	PBS	PBS	PBS	PBS
First antibody 1/500 1h45, 37°C	PBS+BSA 2,5g/l	PBS+BSAd 1g/l	PBS+ BSA 2,5g/l+BSA-G 1g/l+Glycérol 10%	PBSTween+BSA-G 1g/l+Glycerol 5%
Washes	PBSTween	PBS	PBSTween	PBSTween
IgG, M or A 1h, 37°C	PBS+BSA 2,5g/l	PBS+BSAd 1g/l	PBSTween+BSA 2,5g/l	PBSTween +BSA 2,5g/l
Washes	PBSTween	PBSTween	PBSTween	
Color development with OPD (8%), citrate buffer, Stop reaction after 10 min with HCl 2N, read 492 nm				