Massive deep-frozen bone allografts : contamination, immunogenicity and clinical use

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Chapter 3

Human bone allografts can induce T cells with high affinity for donor antigens

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ABSTRACT

We analysed the cellular immune response in ten transplantations of different massive bone allografts, of which five had a poor clinical outcome. Cytotoxic T lymphocytes (CTL) and T helper lymphocytes against mismatched donor antigens were found in all patients. More importantly, CTL with a high affinity for donor antigens were found in five cases. High-affinity CTL need no CD8 molecule to stabilise the antigen binding and are strongly associated with rejection of heart and corneal transplants. Even after removal of most of the bone-marrow cells, we found high-affinity CTL and high TH frequencies. This T-cell response could be detected over a period of years.

We conclude that frozen bone allografts can induce high-affinity donor-specific CTL. The present assay allows qualification and quantification of the levels of CTL and TH in the blood. This approach may be helpful in studying the effect of the immune response on the outcome of the graft.

INTRODUCTION

Frozen bone allografts are used without HLA matching or immunosuppressive drugs. Deep-freezing kills most bone cells and reduces the immunogenicity significantly, but frozen bone allografts may still evoke an immune response. [1-7] The precise mechanism of this and its effect on incorporation of the graft are poorly understood.

Donor-specific antibodies and cell-mediated immunity have been found after transplantation of frozen bone allografts, [3,5-8] as have histological signs suggestive of immunological rejection. [5,9] Animal studies have shown a beneficial effect of histocompatibility matching and the use of immunosuppressive drugs on the incorporation of frozen allografts, [10,11] but in man there are still difficulties in correlating the immunological response with the outcome of the graft.[5,7,8]

Chronic rejection of allografts is considered to be mediated primarily by T cells rather than antibodies. [12-15] Therefore the most direct way to assess the importance of the immune response in transplantation of a bone graft is to analyse T-cell characteristics and incorporation of the graft. This hypothesis is supported by the accepted central role of T cells and their secreted cytokines in the regulation of bone remodelling. [4,16]

T cells can be divided into two subsets with distinctive functions, cytotoxic T lymphocytes (CTL) which recognise HLA class-I antigens and T helper lymphocytes (TH) which recognise HLA class-II antigens. The major function of the CTL in the allograft response is presumably to act as cytotoxic effector cells causing tissue damage. One of the functions of TH cells is to help in the maturation of CTL. Binding of foreign antigen to the T-cell receptor is stabilised by the CD8 molecule on the CTL and by the CD4 molecule on the TH.

Stimulation in vivo with donor antigens (priming) leads to the development of CTL resistant to inhibition by monoclonal antibodies against CD8 (anti-CD8).[17-19] In contrast to naïve CTL, these primed CTL appear to have a high affinity for donor antigens since there is no need for CD8 molecules to stabilise antigen binding. [17] In heart and corneal transplants, the presence of high-affinity CTL in the peripheral blood has been strongly correlated with immunological rejection. [18-20] Increased frequencies of circulating donor-specific TH have also been associated with immunological rejection of heart transplants. [21]

We have studied whether such high-affinity cells are present in human recipients of frozen bone allografts, and if qualification and quantification of the cellular immune response were possible. The frequencies of circulating CTL and TH cells directed against mismatched donor antigens were determined by limiting dilution analysis (LDA) assays. The affinity of donor-specific CTL was determined on the basis of their resistance in vitro to anti-CD8.

PATIENTS AND METHODS

We studied seven patients who had received a total of ten massive frozen allografts after resection of a primary bone tumour (Table I). Recipients of different sizes and types of allograft were selected to evaluate the effect of the antigen load on the immunogenicity. Six grafts were osteoarticular and two were allograft-prosthesis composites (cases 1B, 6A). Of the latter, the cartilage and (most) bone-marrow cells were removed. Two fibular allografts were used for intramedullary fixation of the allograft. The two female patients had not been exposed to alloantigens by pregnancy. Two patients (cases 1B, 5B) received blood transfusions before and four (cases 1A, 3, 5A, 6) after bone transplantation. Four had had chemotherapy but their white blood counts were within normal limits at the time of the study.

All grafts (Netherlands Bone bank Foundation (NBF), Leiden, The Netherlands) were processed under sterile conditions and stored at -80° C without additional sterilisation. The articular cartilage was preserved with 8% dimethyl-sulphoxide. The fibular grafts were processed, defatted and washed in 98% ethanol. The selection of the graft was based on a radiological comparison of the size of the allograft and the resected bone (Table I). All donors and recipients were typed for HLA class-I and class-II antigens before grafting using standard serological methods. Transplantation was performed without any respect to the HLA match (Table II).

The patients were followed for more than three years. We especially looked for complications which were potentially related to an immune response against the allograft, such as inadequate union, massive resorption, fractures and infections.[22] We considered the outcome of the graft to be good if host-graft union took place within one year and no complications occurred. Five transplantations had a good graft outcome and five were poor (Table I). One allograft was resected 28 months after operation

because of a fracture and a new graft was transplanted (case 1B). In one patient (case 5A/B) a chronic infection developed 24 months after refixation and amputation was necessary. Nonunion occurred in four patients (cases 1A, 4, 5A, 6A). They were treated successfully by autogenous grafting and refixation.

Collection of blood samples and isolation of peripheral blood mononuclear cells

Sera for antibody determination and mononuclear cells for the LDA assay were isolated 1 to 41 months after transplantation.

Antibody formation

The sera from each patient were tested against a panel of at least 50 HLA-typed lymphocyte donors. The reactivity was expressed as a percentage of panel reactive-antibodies (PRA).

Combined limiting dilution analysis (LDA) assay

Precursor frequencies of CTL and TH were determined in a combined LDA assay (Fig. 1).[23] Peripheral blood mononuclear cells of patients were used as responder cells. They were cultured in RPMI 1640, Dutch modification (Gibco, Paisley, UK) with 3 nM L-glutamine (Gibco) and 10% pooled human serum (referred to as complete medium). These responder cells were set up in 12-replicate wells at 40 000 cells per well in 50 µl of medium with serial twofold dilution across two 96-well V-bottom plates (Greiner, Frickenhausen, Germany). Concentrations of responder cells therefore ranged from 40 000 to 625 cells per well. Control wells were prepared containing no cells.

As stimulator cells we used cells obtained from the donor's spleen. When splenic cells were not available we used peripheral blood mononuclear cells from healthy volunteers who carried HLA antigens for which the bone donor was mismatched to the patient. The stimulator cells were irradiated with 50 Gy to prevent proliferation. They were added to the responder cells at 50 000 cells per well in 50 µl of complete medium.

After three days of culture at 37°C in a humidified atmosphere containing 5% CO_2, the supernatants were harvested (80 µl per well) and trans-

ferred to U-bottom 96-well plates (Costar, Cambridge, Massachusetts). To determine TH frequencies, the supernatants were tested for the presence of interleukin-2 (IL-2) in the CTLL-2 bio-assay as described below.

The remaining cells were transferred to U-bottom plates and were cultured for another seven days at 37°C in a humidified atmosphere containing 5% CO_2 in the presence of 20 U/ml of IL-2 (human recombinant IL-2; Cetus, Amsterdam, The Netherlands) in 200 µl/well of complete medium. To determine CTL frequencies, a cell-mediated lympholysis (CML) assay was performed as described below.

Table 1. Details of the seven patients who had undergone transplantations of frozen bone allografts

							Graft outcome†			
Case	*Age (yr)*	*Gender*	*Allograft length (cm)*	*Reconstruction*	*Fit & Fixation**	*Adjuvant therapy*	*Union*	*Complications*	*Secondary procedures*	*Follow-up (mth)*
1A	15	F	Tibia prox (12.5)	Osteoarticular	Discrepancy	CT‡	Nonunion	Fatigue fracture graft at 28 mth	Autograft at 12 mth New graft at 28 mth	28
1B	17	F	Tibia prox (12.5)	Allograft-prosthesis	Good	-	<0.5 yr	-	-	37
2	31	F	Femur dist (7)	Osteoarticular	Good	-	<1 yr	-	-	50
3	46	M	Tibia prox (10)	Osteoarticular	Discrepancy	CT	<1 yr	Genu Varum due to subchondral collapse	Correction osteotomy	44
4	29	M	Femur dist (18)	Osteoarticular	Good	-	Nonunion	-	Autograft at 7 mth	43
5A	53	M	Tibia prox (15)	Osteoarticular	Discrepancy Unstable	CT	Nonunion	Traumatic fracture at 11 mth Infection at 35 mth	Refixation plus fibular allo - graft (intramedullary) Amputation at 41 mth	41
5B	54	M	Fibula	Intramedullary	-	-	<1 yr	Infection at 24 mth	Amputation at 30 mth	30
6A	21	M	Humerus (12)	Allograft-prosthesis	Good	CT	Nonunion	Resorption, cortical erosions	Autograft at 4 mth	
6B	21	M	Fibula	Intramedullary	-	-	-	-	-	40
7	22	M	Radius prox (7.5)	Osteoarticular	Good	-	<1 yr	-	-	39

* fit is considered good if discrepancy of articular surfaces is less than 4 mm and the gap at the osteosynthesis site is less than 1.5 mm
† graft outcome is considered good if union took place within one year and no complications occurred
‡ chemotherapy

CTLL-2 bio-assay

To determine the amount of IL-2 present in the supernatants we used an IL-2-dependent murine CTLL-2 cell line.[24] The supernatants were thawed and CTLL-2 cells which had been cultured in IL-2-free medium for 24 hours were added at 3000 cells/well to the plates. After incubation for three days at 37 °C added lying-staining-quenching medium containing Triton-X-100 (Fluka, Buchs, Switzerland) ink (Leitz, Wetzlar, Germany) and propidium-iodide (Sigma, St. Louis, Missouri) in EDTA buffer. The plates were read by automated fluorescence microscopy (Leica-Patimed, Wetzlar, Germany) which measures photometer values (mV) to determine the number of propidium-iodide-stained nucleated cells and thus the pro-

liferation of the CTLL-2 cells. The proliferation of the latter is dependent on the amount of IL-2 present in the supernatants and is a measure of the number of IL-2-secreting cells present in the responder-cell fraction. TH-cells are considered to be mainly responsible for the production of IL-2.[2]

Table II. Frequencies and affinity of T cells directed against mismatched donor HLA antigens, determined by combined limiting dilution analysis. High-affinity CTL are resistant to inhibition by anti-CD8

	Mismatched donor HLA antigens				*CTL freq*			*Panel-reactive anti-bodies (%)*	*Time blood sample (mth)†*
Case	*A*	*B*	*DR*	*Tested HLA mismatches*	*-anti-CD8*	*+anti-CD8 (% inhibition)*	*Th freq**		
1A	2	8	3, 15	A2, B8 ‡	308	258 (16)		50	34
				A2 ‡	53	4 (92)			
				B8 ‡	63	9 (87)			
				DR3, DR15 ‡			1425		
1B	32	17	Unknown	B17	344	13 (96)		50	7
				A32	39	0 (100)	NT1		
2	32	61	4	B61	20	0 (100)		0	41
				DR4§			146		
3	2	44, 38	13, 7	B38, B44	32	13 (59)		0	32
				DR13, DR7§			495		
4	2, 24	44, 38	7	B38, B44	87	43 (51)		86	31
				DR7§			597	—	
5A	24, 26	50	None	A26	8	0 (100)		NT¶1	12
				A24	32	8 (75)	NT¶2		
5B	1	7, 8	15, 17	A1, B7, B8	80	116 (-45)		NT¶	1
				DR15, DR17 §			105		
6A	1, 24	18, 35	15, 11	—		NT¶		77	11
				DR15, DR11 ‡			853		
6B	29, 32	61	4, 7	—		NT¶		77	11
				DR4, DR7 ‡			167		
7	2	7	15	A2, B7	431	313 (27)		67	14
				A2	396	146 (63)			
				B7	271	140 (48)			
				DR15§			51		

* frequencies: per 10⁶ tested peripheral blood cells
† time that has elapsed between transplantation and blood sampling
‡ donor spleen cells used as stimulator
§ DR typing of stimulator is identical to DR typing of donor
¶ NT= not tested; NT1 = DR donor unknown; NT2 = no patient-donor DR mismatches

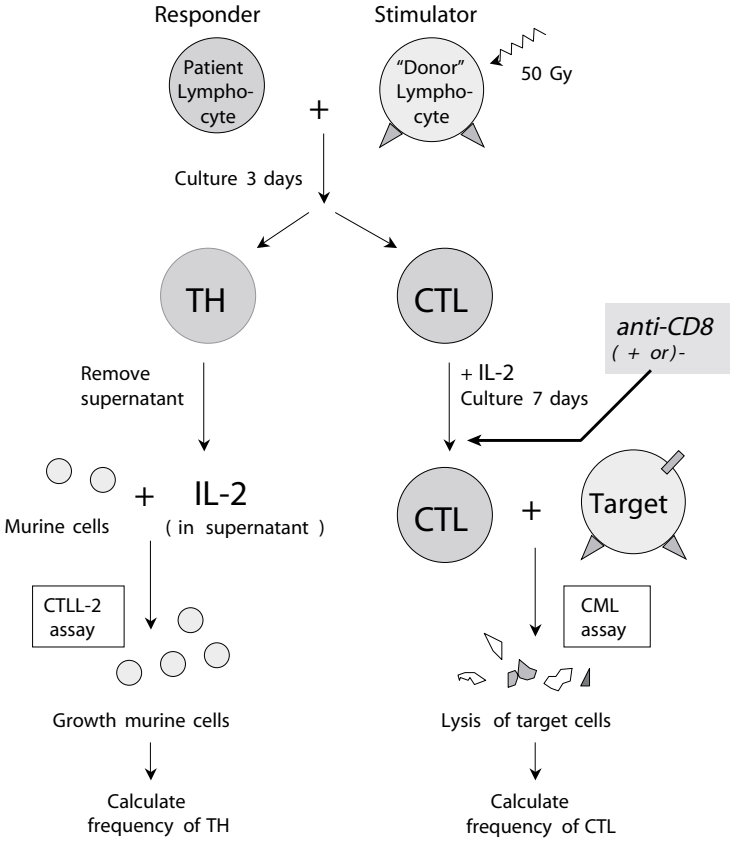

Figure 1. Diagram showing the combined LDA assay, to estimate the fre - quencies of CTL and TH from the peripheral blood. Before the addition of the target cells, half of the culture is incubated by anti-CD8 to test the affinity of CTL for donor antigens.

CML Europium release assay

The target cells which we used were obtained from the donor's spleen or, when not available, peripheral blood mononuclear cells from healthy volunteers which were completely mismatched with the stimulator cells except for the HLA antigens which the stimulator cells shared with the bone donor. These HLA antigens are indicated as 'tested HLA-mismatches' in Table II.

Before addition of the target cells, the cultures were divided into two groups. Half of the wells were incubated with a monoclonal antibody against CD8 (anti-CD8) (FK 18; F. Koning/A.Mulder, Leiden, University Medical Center, The Netherlands).

The Europium release assay was performed as described by Bouma et al.[26] Europium-labelled target cells were added at 5000 cells per well to the responder-stimulator combinations. Each well was tested for cytolytic activity against Europium-labelled target cells in a four hour assay at 37°C in a humidified atmosphere containing 5% CO_2. Supernatants were harvested and fluorescence of the released Europium, due to target-cell cytolysis, was measured in a time-resolved fluorometer (Arcus 1234; Wallac, Turku, Finland).

Evaluation of immunological data

For CTL and TH frequencies the mean counts per second (CTL) or photometer values (TH) and the standard deviation (SD) of the wells in which only stimulator cells were present were calculated. Wells were scored as positive if the counts or photometer values in the well exceeded the mean plus 3 SD of the wells in which only stimulator cells were present. LDA predicts, according to a Poisson distribution, that if 37% of the tested wells are negative then for that given concentration of responder cells there is average of one CTL or TH precursor cell per well.[27]

RESULTS

Frequencies of CTL

In all patients who received a bone allograft, CTL frequencies directed against mismatched donor HLA class-I antigens were detectable (Table II). These varied from 8 to 431 cells per million peripheral blood cells. As expected, the frequencies of CTL against individual HLA antigens were lower than those against a group of mismachted HLA antigens (cases 1A, 7). Frequencies of CTL in patients with good and poor graft outcome were within the same range.

Frequencies of CTL with high affinity

After addition of anti-CD8 in the cytotoxic phase, the inhibition of CTL frequencies varied from 0% to 100% (Table II). The CTL were inhibited less if reactivity was tested against a group of mismatched HLA antigens as compared with individual mismatched HLA antigens (cases 1A, 7). In five patients (cases 1A, 3, 4, 5B, 7) the CTL demonstrated inhibition of less than 60%, showing that a considerable proportion of the CTL had a high affinity for donor HLA antigens. Two of these patients had never been exposed to alloantigens by blood transfusions or pregnancy. All but one of the patients with anti-CD8-resistant CTL had a poor graft outcome (Table I). Of the three patients (cases 1B, 2, 5A) with CTL responses which could be inhibited by anti-CD8 when tested against single HLA antigens, two had a good graft outcome.

Frequencies of TH

The TH frequencies were only determined in patient-donor combinations with at least one HLA-DR mismatch (Table II). In three patients (cases 1A, 6A, 6B) donor spleen cells, instead of peripheral blood mononuclear cells which carried the HLA-DR antigens of the donor, had been used as stimulator. TH frequencies against mismatched donor HLA class-II antigens were detected in all tested patients. The TH frequencies varied from 51 to 1425 per million peripheral blood cells. The four highest frequencies were found in the patients with poor graft outcome (cases 1A, 3, 4, 6A). Relatively low frequencies of TH were found against donor HLA antigens of the defatted fibulae (cases 5B, 6B).

Antibodies

Panel-reactive antibodies were found in four patients (cases 1, 4, 6, 7) (Table II). All had antibodies against donor HLA-A or HLA-B antigens.

There was no clear relationship between CTL and TH characteristics and the presence or absence of an antibody response.

DISCUSSION

We have investigated the frequencies and affinity of cytotoxic T lymphocytes (CTL) and T helper lymphocytes (TH) directed against mismatched donor HLA antigens in the peripheral blood of recipients of frozen bone allografts. Significant frequencies of CTL and TH were found in all recipients. This agrees with the findings of Stevenson [6] and Musculo et al[5] who found a cell-mediated immune response after transplantation of frozen bone allografts. More importantly, we detected CTL with high affinity for donor-specific HLA antigens which were resistant to inhibition by monoclonal antibodies against CD8 (anti-CD8).

We found that the CTL frequencies after transplantation of frozen bone allografts were in the same range as those after corneal transplantation.[20] The clinical significance of absolute CTL frequency determinations in cultures from the peripheral blood is not completely clear. [19,28] In mice, an increase of CTL frequency was associated with allograft rejection. In man, the presence of high-affinity donor-specific CTL, more than absolute frequencies, has been correlated with heart and corneal allograft rejection.[18,20]

In about half of the recipients of frozen bone grafts, a considerable proportion of CTL could not be inhibited by anti-CD8. The occurrence of anti-CD8-resistant, high-affinity CTL is usually the result of activation in vivo by donor-specific HLA class-I antigens. [19] These primed CTL apparently have a high affinity for the target since there is no need for the CD8 molecule to stabilise antigen binding. [17] Thus, our results show that frozen human allogenic bone can prime CTL and that these cells can be found in the peripheral blood.

The maturation of CTL strongly depends on the presence of IL-2, mainly produced by TH. [25] In heart transplants, there is a correlation between increased frequencies of circulating donor-specific TH and graft rejection.[21] We found the highest TH frequencies in the patients with a poor graft outcome, which may suggest that a high TH frequency is predictive of rejection of bone graft. The TH can be stimulated by the direct presentation of class-II antigens on surviving donor cells, or by the indirect presentation of donor-derived antigens (peptides) by host antigen-presenting cells. After transplantation of non-viable frozen bone allografts, antigens are most likely presented by the indirect route and hereby primarily activate TH. [12] The activated TH secrete cytokines which can induce resorption of osteoclastic bone and failure of graft incorporation. [4,16]

In our study, the presence of HLA-specific antibodies did not always correlate with T-cell characteristics. For example, we found primed CTL and high frequencies of TH in a patient who did not develop specific HLA antibodies. The correlation between the humoral and cellular response to alloantigens is not completely clear.[28-30] Roelen et al[19,31] found that the presence of high-affinity CTL may be related to the type of HLA mismatch ant the immunoglobulin class of the antibodies. Strong et al [7] observed formation of antibodies in about 70% of recipients of massive osteochondral allografts. None the less, most sensitised patients had a good clinical outcome. On the other hand, Nelson et al [8] have found a correlation between donor-specific IgG antibodies and bone graft outcome. Many, however, consider, the formation of antibodies to be not directly relevant to the chronic rejection process of non-viable frozen bone allografts, which is primarily T-cell mediated.[12,13]

We found high-affinity CTL and high TH frequencies even after transplantation of grafts, from which most cells had been removed. Primed CTL were found after transplantation of a processed fibular graft (case 5B). The interpretation of this T-cell response was complicated by the development of an infection, which was noticed clinically months after the immunological testing. Although an infection will cause massive infiltration of the graft with immune cells, [16] the presence of high-affinity donor-specific CTL is clearly the result of a specific immune response. A high frequency of TH was detected after transplantation of a graft from which most bone-marrow cells and cartilage had been removed (case 6A). In these cases, the cellular immune response may be evoked by the remaining cells in the endosteal surface or perhaps by non-cellular antigens such as collagen and matrix proteins. [16,32,33]

The cellular response could be measured over a long period. We found high-affinity CTL and high frequencies of TH more than two years after transplantation. A massive bone allograft is a so-called depot of antigen which may provide a slow but continuous release of antigens over a long period of time, probably facilitated by low-grade, persistent resorption.[30]

The interpretation of our data on the induction of an immune response by frozen bone allografts is complicated by the exposure of some patients

to alloantigens by previous blood transfusions. HLA-mismatched blood transfusions can also induce high-affinity CTL against blood donor-specific antigens.[34] However, considering the enormous polymorphism of the HLA-system, the probability of the same mismatch between the patient and bone or blood donor is extremely small. On the other hand, the presence of donor-specific CTL with high affinity in two men (cases 4, 7), who had never received blood transfusions, shows clearly the induction of high-affinity CTL by frozen bone allografts. These high-affinity donor-specific CTL appear after stimulation in vivo (priming) with donor antigens and are not present before transplantation.[17-19]

Although not the primary goal of our study, we also looked for a correlation between T-cell characteristics and graft outcome. Patients with high frequencies of TH and those with high-affinity CTL more often showed poor graft outcome. No clear relationship was found in our diverse group. Poor fit and fixation may be responsible for some of our observed nonunion as may the adjuvant chemotherapy. [35] Each can lead to failure in the absence of a considerable immune response. On the other hand, of the three patients with good fit and fixation and no chemotherapy, only the one with high-affinity CTL and high TH frequency showed no incorporation of bone graft. A multi-factorial analysis of a larger and more uniform group is necessary to establish the role of the immune response on the incorporation of the graft.

The in vitro assay which we used allows the quantification and qualification of the T-cell response in recipients of frozen bone grafts. The current approach is attractive because it is non-invasive and can be carried out as often as required. Although the graft itself is considered to be the most informative site for the detection of cells which are relevant for rejection,[5,18,28] a bone transplant biopsy is difficult to obtain and the cellular infiltrate is generally not uniform and difficult to interpret.[9,30]

In conclusion, frozen massive bone allografts are capable of inducing CTL with high affinity for donor-specific HLA antigens. Participation of donor-specific TH in the immune response was also shown. The present in vitro assay allows quantification and qualification of these donor-specific CTL and TH responses in the peripheral blood. Since the chronic rejection of the allograft is considered to be primarily mediated by T-cells, [12-15] with an important role for high-affinity CTL and TH, [18,20,21] our approach may be helpful in establishing the effect of the immune response on the incorporation of bone graft.

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REFERENCES

1. *Burwell RG.* Studies in the transplantation of bone: V. The capacity of fresh and treated homografts of bone to evoke transplantation immunity. *J Bone Joint Surg [Br]* 1963;45-B:386-401.
2. *Elves MW.* Cell mediated immunity to allografts of fresh and treated bone. *Int Orthopaedics* 1978;171-5.
3. *Friedlaender GE.* Immune responses to osteochondral allografts: current knowledge and future directions. *Clin Orthop* 1983;174:58-68.
4. *Friedlaender GE, Horowitz MC.* Immune responses to osteochondral allografts: nature and significance. *Orthopaedics* 1992;15:1171-5.
5. *Musculo DL, Ayerza MA, Calabrese ME, Redal MA, Araujo ES.* Human leukocyte antigen matching, radiographic score, and histologic findings in massive frozen bone allografts. *Clin Orthop* 1996;326:115-26.
6. *Stevenson S.* The immune response to osteochondral allografts in dogs. *J Bone Joint Surg [Am]* 1987;69A:573-82.
7. *Strong DM, Friedlaender GE, Tomford WW, Springfield, DS, Shives TC, Burchardt H, Enneking WF, Mankin HJ.* Immunologic responses in human recipients of osseous and osteochondral allografts. *Clin Orthop* 1996;326:107-14.
8. *Nelson K, Hoekema J, Yaldock W, Chloupek S, Bazzano R, Sundling NA, Strong DM, Conrad III EU.* Humoral immunity to bone allografts: development of IgG to donor alloantigens is associated with delayed union and poor function. *Hum Immunol* 1994;40 (Suppl 1):8.
9. *Enneking WF, Mindell ER.* Observations on massive retrieved human allografts. *J Bone Joint Surg [Am]* 1991;73A:1123-42.
10. *Stevenson S, Li XQ, Martin B.* The fate of cancellous and cortical bone after transplantation of fresh and frozen tissue-antigen-matched and mismachted osteochondral allografts in dogs. *J Bone Joint Surg [Am]* 1991;A:1143-56.

11. Welter JF, Shaffer JW, Stevenson s, Davy DT, Field GA, Klein L, Li XQ, Zika JM, Goldberg VM. Cyclosporin A and tissue antigen matching in bone transplantation: fibular allografts studied in the dog. *Acta Orthop Scand* 1990;61:517-27.

12. Czitrom AA. The immune response: the afferent arm. *Clin Orthop* 1996;326:11-24.

13. Horowitz MC, Friedlaender GE, Qian H-Y. The immune response: the efferent arm. *Clin Orthop* 1996;326:25-34.

14. Sayegh MH, Watschinger B, Carpenter CB. Mechanism of T cell recognition of alloantigen: the role of peptides. *Transplantation* 1994;57:1295-302.

15. Tilney NL, Kupiec-Weglinski JW. Advances in the understanding of rejection mechanism. *Transplant Proc* 1989;21:10-3.

16. Horowitz MC, Friedlaender GE. The immune response to bone grafts. In: Friedlaender GE, Goldberg VM, eds. *Bone and cartilage allografts.* Park Ridge: AAOS, 1991:85-101.

17. MacDonald HR, Thiernesse N, Cerottini JC. Inhibition of T-cell mediated cytolysis by monoclonal antibodies directed against Lyt-2: heterogeneity of inhibition at the clonal level. *J Immunol* 1981;126:1671-5.

18. Ouwehand AJ, Baan CC, Roelen DL, Vaessen LM, Balk AH, Jutte NH, Bos E, Claas FH, Weimar W. The detection of cytotoxic T cells with high-affinity receptors for donor antigens in the transplanted heart as a prognostic factor for graft rejection. *Transplantation* 1993;56:1223-9.

19. Roelen D, Datema G, Van Bree S, Zhang L, Van Rood J, Claas F. Evidence that antibody formation against a certain HLA alloantigen is associated not with a quantitative but with a qualitative change in the cytotoxic T cells recognizing the same antigen. *Transplantation* 1992;53:899-903.

20. Roelen DL, Van Beelen E, Van Bree SP, Van Rood JJ, Völker-Dieben HJ, Claas FH. The presence of activated donor HLA class I-reactive T lymphocytes is associated with rejection of corneal grafts. *Transplantation* 1995;59:1039-42.

21. DeBruyne LA, Ensley RD, Olsen SL, Taylor DO, Carpenter BM, Holland C, Swanson S, Jones KW, Karwande SV, Renlund DG, Bishop DK. Increased frequency of alloantigen-reactive helper T-lymphocytes is associated with human cardiac allograft rejection. *Transplantation* 1993;56:722-7.

22. Murphey MD, Sartoris DJ, Bramble JM. Radiographic assessment of bone grafts. In: Habal MB, Reddi AH, eds. *Bone grafts and bone substitutes.* Philadelphia: WB Saunders Company, 1992:9-36.

23. Bouma GJ, Van der Meer-Prins EM, Van der Woude FJ, Van Rood JJ, Claas FH. Relevance of pre-transplant donor-specific T cell allorepertoire for human kidney graft survival. *Transplantation* 1995;59:969-76.

24. Schanz U, Roelen DL, Bruning JW, Kardol MJ, Van Rood JJ, Claas FH. The relative radio-resistance of interleukin-2 production by human peripheral blood lymphocytes: consequences for the development of a new limiting dilution assay for the enumeration of helper T lymphocyte precursor frequencies. *J Immunol Methods* 1994;169:221-30.

25. Lin Y, Langman R, Cohn M. The priming of cytotoxic T-cell precursors is strictly helper T cell dependent. *Scand J Immunol* 1992;35:621-6.

26. Bouma GJ, Van der Meer-Prins PM, Van Bree FP, Van Rood JJ, Claas FH. Determination of cytotoxic T-lymphocyte precursor frequencies using Europium labeling as a nonradioactive alternative to labeling with Chromium –51. *Hum Immunol* 1992;35:85-92.

27. Sharrock CE, Kaminski E, Man S. Limiting dilution analysis of human T-cells: a useful clinical tool. *Immunol Today* 1990;11:281-6.

28. Orosz CG, Zinn NE, Sirinek L, Ferguson RM. In vivo mechanisms of alloreactivity. I. Frequency of donor-reactive cytotoxic T lymphocytes in sponge matrix allografts. *Transplantation* 1986;41:75-83.

29. Nossal GJ. Immunologic tolerance: collaboration between antigen and lymphokines. *Science* 1989;245:147-53.

30. Stevenson S, Horowitz M. Current concepts review: the response to bone allografts. *J Bone Joint Surg [Am]* 1992;74-A:939-50.

*31. Roelen DL, Van Bree J, Witvliet MD, Habraken I, Van Beelen E, Van't Sant PG, De Waal LP, Van Rood JJ, Claas FH.*IgG antibodies against an HLA antigen are associated with activated cytotoxic T cells against this antigen, IgM are not. *Transplantation* 1994;57:1388-92.

32. Champion BR, Sell S, Poole AR. Immunity to homologous collagens and cartilage proteoglycans in rabbits. *Immunology* 1983;48:605-16.

33. Trentham DE, Townes AS, Kang AH, David JR. Humoral and cellular sensitivity to collagen in type II collagen-induced arthritis in rats. *J Clin Invest* 1978;61:89-96.

34. Van Twuyver E, Mooijaart RJ, Ten Berge IJ, Van der Horst AR, Wilmink JM, Claas FH, De Waal LP. High affinity cytotoxic T lymphocytes after non-HLA-sharing blood transfusion: the other side of the coin. *Transplantation* 1994;57:1246-51.

35. San-Julian-Aranguren M, Leyes M, Mora G, Canadell J. Consolidation of massive bone allografts in limb-preserving operations for bone tumours. *Int Orthop* 1995;19:377-82.