COMPARISON BETWEEN TWO METHODS FOR IMMUNOTOLERANCE INDUCTION IN DISCORDANT COMBINATION (DOG-POULTRY)

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Summary

The purpose of this study was comparative assessment of two methods for inducing immunotolerance to discordant xenografts, inoculation in allantoic sac and *in ovo*. Biological material was represented by two donor dogs and two experimental groups of COBB 500 embryos, matched by two control groups with an equal numbers of birds. Inoculation of antigenic material - blood mature mononuclear cells - was performed in the sixth day (in the allantoic sac) and fifth (in ovo) day of incubation.

At the age of three weeks, the resulting birds were tested for donor-recipient compatibility using mixed lymphocytic reaction; also, at this age, lymphocyte profile of the recipient individuals has been evaluated. Further checking was done by skin transplantation at the age of four weeks.

The results show insignificant differences between the two methods applied, both in terms of paraclinical and clinical aspects.

Key words: immunotolerance, xenotransplant, discordant combination

Xenotransplant, grafting of cells, tissues and organs at individuals from different species, is seen in recent years as a potential solution to the problem of human donors' reduced availability. Limited sources of cells, tissues and organs make transplant possible only in 15-59% of patients (1, 17), this aspect being the major reasons of research focused on removing xenogeneic barriers. Other arguments can be also mentioned, such as the possibility that transplantation of animal tissue and organs prevents recurrence of the disease that induced the need for transplantation, including in this class genetic, metabolic, infectious or neoplastic diseases (8, 12). It was also outlined the hypothesis that use of xenografts as "vehicle" for different genes or sets of genes could have a greater success than the traditional approach of gene therapy (8, 10).

This experiment, based on clonal selection theory of Burnet Macfarlane (2), aimed to bring recipient organism, found in an early stage of ontogenesis, in contact with the antigenic information of the future donor organism using two methods for inoculation: *in ovo* and allantoic sac. Thus were created the premises to induce immunotolerance for non-self phenotypes.

Materials and methods

Biologic material. 120 COBB 500 embryonated eggs divided in two groups of 60 embryos used for blood mononuclear cells inoculation in allantoic sac and *in ovo*. Two crossbreed male dogs aged 3-4 years.

Obtaining the antigenic material

The antigenic material was obtained by blood aspiration from dogs' cephalic vein and by separation of mononuclear cells at Immunophysiology and Biotechnology Center, Univ. "V. Babes" Timisoara by three centrifugations, after Ficoll-Paque solution addition.

The resulting antigenic material, mononuclear cells, was inoculated *in ovo* in the fifth day of embrionary development and in allantoic sac in the sixth day of incubation.

Inoculation of the antigenic material. The mature mononuclear cells concentrates (n = 2) were inoculated in 2 groups of 60 embryonated eggs as follows – table 1.

Table 1

Experimental scheme

Donor	Recipients	Inoculation method	Intervention day (incubation day)
Dog 1	60 embryonated eggs / **E1 group**	in allantoic sac	6
Dog 2	60 embryonated eggs / **E2 group**	*in ovo*	5

After hatching, each experimental group was matched by a control group comprising an equal number of birds.

Assessment of donor-recipient compatibility using mixed lymphocyte reaction. We made 10 mixed lymphocyte cultures (5 for experimental groups and 5 for control groups) using Sigma PK Linker modified protocol, respectively a reaction in which proliferative and lytic responses of both cell populations (donor and recipient T cells) is measured. The blood samples (source of T lymphocytes) were gathered from donor dogs and three weeks age recipient and control birds. After separation of mononuclear cells using the Ficoll-Paque solution, PKH26 Red fluorescent cell linker mini kit, PKH2 Green fluorescent cell linker kit (Sigma Aldrich®) were used.

Mixed lymphocyte cultures were analyzed by flow cytometry after marking dead cells with 7-aminoactinomicin D (read the FL3 detector, wavelength 680 nm, with fluorescent population differentiation, respectively PKH26 - red and PKH2 - green).

Evaluation of the lymphocyte T subsets. At the age of three weeks, from both experimental and control birds (n = 5 per group), peripheral blood was

gathered for obtaining the T lymphocytes. Cells were labeled with monoclonal antibodies as follows: antibodies anti-CD3 for label T lymphocytes, antibodies anti-CD4 for T helper lymphocytes, antibodies anti-CD8 for cytotoxic T cells, antibodies anti-CD45RO for differentiating between memory and naive T cells, antibodies anti-CD28 for differentiating between memory and effector T lymphocytes, and antibodies anti-CD25 for label the eventual activate subset of T cells from subpopulation Treg - regulatory T cells. This labeling served in determining the lymphocytes T profile in experimental and control groups.

For assessment of T cells subpopulation flow cytometer FACScan (Becton Dickinson, USA) was used. For data acquisition and analysis, Cell Quest and Win MDI2.9 softwares were used.

Skin grafts transplant. Transplantation of the split-thickness skin xenografts was made at the age of four weeks of recipient birds and surgical procedure followed the principles suggested by literature (5, 11).

Clinical monitoring. The subjects were examined daily, paying attention to the 21 macroscopic characteristics of the skin grafts among the most important being: color, aspect and adherence of the grafts to the recipient bed, as well as the aspect of the sides of the wound, making different measurements and taking pictures.

Results and discussions

Following isolation of mononuclear cells by Ficoll-Paque method were obtained cells concentrates with viability (checked by counting cells in hemocytometer after Trypan blue staining) between 89 and 96%.

As shown in Table 2, inoculation of blood mature mononuclear cells yielded similar results regardless of inoculation method used.

It was considered that mortality in the first two-three days after inoculation is the result of technical errors errors of inoculation and the negative effect of mature mononuclear cells and was estimated at 45% for *in ovo* inoculation, respectively 43.33% for inoculation in allantoic sac.

Embryonic loss in the first three days after inoculation were not associated with visible macroscopic changes, which is why we suspected a mechanism in which the antigenic material used (mature lymphocytes and monocytes) has played a major role, especially because of genetic differences between classes that have been object of the transplantation. Mortality occurred also after hatching (6.66% in both experimental groups).

Compatibility test performed on mixed lymphocyte cultures revealed 100% incompatibility between donor dogs the recipient birds evaluated, from both experimental and control groups. In both types of cultures following analysis by flow cytometry of lymphocytes marked PKH2/PKH26 (FL1/FL2), T cell proliferation of recipient birds and lysis donor cells was observed. Differences between cultures from experimental and control groups did not have statistical significance.

Table 2

Results obtained after blood mononuclear cells in chicken embryos

	Method of inoculation	Embrionary mortality	Donor-recipient compatibility	Lymphocyte profile	Day of skin grafts' rejection
Dog 1	in allantoic sac (6^{th} ID)	80% (12 viable birds in **E1** group)	100% incompatibility	dominated by naive T cells, similar to control group	4.83 ± 0.71
Dog 2	*in ovo* (5^{th} ID)	81.66% (11 viable birds in **E2** group)	100% incompatibility	dominated by naive T cells, similar to control group	4.36 ± 1,36

Legend: ID = incubation day

After lymphocyte profile analysis using flow cytometry, the lymphocytes subsets were defined as follows: naive $CD4^+$ helper T cells with phenotype $CD3^+CD4^+CD45RO^-CD28^+$; memory $CD4^+$ helper T cells with phenotype $CD3^+CD4^+CD45RO^+CD28^+$; effector $CD4^+$ helper T cells with phenotype $CD3^+CD4^+CD45RO^+CD28^-$; effector $CD4^+$ helper T cells with phenotype $CD3^+CD4^+CD45RO^-CD28^-$; naive $CD8^+$ cytotoxic T cells with phenotype $CD3^+CD8^+CD45RO^-CD28^+$; memory $CD8^+$ cytotoxic T cells with phenotype $CD3^+CD8^+CD45RO^+CD28^+$; effector $CD8^+$ cytotoxic T cells with phenotype $CD3^+CD8^+CD45RO^+CD28^-$; effector $CD8^+$ cytotoxic T cells with phenotype $CD3^+CD8^+CD45RO^-CD28^-$; regulatory T cells with phenotype $CD3^+CD4^+CD25^+$; and regulatory T cells with phenotype $CD3^+CD8^+CD25^+$.

Naive T cells were represented in a superior proportion in all the individuals of the experimental (E1 – 61.55 ± 7.04%; E2 – 62.93 ± 6.84%) and control groups (C1 – 64.32 ± 8.15%; C2 – 63.77 ± 7.05%), the differences being statistically insignificant both between experimental and control groups and between experimental groups.

Xenografts' rejection occurred in approximately four days after transplant (Table 2, fig.1), this moment being registered in both experimental and control groups. Unfavorable clinical evolution of the concordant and discordant xenografts confirmed results of the paraclinical exams.

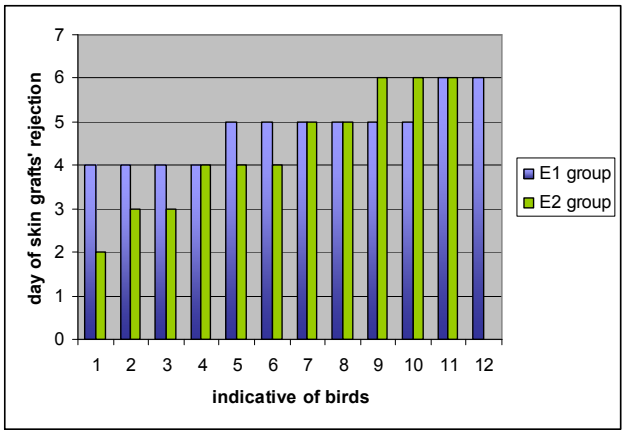

Fig. 1. Post-surgery days of skin grafts' rejection

The results obtained shows insignificant differences between inoculation of blood mononuclear cells in allantoic sac and *in ovo*.

Transplant performed in species belonging to different classes, in this case dog (donor) and poultry (recipient), is accompanied by similar problems to those reported for the transplantation of cells, tissues and organs belonging to the lower mammals to humans. This phenomenon is due to the fact that lower mammals have Galα1-3Gal epitopes on the surface of all cells, and man and birds have natural antibodies directed against this structure (3, 7, 12, 16). The presence of these natural antibodies are a major barrier of xenotransplantation, especially in chicken, in which was already demonstrated their passive transfer by vitelus (16).

For this reason, plus much higher avidity of anti-Galα1-3Gal avian antibodies *versus* human ones (7) and as a consequence, extremely high risk of complement-mediated rejection of mononuclear cells inoculated in the purpose of immunotolerance induction we choused an earlier intervention on embryos. Methods of inoculation in the allantoic sac and *in ovo*, in the sixth, respectively fifth day of incubation, are supported by the encouraging results obtained in concordant combinations (duck - chicken), at least for inducing a specific tolerance to some cell types (13, 15).

Given the existing information in literature about α-galactosyl density sites, the dog was chosen as a donor specie because it possesses about 1.5×10^4 sites/cell (6). Although rat, guinea pig or rabbit would have been more appropriate species in terms of thickness of skin tissue, immunological considerations (4.5×10^4 to 1.5×10^5 sites α-galactosyl/cell) have excluded them from the outset.

The choice of antigenic material used in the present experiment and the method of obtaining them was motivated by several aspects:

- gathering of the blood samples is a quick and easy method;

- Ficoll-Paque separation technique of blood mononuclear cells is one of the standard methods for obtaining lymphocytes for growing (4), so it is not toxic to cells and organisms;
- mature blood mononuclear cells have a very wide range of antigens, which facilitates the immunotolerance induction feature a higher level than the specific ones.

The two conditions in inducing a state of immunological tolerance are *initiation*, by exposing the body to antigens, and *providing persistence*, by continued presence of the antigens (12). The applied inoculation methods have provided the first condition, making contact recipient organisms with antigenic information of the donors as late as the sixth day of incubation and early, within certain immaturity of the immune system. The second condition, ensuring the persistence of antigenic information was accomplished by the lifetime of lymphocytes – between 21 days for mature B lymphocytes (9) and months or even years for T lymphocytes (14).

However, despite ensuring these conditions, inoculation blood mononuclear cells, regardless of method, not allowed to induce immunological tolerance in the dog-chicken discordant combination. This could have two explanations which converge to same result - the survival of embryos that have received the best passive immunity. On the one hand those embryos that survived were able to counteract negative effects of mature mononuclear cells (graft *versus* host reaction) and, on the other hand, antigenic material was neutralized by the presence of anti-Galα1-3Gal antibodies.

Conclusions

Inoculation of the blood mononuclear cells in allantoic sac and *in ovo* not ensure induction of immunological tolerance in dog-chicken discordant combination, the results obtaining after these methods being similar.

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It is well established that persons with lactose maldigestion experience better digestion and tolerance of the lactose contained in yogurt than of that contained in milk (3). The mechanisms involved have been extensively investi-gated. The importance of the viability of lactic acid bacteria was speculated as pasteurization reduced the observed digestibility. At least 2 mechanisms, which do not exclude each other, have been shown: digestion of lactose in the gut lumen by the lactase con-tained in the yogurt bacteria (the yogurt bacteria deliver lactase when lyzed by bile acids) and slower intes Comparison between two methods for immunotolerance induction in discordant combination (dog-poultry). Article. Full-text available.Â Reference: APPLICATION OF MODERN BIOSENSORS METHODS IN ECOTOXICOLOGICAL MONITORING OF SOME TOXINS OF NATURAL (MICOTOXINS) AND ANTROPOGENIC (PESTICIDES) ORIGIN. PART 1. MICOTOXINS. Anatomopathological Changes Induced by Mycotoxins. â€¢ Comparison Between Two Methods for Immunotolerance Induction in Discordant Combination (Dog-Poultry) more. by Emil Tirziu. and Ciceronis Cumpanasoiu. The purpose of this study was comparative assessment of two methods for inducing immunotolerance to discordant xenografts, inoculation in allantoic sac and in ovo. Biological material was represented by two donor dogs and two experimental more.Â The results show insignificant differences between the two methods applied, both in terms of paraclinical and clinical aspects. Xenotransplant, grafting of cells, tissues and organs at individuals from different species Download (.pdf). In poultry, stress-induced immunosuppression is manifested by failure in vaccination, and increased morbidity and mortality of flocks. Immunosuppressive agents can have cytolytic effects on lymphocyte populations leading to atrophied and depleted lymphoid organs. Immunosuppression can be due to infectious agents or non-infectious agents or due to a combination of them. At present, several modern cellular and molecular approaches are being used to determine the status of the immune system during stress and disease. Comprehensive methodologies for the evaluation of immunosuppression by combined