IMMUNOLOGIC AND OSTEOGENEIC PROPERTIES OF XENOGENEIC AND ALLOGENEIC BONE IMPLANTS

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ABSTRACT

Immunogenicity of bone xeno and alloimplants

Xenografting is increasingly being developed as a response to the shortage of human tissues. However, antigenic components of bone material eliciting immune responses – particularly of cellular nature – are blamed for the reduction of the osteoinductive properties of bone and bone-derived implants. The aim of our study was to compare the immunologic response and osteogenesis induced by antigen-depleted allogeneic and xenogeneic bone-derived implants to that induced by partially antigen-depleted material heterotopically placed (muscular pouch) in rats. Wistar rats received bone-derived implants of different antigeneic condition, from both xenogeneic (rabbit) and allogeneic (rat) origin. After sacrifice, animals were evaluated for osteogenesis and immune response. New bone formation was observed around all bone-derived implants, whether fully or partially antigen-extracted, and from both xenogeneic and allogeneic origin. No significant humoral response resulted following bone implantation. No qualitative difference was found in the newly formed bone or in the immune (cellular) response to partial and fully antigen-extracted bone of both allogeneic and xenogeneic origin.

KEY-WORDS: Osteogenesis, xenografting, allografting, immune response

1 – INTRODUCTION

Bone allografts and xenografts may elicit immune responses in the host that interfere with new bone formation (osteoinduction) around the

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implants[9]. However, using processed bone implants rather than fresh material[6] can minimize the host's immune response.

Different methods of antigen depletion in both allogeneic and xenogeneic bone implants have been utilized to reduce the host immune response while preserving the osteoinductive properties of the implants[33,17,18]. Bone immunogenicity resides chiefly in its marrow component in fresh material[6]. Other cellular debris in processed bone tissue can elicit an immune response through an indirect antigen presentation[15]. Implants of bone matrix collagen have been blamed for serologic activity (humoral immune response) in the host. Several authors have demonstrated that collagen immunogenicity resides in its telopeptide regions[7,23,28]. Thus, extracting collagen telopeptides in processed bone implants may minimize or even abolish immune responses in the host.

To compare the characteristics of a potential immune response to autolyzed, antigen-extracted allogeneic (AAA) and xenogeneic (AAX) bone implants versus telopeptide-extracted AAA (AAAp) and AAX (AAXp) bone implants, we designed an experiment in rats (host) in which differentially processed allogeneic (rat) and xenogeneic (rabbit) bone was heterotopically implanted. Normal bone healing process in fractures shows the local recruitment of immunocompetent cells (CD4+ lymphocytes, dendritic cells expressing HLA-DR, macrophages)[2,13]. Thus, local cellular recruitment in the healing process of allogeneic or xenogeneic, orthotopically-placed bone implants may interfere with an accurate interpretation of true immunologic events in the graft-host interface. To avoid this bias, we used a heterotopic bone implantation model in our experiment.

2 – MATERIALS AND METHODS

2.1 – Implant preparation

Femora from two New Zealand rabbits and 5 Wistar rats were obtained under sterile conditions immediately after sacrificing the animals. Rat and rabbit bones were processed separately. The epiphyses were cut out and discarded. The bones were thoroughly cleaned, freed of soft tissues, washed and cut into pieces measuring 0.8 x 0.3 x 0.1cm. They were immediately processed according to Urist's technique[33]. Half of the total amount of bone material was weighed and further incubated at 37°C for 72 hours in a bath containing buffer phosphate. Then, the bones were treated with pepsin (Pepsin, Sigma, 1:100 w/w enzyme-matrix) and 0.01N chlorhidric acid at 22°C for 6 hours. Pepsin activity was stopped by increasing the solution's pH to 8.0 with 0.1N Sodium hydroxide for 6 hours at 4°C. All the bones were further washed with distilled water, freeze-dried (Labconco 5 Freeze-

Dryer) and sterilized (25 KGy, Co60).

2.2 – Surgical model

Fifty young adult male Wistar rats, each weighing 300g at the beginning of the experiment, were separated into 5 groups of 10 animals each. The animals were kept in cages without any restriction of mobility and fed ad libitum. After being anesthesized (i/m ketamine at a dose of 50 mg/kg and xylazine 8 mg/kg), 0.8 cubic cm of blood was obtained from the tail vein of each rat. Under sterile conditions, rats were operated through a dorsal incision in the right thigh, and implants were placed in an intraquadricipital pouch. The wound was closed with staples. Each group of rats received, respectively, implants of AAA rat bone, AAAp rat bone, AAX rabbit bone, AAXp rabbit bone, and gelatin capsules (controls). After the surgery, each rat received i/m cephradine (Sefril, Urufarma), at a dose of 25 mg/kg/day for seven days.

Animals used in this experiment were treated, kept and handled according to the international rules for the use of animals in scientific experiments (Council of International Organizations of Medical Sciences), and the guidelines of the California Animal Subjects Committee.

2.3 – In vivo studies

At 10, 20, 30, 45 and 60 days after surgery, two animals from each group were sacrificed. Blood was obtained (0.8 cubic cm) from each rat for electrophoretic study of serum proteins and an X-Ray study of the implant area was performed. The spleen was excised, weighed and fixed in Bouin's solution. The draining lymph node of the implant area (right cranial inguinal main lymph node)[30] was carefully excised, measured, weighed and fixed in Bouin's solution. The implant and surrounding tissues were excised en block and fixed in Formalin. Specimens containing mineralized tissues were demineralized in disodium ethylenediaminetetraacetate. All specimens were embedded in paraffin, cut and stained for microscopic evaluation. All tissues were stained with Hematoxilin-Eosin; tissues from the implant site were also stained with Azure II-Eosin. These latter tissues and the lymph nodes were also processed for immunostaining. Anti-rat monoclonal antibodies from mouse source (CD45RC, cloneOX-22, CD45RC, clone HIS25, $\alpha\beta$ T-cell Receptor, clone R73, and CD8a, clone OX-8, PharMingen Int.) were used to characterize B and T lymphocytes and to further distinguish T subsets, both in the lymph nodes and at the implant sites.

2.4 – Statistical analysis

Results from the serum proteins values were analyzed using chi-square test for significance. Pre and postoperative values were

compared, and the latter were expressed as a percentage of variation from the initial values.

3 – RESULTS

3.1 – Chondro-osteogenesis

Histologic evidence of cartilage formation was detected in all animal groups except the control group at 10 days postoperative. After day 20, all animals with bone-derived material implants showed osteoid seams within the implants, sparse osteoblasts and mineral deposits mainly at the center of the implants. Some specimens still showed chondrocyte groups co-existing with osteoblasts surrounded by osteoid. By day 30, all the implants showed abundant osteoid and mineralized areas surrounding lacunae containing osteoblasts. The implants showed areas of peripheral resorption. By day 45, specimens showed partial resorption of the implants with abundant osteocytes surrounded by mineralized bone matrix. Osteoid could still be observed in some implants. By day 60, all the specimens obtained from animals implanted with bone-derived material showed conspicuous remodeling of the implant (peripheral resorption), with full mineralized, true ossicles containing bone marrow cells at the center of the implant area (Fig.1).

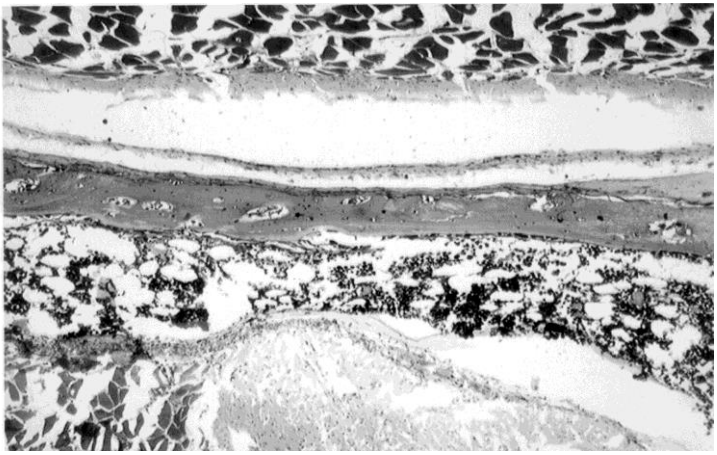

FIGURE 1 – Photomicrograph of histologic section of a day 60 specimen of intramuscular AAX bone implant. Newly formed bone cells and calcified matrix surround bone marrow cells (ossicle). (Stain, Azure II; original magnification X 400).

X-Ray studies showed radiopacity at all implant sites except for the gelatin capsules at day 20 postoperative. Radiopaque images corresponded to the original implant's shape in all animal groups. X-Ray

studies on postoperative days 30 and 45 also showed similar mineralized images. Day 60 studies showed round or oval-shaped radiopaque images at all bone-derived implant sites.

3.2 – Immune response

Serum protein electrophoresis

Pre and postoperative serum electrophoresis studies showed a normal pattern of protein levels during the whole course of the experiment in all groups of animals, including controls. Albumin/globulin ratio also showed a normal pattern[22]. Preoperative total protein, albumin, $\alpha 2$, β, γ globulin values and Albumin/Globulin ratio compared to postoperative values (day 10, 20, 30, 45 and 60) did not show any statistically significant differences between all groups of animals, including controls. Conversely, $\alpha 1$ globulin values showed a significant increase ($p \leq 0{,}001$) in the postoperative period in all groups of animals, including controls. Postoperative values of $\alpha 1$ globulin increased in 12.4% (mean value) compared to preoperative mean percentage of values. These values did not significantly influence the mean values of total globulin proteins.

Spleen histology

No modification of the normal aspect of the spleen could be detected in the postoperative period in any group of animals. Total spleen weight and white pulp volume was considered similar to normals; gross anatomy and histology did not show any abnormality.

Regional lymph node histology

Regional lymph nodes draining the implant site in all groups of animals (including controls) showed a consistent enlargement and weight increase compared to those from normal rats of the same body weight and age, at 10, 20 and 30 days postoperative. Lymph nodes excised on days 45 and 60 showed normal weight in all animals.

Monoclonal antibody technique showed CD45RC (OX-22) + cells at the follicular area (outer cortex) of all lymph nodes during all the postoperative period. Deep cortex and germinal centers of lymph nodes lacked CD45RC (OX-22) + cells, but the former area showed abundant CD8a (OX-8) + cells in every case and during all the postoperative period (Fig. 2).

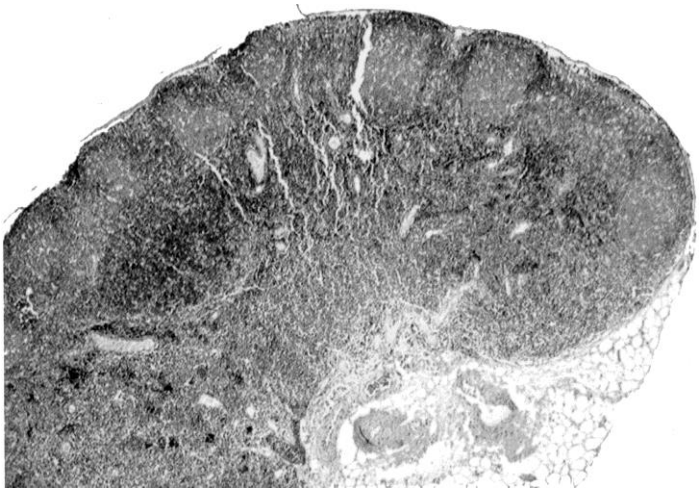

FIGURE 2 – Photomicrograph of histologic section of the draining lymph node corresponding to day 30 postoperative intraquadricipital AAX bone implant. OX-22 + lymphocytes are observed at the follicular area (outer cortex) surrounding germinal centers. (Stain, Anti-CD45RC (OX-22) monoclonal)

Anti-rat αβTCR monoclonal antibody was positive in cells at the peripheral cortex from day 10 to day 30 postoperative in all excised lymph nodes, including those from controls. All day 60 specimens, however, failed to stain positive for anti-rat αβTCR monoclonal antibody.

Implant site histology

At postoperative day 10, all implant sites showed a local infiltrate at the periphery of all the implants (allogeneic, xenogeneic, and gelatin capsules), consisting of lymphocytes, histiocytes, neutrophils, eosinophils and plasma cells. Round lymphocytes were more abundant around allogeneic and xenogeneic bone implants.

Day 20 and 30 studies showed an infiltrate around the bone-derived implants consisting of round lymphocytes and some plasma cells predominantly around xenogeneic non-pepsinized implants. A thin layer of this infiltrate could also be observed around the gelatin capsule structure.

The outer layer of infiltrate was surrounded by fibroblasts that on day 30 already formed a layer of fibrous tissue that persisted on specimens from days 45 and 60.

The cell infiltrate at days 45 and 60 was scarce – represented by round lymphocytes lining the partially resorbed, outer part of the bone

implants. A thin fibrous layer lined the surface of the bone implant, separating it from surrounding muscle fiber.

Monoclonal antibody technique showed anti-αβTCR + cells in the infiltrate around all the implants at day 10 postoperative. Day 20 and 30 specimens showed anti-αβTCR + and also some anti-CD45RC (OX-22) + cells infiltrating the periphery of all bone-derived implants.

Day 45 and 60 implants grossly showed a decreased anti-αβTCR + and anti-CD45RC (OX-22) + cell infiltrate along with the presence of anti-CD8a (OX-8) cells lining the periphery of the bone-derived implants.

4 – DISCUSSION

Our results showed that all animals receiving heterotopic implants of bone-derived material formed new bone within the implants no matter what the animal source (xenogeneic or allogeneic) and no matter what the antigen-depletion technique (telopeptide-depleted or non depleted) used in our experiment.

Other authors, including Guizzardi, et al.[12], found similar results in bone formation comparing xenogeneic demineralized bone matrix implants to autologous grafting in a spinal fusion model in rats. Other authors[24] found that the loss of immunogenicity of the processed bone implants coincided with a decrease of graft incorporation in a tibial osteotomy model in rats.

To the contrary, early studies on xenografts and allografts[9] indicate that the effectiveness of the graft in terms of osteogenesis could be limited or impaired by the immunologic reactivity of the host. But, in those studies composite grafts were used and -- according to the processing method employed -- most probably all types of implants lacked BMP activity. To relate osteogenesis to immune reactivity, Fukunaga et al.[11] implanted fresh and frozen xenogeneic (rabbit) bone to immunosuppressed rats. The implants induced new bone formation, while non-immusuppressed rats showed necrotic bone xenoimplants. Although those authors reported early new bone formation within the non-demineralized bone implants in the immunosuppressed group, strong evidence based on the analysis of a profuse bibliography doesn't seem to match with the finding of conspicuous new bone formation induced by non-demineralized tissues. Besides, it has not been demonstrated that the immunosuppressive agent used in that experiment (FK 506) could modify the mechanism of the osteoinductive process and, therefore, modify new bone yield.

Urist[31] and Janovec[18] demonstrated the ability of AAA and AAX bone to induce bone formation in rodents. Pepsinizing the bone matrix

as a process to further reduce antigenicity of bone through the extraction of collagen's telopeptides did not alter new bone formation in our experiment. Testing different enzyme-treated bone matrices, Urist and Iwata[32] found that pepsin does not degrade BMP or reduce bone yield in rat bioassays. Pepsin removes a fragment of approximately 15 amino acids from the C-terminal part of the collagen molecule, which carries the P-specific (antigenic) determinant[21].

Major antigenic determinants of collagen are located in the telopeptide region. Furthermore, Rubin et al.[23] demonstrated that the antigenic response to injected heterologous tropocollagen was detected against the peptide appendages (telopeptides) external to the triple helix, and Davison showed that those telopeptides could be largely removed by enzyme treatment, though preserving the collagen triple helix structure unaltered[7].

Much has been published about immune response to bone and bone-derived allo and xeno-implants, both in the clinical and the experimental arena. In the present experiment, we could demonstrate that both xenogeneic and allogeneic material, either pepsinized or not, elicited a qualitatively similar cellular response. This immune reaction did not impair osteoinduction.

The antigeneic determinants in bone have long been discussed. Elves and Salama[9], who studied immune responses to different antigen-depleted bone allo and xenografts, claimed that the immunogenicity of those preparations (D) was due to their cellular or plasma protein content, and not to the purified collagen-mineral matrix[8]. Conversely, Davison et al.[7] affirm that tropocollagen is immunogenic and the serologic activity is species specific.

Recent studies seem to point out that the host response in bone allografting is predominately a cell-mediated response to cell-surface antigens carried by cells in the allograft[26]. The cells of the musculoskeletal tissues display Class I MHC antigens, and frequently a subset of cells displaying class II MHC antigens[25]. According to Horowitz et al[14]., antigens recognized by the T cells are expressed on the cell surface and not in the mineral or bone matrix.

An interesting issue arises when considering the immunocompetent cell's role in skeletal repair. Mice lacking T cells were unable to regenerate bone in a fracture model[3]. Allografted and autografted tibial defects in rats, and also in control rats (tibia osteotomy) showed lymphoid tissue activity following the procedure (enlargement of spleen's white pulp, thickening of regional lymph nodes and increasing of the secondary follicles)[24]. Thus, to avoid the immunocompetent cell response to osteotomy or the bone healing process in the orthotopic

placement of the grafts, we decided to use a heterotopic implant model.

Humoral immune response to implanted materials could not be confirmed in our study, although our approach to studying this process was very simple (electrophoretic study of serum proteins). During the whole course of their experiment (5 weeks in total), Burwell and Gowland[4] found no change in any component of the sera of rabbits receiving heterotopically-placed primary and secondary fresh cancellous bone allografts. Our results also failed to show any statistically significant increase in the postoperative values of the different serum proteins compared to the preoperative values, which were consistent with normal serum electrophoresis values of normal rats[22]. Only α1 globulin showed a consistent increase compared to the preoperative values that persisted from postoperative days 10 to 60 in all animals, including controls. The average increase in those values (12.4%) was statistically significant ($p \leq 0.001$). To our knowledge, altered values of serum α1 globulin have not been related to immunologic reactivity. Even though the control animals showed increased values of α1, with the only variable among the groups being the surgical procedure itself, we hypothesize that this phenomenon could be related to the inflammatory and/or reparative process involved in wound and soft tissue healing. We have not found any reference to this finding in our literature search.

Stevenson et al.[27] think that sustained exposure to donor MHC (that resides in cell membranes) is required for a prolonged production of antidonor antibody. Our processed bone implants lacked any cell component, which favors a weak host reactivity.

Although all implants in our experiment (including gelatin capsules) showed a non-specific cell infiltrate during the first 10 days postoperative, bone-derived implants elicited a cellular immune response that was quite characteristic after day 20. Lymphocyte infiltrate around the implants consisted mainly of peripheral T-cell receptor+ lymphocytes during the first 30 days postoperative and of T helper lymphocytes between day 20 and day 45 postoperative. The immune cellularity around the bone-derived material implants changed to a killer/suppressor type after postoperative day 45. This last infiltrate was consistent with the resorptive-remodeling phenomenon within the bone implants and the ossicle formation.

Friedlander et al.[10] established a direct relationship between the immunologic activity both in allografting events and bone remodeling phenomena. The authors hypothesize that interaction of primed immunocompetent cells (T cells activated by alloantigens) with their targets (bone cells) results in cytokine release, some of which are known to have

regulatory roles in the bone remodeling cycle. Thus, they established a direct relationship between those immunologic events and their biological consequences with respect to skeletal repair. Horowitz et al.[16] postulate that activated T cells secrete a genetically programmed set of cytokines that in turn activate macrophages to secrete interleukin-1, which is also a potent stimulator of osteoblasts. Osteoblasts can subsequently induce osteoclastic bone resorption. This phenomenon can be observed in a heterotopic model, as the one we used in our experiment.

Our anatomic studies of the draining lymph nodes showed a conspicuous enlargement in all animals, including controls, during the first 30 days postoperative. This enlargement subsided later on. Ager et al.[1] found that in the draining lymph nodes of rats an increase in lymphocyte traffic can be elicited by the trauma of injection of a substance that needs not to be antigenic. An increase in blood flow to the whole lymph node is not essential to all increases in lymphocyte influx. Thus, our finding of node enlargement in all animals, including controls, up to day 30 does not necessarily represent a true immunologic activity at the node level but a reactive pattern of response to surgical implantation of materials. Moreover, others found that there is no linear relationship between rat lymph node weight changes and the extent of lymphocyte recruitment[19]. Schratt et al.[24] found lymph node thickening in bone autografted rats.

Our monoclonal antibody studies of the draining lymph nodes showed a normal pattern of distribution of B and T cells[5]. After day 45, however, a decrease in TCR + lymphocytes was observed at the peripheral cortex area, and a parallel decrease was observed at the implant site of all bone-derived material, along with a decrease in weight and volume of the lymph nodes. The simultaneous occurrence of these two facts may suggest a decrease of the lymphocyte traffic to the lymph node. It is important to point out that none of the animals became infected or showed any sign of inflammation at the operated limb during the course of the experiment.

There was no change in spleen anatomy or histology in any group of animals, although some studies suggest that the spleen may have a primary role in xenograft rejection in rodents[29]. Schratt et al.[24] found some changes in the spleen of rats after surgical implants of bone derivates, even after a simple osteotomy, but their experiments consisted of the orthotopic placement of implants which involves the mechanisms of fracture healing in the grafting process.

We did not observe any qualitative difference in the local cellular immune response to the implanted bone-derived materials, whether of allogeneic or xenogeneic origin. Moses et al.[20] consider that many of the

cellular and antigenic requirements of the effector phase of a xenogeneic response are similar to those of allogeneic response. Furthermore, the cell population mediating xenogeneic responses and the target antigens recognized by xenoreactive T cells are largely the same as those found in allogeneic responses. They found that a difference of potentially major importance is the possibility of a potent NK-like cytotoxic effector pathway for xenoantigens.

In our study, we could not find any conspicuous difference in new bone formation or in the pattern of immunocompetent cellularity responding to differentially antigen-extracted bone derivates taken from either an allogeneic or xenogeneic source.

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