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Evaluation of plasma levels of tumour necrosis factor alpha and interleukin-6 as rejection markers in a cohort of 142 heart-grafted patients followed by endomyocardial biopsy

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The rejection reaction after cell or organ transplantation has to be detected as early as possible in order to conduct optimal immunosuppressive treatment. Among the numerous events leading to rejection, cytokine production, especially of tumour necrosis factor alpha, is particularly important. Interleukin-6 and tumour necrosis factor alpha were investigated in 142 heart-grafted patients in order to define an early peripheral non-invasive marker of an acute rejection that could fit well with myocardial biopsy results. Cytokines were immunoenzymatically measured in blood specimens collected on the day of the endomyocardial biopsy. The values were compared to the grade of heart graft rejection established according to pathological criteria. Plasma interleukin-6 and especially tumour necrosis factor alpha determined on the day of the rejection diagnosis were significantly increased in the patient sample with moderate or severe rejection when compared with mean values of interleukin-6 and tumour necrosis factor alpha in the patient sample without rejection or with mild rejection (P=0·04 and 0·001 respectively). Because high levels of tumour necrosis factor alpha may appear before histological signs, this biological marker could be useful in the follow-up of heart-grafted patients.

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Key Words: Cardiac transplantation, heart-graft rejection, tumour necrosis factor alpha, interleukin-6.

Introduction

The immunological mechanism of rejection of a transplanted heart begins with the recognition of graft antigens and the recruitment of cytotoxic T lymphocytes. Then, together with the release of inflammatory cytokines (interferon-γ, tumour necrosis factor), the proliferation and differentiation of T and B lymphocytes occur, leading to macrophage activation and the release of tumour necrosis factor alpha and beta, interleukin-1 and other messengers. Finally, graft lysis occurs, as a result of the direct action of macrophages, T cells, K cells and of cytotoxic effects mediated by cytokines and other molecules. These mediators might not only be biological markers of rejection but also accurate parameters demonstrating the effectiveness of new immunosuppressive treatments specifically directed towards anti-allograft immunity[1].

Several biological actions are assigned to interleukin-6, particularly regarding immune response, inflammatory reaction and, recently, graft rejection. The latter role has not yet been fully assessed. Studies in vitro have shown that interleukin-6 is involved in T-cell proliferation and activation, in the induction of cytotoxic T lymphocyte clonal expansion[2] and in B-cell differentiation with the development of alloantibodies[3]. Several human clinical studies have described a large increase in serum interleukin-6 levels after organ transplantation, with a subsequent decrease to normal range within 2–3 weeks in uncomplicated cases. Serum interleukin-6 levels substantially increased a few days before or during the episodes of clinical rejection[4].

The pro-inflammatory nature of tumour necrosis factor can account for its participation in the rejection mechanism. Tumour necrosis factor is known to regulate the immunogenicity of the transplanted tissue by amplification of class I[5] and II[6] human leukocyte

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antigen gene expression. Tumour necrosis factor can also regulate stimulation of neoantigen expression on human endothelial cells, which could explain the increase in anti-endothelial cell antibodies in patients undergoing humoral allograft rejection[7]. Moreover, tumour necrosis factor amplifies rejection by a direct cytotoxic effect[8] or via T cell[9] or macrophage[10] action. Tumour necrosis factor appears to induce the release of other cytokines and the appearance of many cytotoxic effector cells. Elevated tissular or plasmatic levels of tumour necrosis factor alpha were found at the time of, or a few days before, a clinical diagnosis of heart rejection[7,11,12]. These data suggest that increased plasma tumour necrosis factor levels in cardiac allograft recipients may be used as a peripheral marker for predicting severe allograft rejection[13]. Treatment with anti-tumour necrosis factor antibodies is known to significantly inhibit cardiac allograft rejection in animals[14,15]. Measurement of plasma tumour necrosis factor levels is quite difficult owing to poor correlation with immunological or inflammatory events, the cyclic release of tumour necrosis factor from stimulated immuno-competent cells and tumour necrosis factor instability in serum and plasma. Thus, continuous monitoring is required to display the peak level[16].

Both interleukin-6 and tumour necrosis factor present the same technical difficulties of measurement[17]. Furthermore, the primary release of pro-inflammatory cytokines is probably triggered by the tissue or the organ of interest, but only hyperactivation of the immune system induces a generalized reaction with high plasma tumour necrosis factor levels. The use of serum cytokines in predicting allograft rejection is under various influences. The nature of these are the immunosuppressive protocols used by various transplantation programmes, the occurrence of opportunistic infections, or the development of ischaemic myocardiopathy.

Up to now, the diagnosis and grading of rejection have been mainly based on endomyocardial biopsy[18] which is invasive and sometimes difficult to interpret. An alternative procedure (or at least a complementary one) involving cytokine evaluation would be of great benefit for early diagnosis, monitoring of cardiac allograft rejection and modulation of immunosuppressive treatment.

This paper aims at studying how interleukin-6 and tumour necrosis factor alpha act as plasmatic biochemical markers for the occurrence and severity of allograft rejection, defined according to clinical and pathological data in a cohort of heart-grafted patients.

Methods

Patients

One hundred and forty-two heart-transplanted patients (129 males, 13 females), 18 to 67 years old (mean=51) were included. The patients presented with end-stage heart disease and were grafted between August 1986 and October 1994 in the Cardiologic University Hospital of Bordeaux. Study A was a 36-month (July 1992–June 1995) clinicobiological and pathological follow-up to determine plasma tumour necrosis factor alpha and interleukin-6. Study B comprised 27 of the 142 patients. They were grafted after July 1992 and had weekly tumour necrosis factor alpha and interleukin-6 determinations during the early post-transplantation period.

Immunosuppressive therapy

Post-transplantation immunosuppression consisted of cyclosporine (6–8 mg . kg^{-1} . day^{-1} in order to maintain plasma levels at 150–200 ng . ml^{-1}), azathioprine (2–3 mg . kg^{-1} according to white blood cell counts) and prednisolone (30–50 mg . day^{-1}).

Endomyocardial biopsies

These were performed weekly for the first 4 weeks after transplantation in patients who developed an allograft rejection, in order to monitor responses to immunosuppressive therapy. Thereafter, endomyocardial biopsies were carried out with decreasing frequency. The grading of allograft rejection, according to the International Society for Heart and Lung Transplantation criteria[18] was determined after histopathological examination for four endomyocardial biopsy specimens: grade 0: absent rejection; grade IA: focal mild rejection; grade IB: diffuse mild rejection; grade II: focal moderate rejection; grade IIIA: multifocal moderate rejection; grade IIIB: diffuse moderate rejection; grade IV: severe rejection. For analysis of our data, we used a simplified clinicotherapeutic classification: on the one hand, no or mild rejection (corresponding to grades 0, IA and IB) and, on the other, moderate or severe rejection (corresponding to grades II, IIIA, IIIB and IV). Table 1 gives the distribution of the 142 allografted patients investigated (i) according to the criteria of the International Society for Heart and Lung Transplantation and (ii) according to our simplified clinicotherapeutic classification. No specimen was classified grade IIIB or IV.

Cytokine determinations

Blood samples from cardiac allograft recipients were collected in sterile EDTA-treated vacuum tubes. After immediate centrifugation, plasma supernatants were stored at −80 °C before analysis. Plasma samples were analysed using ELISA kits for interleukin-6 (Eurogenetics, Belgium) and tumour necrosis factor alpha (Immunotech, France) with specific monoclonal antibodies and labelling systems (biotin-streptavidin for interleukin-6 and alkaline phosphatase for tumour necrosis factor alpha). Interpretation of interleukin-6 and tumour necrosis factor alpha values was done by

Table 1 Distribution of the 142 allografted patients (in number and percentage)

Grade	International Society for Heart and Lung Transplantation classification							Simplified clinicotherapeutic classification	
	0	IA	IB	II	IIIA	IIIB	IV	*No or mild rejection* 0+IA+IB	*Moderate or severe rejection* II+IIIA+IIIB+IV
n	26	28	32	24	32	0	0	86	56
%	18·3	19·7	22·6	16·8	22·6	0	0	60·6	39·4

Table 2 Plasma tumour necrosis factor alpha (TNF-α) and interleukin-6(IL-6) levels in heart-grafted patient groups divided into no rejection or mild rejection (NR) and moderate or severe rejection (R)

	NR group (n=86)			R group (n=56)	
	Mean value (from several specimens of each patient)	Maximum value (during the follow-up)		Simultaneous value (on the day of rejection diagnosis)	Maximum value (during the follow-up)
TNF-α (pg . ml^{-1})	11·0 ± 1·7	28·6 ± 5·1	*0·02* ← *0·05* → *0·001*	95·9 ± 34·0	231·7 ± 49·6
IL-6 (pg . ml^{-1})	8·3 ± 0·8	20·4 ± 3·6	*0·05* ← ns → *0·04*	13·7 ± 2·4	39·8 ± 7·9

Variables are expressed as mean and standard error. *P* values are indicated in italics.
ns=no significant. There is significance when $P<0·05$.

reference with our usual normal range (<10 pg . ml^{-1} and <20 pg . ml^{-1} respectively).

Statistical analysis included the use of the Student t-test and the Gauss Z test for comparison of mean values. The Cox proportional hazards model was used for analysing patients in the 3-month period after transplantation. Variables were expressed as mean and standard error. Results were considered significant when *P* was less than 0·05.

Results

In study A, we included 142 patients. Eighty six experienced no or mild rejection (NR group) and 56 moderate or severe rejection (R group), with repeated tumour necrosis factor alpha and interleukin-6 determinations. For each cytokine we defined a maximum level in the two groups, a mean level in the NR group and a simultaneous level (i.e. the level on the day of the first rejection diagnosis) in the R group. Interleukin-6 and tumour necrosis factor alpha were more raised in the R group than the NR group (Table 2). There was a significant difference between maximum values for interleukin-6 and especially for tumour necrosis factor alpha ($P=0·04$ and $P=0·001$). The significant difference remained when the mean values of the NR group were compared with simultaneous levels ($P=0·05$ and 0·02 for interleukin-6 and tumour necrosis factor alpha, respectively). Nevertheless, only for tumour necrosis factor alpha was the simultaneous level of the R group significantly higher ($P=0·05$) than the maximum level of the NR group. The positive predictive value calculated from plasma tumour necrosis factor alpha higher than 20 pg . ml^{-1} and interleukin-6 higher than 10 pg . ml^{-1} was 65% for each cytokine; the negative predictive value was 68% and 71% with tumour necrosis factor alpha and interleukin-6, respectively. There was no significant increase in predictive values when tumour necrosis factor alpha and interleukin-6 were combined. (Positive predictive value=68%; negative predictive value=72%). We noted among the false positive patients: cyto magalovirus infections, myocardial ischaemia, chronic active hepatitis and thrombotic complications.

Study B comprised the 27 patients followed-up weekly for 3 months since the graft day. There were 8 NR and 19 R, and they constituted a patient sample representative of the 142 patients of study A. The aim was (i) to compare cytokine evolution profiles between NR and R groups and (ii) to define a possible predictive value of tumour necrosis factor alpha and/or interleukin-6 as regards the occurrence of rejection in the first 3 months after transplantation. Figure 1 shows the tumour necrosis factor alpha and interleukin-6 profiles of the two groups. There was no significant difference for interleukin-6, but tumour necrosis factor alpha appeared significantly elevated in the 4 first weeks (and in week 8) after transplantation in the R group. Positive predictive value and negative predictive value calculated with tumour necrosis factor alpha and interleukin-6

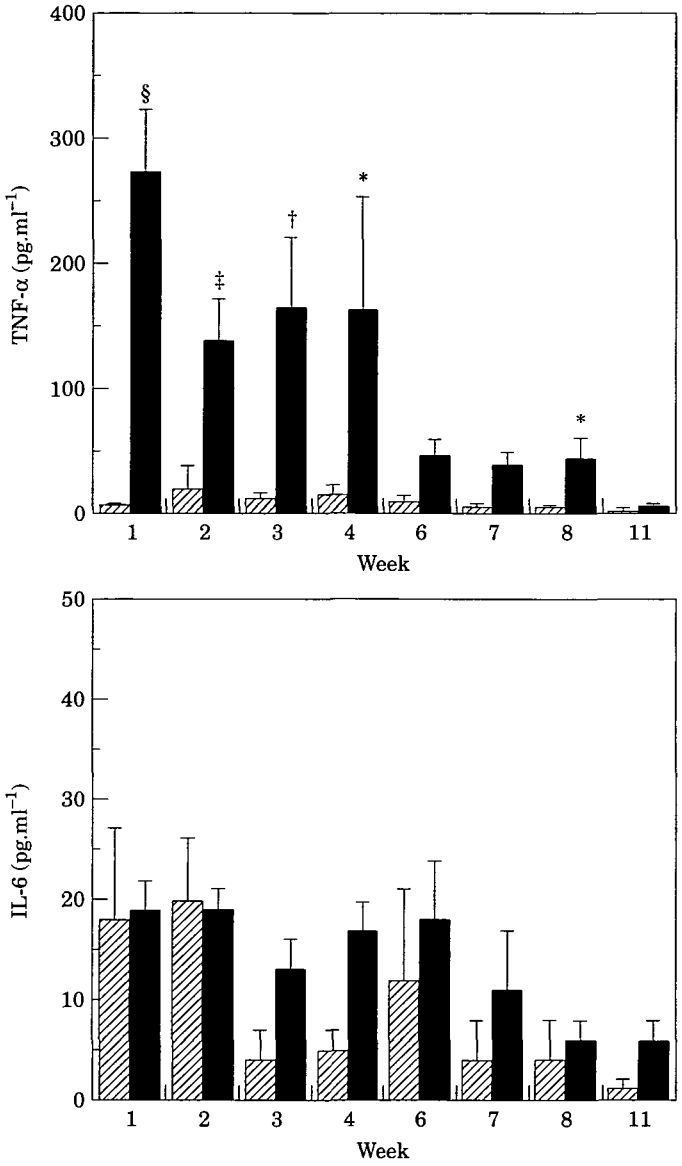

Figure 1 **Comparison between NR (▨) and R (■) groups of weekly plasma levels of tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) for 3 months after cardiac transplantation (in weeks 1, 2, 3, 4, 6, 7, 8, and 11). Not enough data were available in weeks 5, 9, 10 and 12. Vertical bars correspond to standard error. Significance (R vs NR) is as follows: *P=0·05, †P=0·02, ‡P=0·002, §P=0·001.**

associated plasma levels (in the same conditions as before) were, respectively, 90% and 88%, showing an increased predictive accuracy. Nevertheless, by using the Cox model with a time-dependent variable, we noted an absence of significance, meaning that at neither time did tumour necrosis factor alpha have an influence on instantaneous moderate or severe rejection risk. In our sample, the first rejection episode appeared within the 2 months after transplantation, nearly two-thirds of patients rejecting in weeks 2, 3 or 4 (Fig. 2).

In nine patients who developed several episodes of moderate or severe rejection, plasma cytokine levels remained very high (tumour necrosis factor alpha from 100 to more than 1000 pg . ml^{-1} and interleukin-6 from 20 to 100 pg . ml^{-1}) for a long time (from 1 month to 1 year).

Discussion

Up to now, many investigations have been carried out on the role of cytokines in mediating humoral allograft rejection. Only a few have focused on the use of cytokine

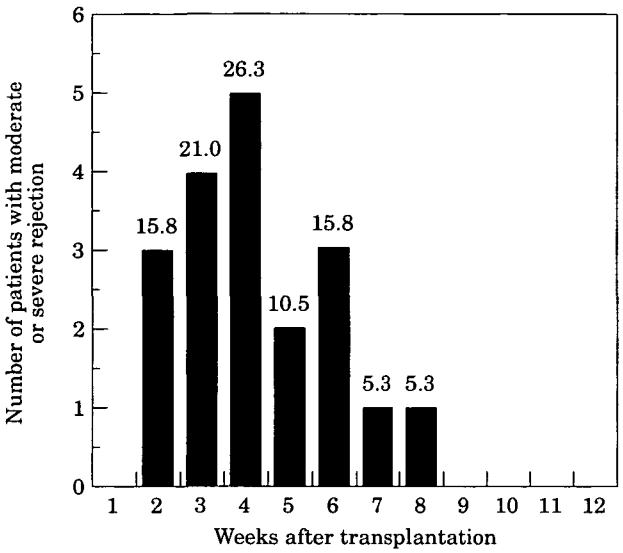

Figure 2 **Distribution of patients with moderate or severe rejection according to time of rejection after transplantation. Percentages are shown in the columns.**

measurements for the biological supervision of heart graft. Some authors[19–22] showed no correlation of plasma cytokine levels with grade of allograft rejection. On the other hand, previous studies reported rises of interleukin-6[4,23] and tumour necrosis factor alpha[7,11,12] in allograft tissue or in peripheral blood, either at the same time or some days before the appearance of pathological and clinical signs of rejection. Our data confirm tumour necrosis factor alpha involvement in moderate or severe allograft rejection episodes. We agree with the observation that after a recent graft, a transitory increase in interleukin-6 could be linked with stress, inflammatory stimulus, 1 severe infection, or myocardial ischaemia[24].

The presence of significantly high levels of tumour necrosis factor alpha only in patients suffering from moderate or severe heart graft rejection indicates that tumour necrosis factor alpha could be an interesting marker of rejection, even if the demonstration of its predictive value is not definitely provided here.

We noted that, although the majority of patients with moderate or severe rejection presented with high levels of tumour necrosis factor alpha, a minority (three out of 19) had normal or slightly elevated tumour necrosis factor alpha. A possible explanation could be inter-individual differences in tumour necrosis factor alpha gene expression[25]. We undertook studies on the tumour necrosis factor alpha gene in heart-grafted patients in order to search for a possible association between tumour necrosis factor alpha gene variation and risk for rejection, as expressed by plasma tumour necrosis factor alpha values.

In conclusion, plasma tumour necrosis factor alpha determination is important and may be a reliable marker of cardiac allograft rejection. This can be checked weekly in the early post-transplantation follow-up period, then monthly. However, patient supervision still requires the use of biopsies. Further investigations, in larger patient cohorts, linking new plasma cytokine determinations with tumour necrosis factor alpha are necessary to specify the use of a relevant cytokine profile in the biological supervision of cardiac transplantation. To explain differences in cytokine production between allografted patients, and in predicting allograft rejection, further studies are needed. Studies on tumour necrosis factor alpha polymorphism and cytokine gene expression in endomyocardial biopsy tissue can contribute to this work.

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