American Journal of Pathology, Vol. 139, No. 4, October 1991
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Rapid Communication

Expression of Tumor Necrosis Factor in Human Acute Cardiac Rejection

An Immunohistochemical and Immunoblotting Study

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The authors performed an immunohistochemical study on expression of tumor necrosis factor alpha (TNFα) in endomyocardial biopsies from human cardiac allografts. TNFα immunoreactivity was found in 45% biopsies with mild acute rejection, in 83% biopsies with focal moderate rejection, in 80% biopsies with diffuse moderate rejection. Biopsies with absent rejection did not show immunoreactive cells. In mild rejection, positive cells were few and scanty monocytes and macrophages (MAC-387 and LN5 positive cells) and T lymphocytes (UCHL-1/CD45 RO positive cells) (up to 20% of all infiltrating cells). Expression of major histocompatibility complex (MHC) class II antigens on infiltrating and endothelial cells occurred earlier and independent of TNFα reactivity. Number of immunoreactive cells increased in moderate rejection (up to 50%). Immunoreactivity was also present in nonpigmented macrophages in part of the biopsies with resolving rejection (45%). The authors conclude that TNFα is expressed in acute cardiac rejection by immunologically activated inflammatory cells. Immunoreactive cells increase in number with increasing severity of the reaction. (Am J Pathol 1991, 139:709–715)

Tumor necrosis factor alpha (TNFα) is a macrophage-derived, 17KDa cytokine naturally occurring in mono or multimeric forms.[1] Previous *in vitro* studies have demonstrated that it is involved in multiple cell-mediated reactions[2–4] such as sepsis[5–7] or immunity.[8–10] TNFα function in immunity is actually under investigation. Chavin et al. demonstrated that antibodies targeted to this cytokine administered *in vivo* at the time of antigen priming can interfere with generation of both CD4 positive, class II restricted T cells and CD8-positive class I-restricted cytotoxic T cells.[11] In fact, in experimental animals TNFα also enhances effects of IFNγ on class I and II antigens expression.[12] TNFα serum levels have been shown to increase during allograft rejection[13] and during antirejection therapy with OKT3.[14] In fact, monotherapy with anti-TNFα antibodies was shown to prolong survival of heart allografts in rats.[15] Anti-TNFα serum can also prevent development of graft versus host disease[16] and abrogates cytokine-related CD3-induced syndrome on BALB/c mice.[17] The authors immunohistochemically investigate TNFα expression, distribution, and relation to infiltrating cells as well as MCH class II antigen expression in a large series of endomyocardial biopsies with acute rejection from patients who had undergone cardiac transplantation.

Materials and Methods

Endomyocardial Biopsy

One hundred fifty biopsies from 43 patients were processed. Forty-one patients had heterotopic and two patients had orthotopic transplants. Endomyocardial biopsies were performed according to Stanford technique.[18] Four to six adequate samples for each procedure were

Supported by grant "Trapianto Cardiaco" from Health Ministry to IRCCCS Policlinico San Matteo, Pavia, Richerche Finalizzate 1989–90.

Accepted for publication August 5, 1991.

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Table 1. *Endomyocardial Biopsies Investigated for TNFα*

	N	All biopsies (%)
Absent acute rejection		
WF-ISHT Grade 0	30	20
Mild rejection		
WF-ISHT Grade 1A-1B	45	30
Moderate rejection		
WF-ISHT Grade 2-3A-3B	63	42.3
Severe rejection		
WF-ISHT Grade 4	1	0.7
Resolving rejection	11	7
	150	100

fixed in 10% buffered formalin (20 minutes), dehydrated in a modified alcohols series (95% alcohol: 15 minutes, and 100% alcohol: 15 minutes) and xilene (15 minutes) and embedded in a single paraffin block. One hundred twenty sections were serially cut, consecutively collected on 40 slides and stained at six cutting levels (every 75 μ) with hematoxylin–eosin (H&E) and Masson's Trichrome. Unstained sections were used for immunohistochemical studies.

Acute Rejection Diagnosis and Immunologic Surveillance Protocol

Acute cardiac rejection was diagnosed according to Stanford criteria and grading.[19] We only added a distinction between focal (type A) and diffuse (type B) moderate rejection with special reference to extension of myocyte damage and necrosis. In January 1991, we introduced the WF-ISHT new grading for acute rejection.[20] However in the present study, most biopsies were investigated before introduction of the new grading and are reported herein with the original diagnosis and grade (Table 1). Immunosuppressive protocol used for patients reported in the present series consists in a triple drug therapy with steroids, azathyoprine, cyclosporine A. Anti-thymocytidc globulin (ATG) is given for a week immediately after surgery. None of the biopsies were performed with patients receiving OKT3 profilaxis. Two patients had concomitant recurrent human cytomegalovirus infection (HCMV). The results of their biopsies were negative for both rejection and myocardial HCMV localization documented by *in situ* hybridization with a biotin-labeled probe (Enzo Biochem. Inc., New York, NY) and immunohistochemical search for early and late viral antigens.

Light Microscopy Immunohistochemistry

Immunohistochemical stain was performed on sections consecutive to those stained with H&E and Masson's Trichrome. Sections were deparaffinized and brought to Tris-phosphate-buffered saline solution (TBS 0.15 M, pH 7.35). After blocking endogenous peroxidase with 3% H_2O_2 and pretreatment with type XXVII protease (Sigma Chemicals) or tripsin, the slides were incubated overnight with the primary antisera. Immunohistochemical staining was performed with the proxidase-antiperoxidase method[21] and avidin-biotin complex technique.[22] Diaminobenzidine (Sigma Chemicals) was used as chromogen substrate. Specificity tests, performed by omitting the first layer, had negative results. Antisera used throughout the study are detailed in Table 2. The percentages of TNFα positive cells were semiquantitatively eval-

Table 2. *Antisera and Working Dilutions Used for Immunohistochemical Study*

Antibodies	Cell specificity	Dilution	Source
α-TNF	α-TNF containing cells*	1:500	Boehringer
α-TNF	α-TNF containing cells[4]	1:500	Sera-lab
LN5	Macrophages	1:100	Clonab-Biotest
LN3	HLA DR expressing cells	1:100	Clonab-Biotest
MAC-387	Monocytes–macrophages	1:150	Dakopatts
L26	B-lymphocytes (CD 20)	1:100	Dakopatts
UCHL-1	T-lymphocytes (CD 45 RO)	1:1000	Dakopatts
LCA	Leukocytes (CD 45)	1:100	Dakopatts

* Monoclonal antibody against human tumor necrosis factor purified from PHA-stimulated human peripheral blood mononuclear cells.
† Monoclonal antibody against human recombinant tumor necrosis factor alpha.

Table 3. *Incidence of AntiTNFα Immunoreactive Endomyocardial Biopsies with Acute Rejection*

Grade of rejection	No. of biopsies	Positive cases (%)	Negative cases (%)
Negative O WF-ISHT	30	0 —	30 (100)
Mild 1A-1B WF-ISHT	45	20 (44.4)	25 (55.6)
Moderate (A) 2-3A WF-ISHT	54	45 (83.3)	9 (16.7)
Moderate (B) 3B WF-ISHT	9	7 (78)	2 (20)
Severe 4 WF-ISHT	1	1 (100)	0 (0)
Resolving rejection	11	5 (45.5)	6 (54.5)
	150	78 (52)	72 (48)

uated in all samples of each biopsy using ×25 objective lens.

Immunoblotting

Presence and specificity of anti-TNFα antibodies were also tested by immunoblotting study in multiple samples from one normal and two transplanted hearts with acute rejection obtained either at retransplantation (n = 1) or at autopsy (n = 1). Biopsy samples are in fact too small to extract adequate amounts of protein for blotting. Methods have been previously described in detail.[23] Polyacrylamide gel electrophoresis was performed according to the Laemmly method.[24] Proteins were transferred to nitrocellulose according to Towbin et al.[25]

Results

Most of endomyocardial biopsy results that showed acute rejection displayed positive reaction to TNFα antibodies. Biopsy results that were negative for rejection did not show any immunoreactivity. Results are summarized in Table 3. Only part of the biopsy sections with mild rejection (40%) showed TNFα positive reaction limited to a few monocytes—macrophages (MAC 387 and LN5 positive cells) and T lymphocytes (UCHL1/CD45 RO positive cells). Immunoreactive cells were found either in the small clusters of infiltrates or sparse and isolated in the interstitium (Figure 1). Eighteen of the 20 TNFα positive mild rejections (90%) progressed to a higher grade, whereas only 5 of the remaining 25 negative cases (20%) had moderate rejection ($P < 0.001$). Progression occurred during the following week in three of the latter five

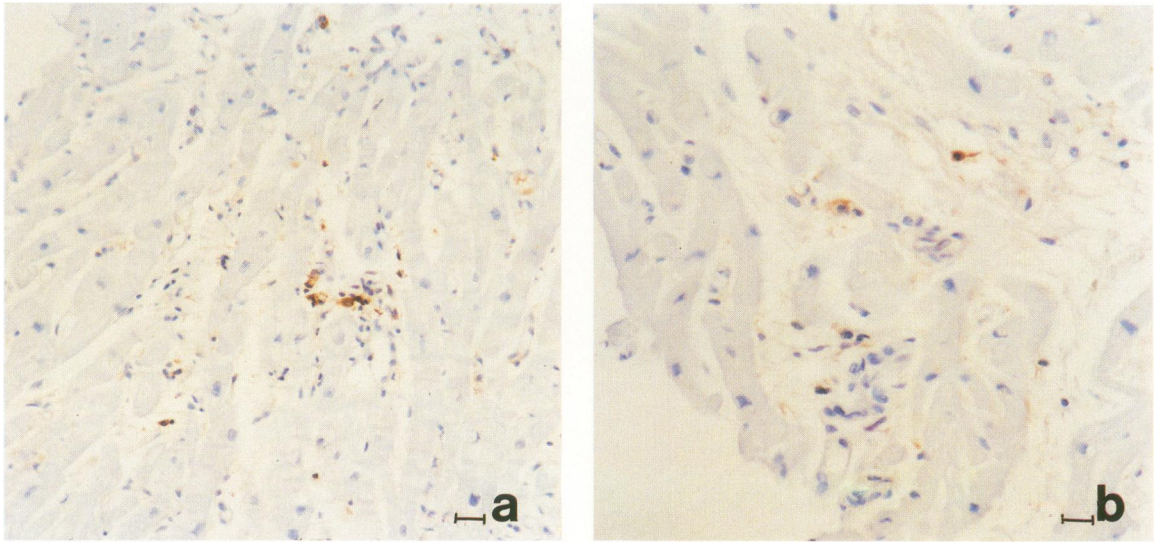

Figure 1. *Immunostaining using peroxidase-antiperoxidase technique.* **a:** *Interstitial cells displaying TNFα immunoreactivity in an endomyocardial biopsy with mild acute rejection (Bar = 20 μm).* **b:** *Isolated TNFα immunoreactive inflammatory cells in the above endomyocardial biopsy: area free from significant interstitial inflammatory infiltrates (Bar = 16 μm).*

cases and in 2 weeks in remaining cases. Persistent mild rejection occurred in two additional TNFα negative cases. TNFα immunoreactivity was more frequently observed in moderate than in mild acute rejection (80 vs. 40%). In moderate-rejection positive cells were mostly monocytes—macrophages and T lymphocytes (Figure 2). TNFα positive cells ranged from 10 to 55% (38 ± 16) of all infiltrating cells.

MHC class II antigens were always expressed by endothelial cells and by more than 70% of infiltrating cells (B and activated T lymphocytes and macrophages) in all biopsy results that showed any grade of acute rejection. However, MHC class II antigens were also expressed by endothelial cells as well as sparse interstitial cells in biopsy results that showed no features of rejection. TNFα positive cells were often close to the site of myocyte damage, which consisted of myofibrillar lysis and membrane irregularities with nuclear preservation (Figure 3); 45% of biopsy sections with features of healing rejection with focal persistence of inflammatory infiltrates, showed positive reaction in few mostly nonpigmented macrophages. Both antibodies to TNFα recognized a 17 KDa band polypeptide (Figure 4) by immunoblotting study of large samples with acute rejection. A 34 KDa band corre-

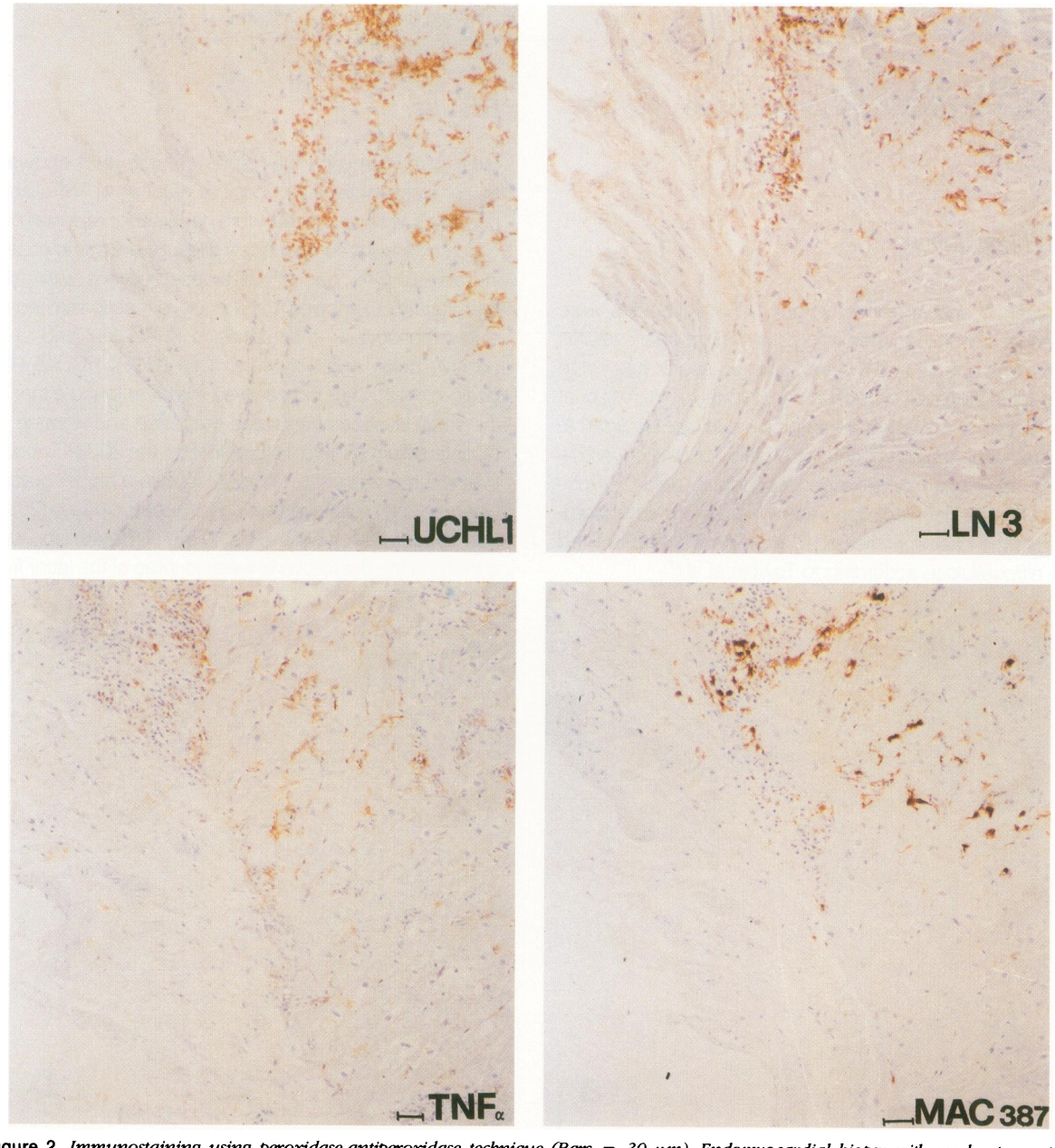

Figure 2. *Immunostaining using peroxidase-antiperoxidase technique (Bars = 30 μm). Endomyocardial biopsy with moderate acute rejection showing distribution of UCHL 1, LN3, TNFα, and MAC387 immunoreactivity and distribution.*

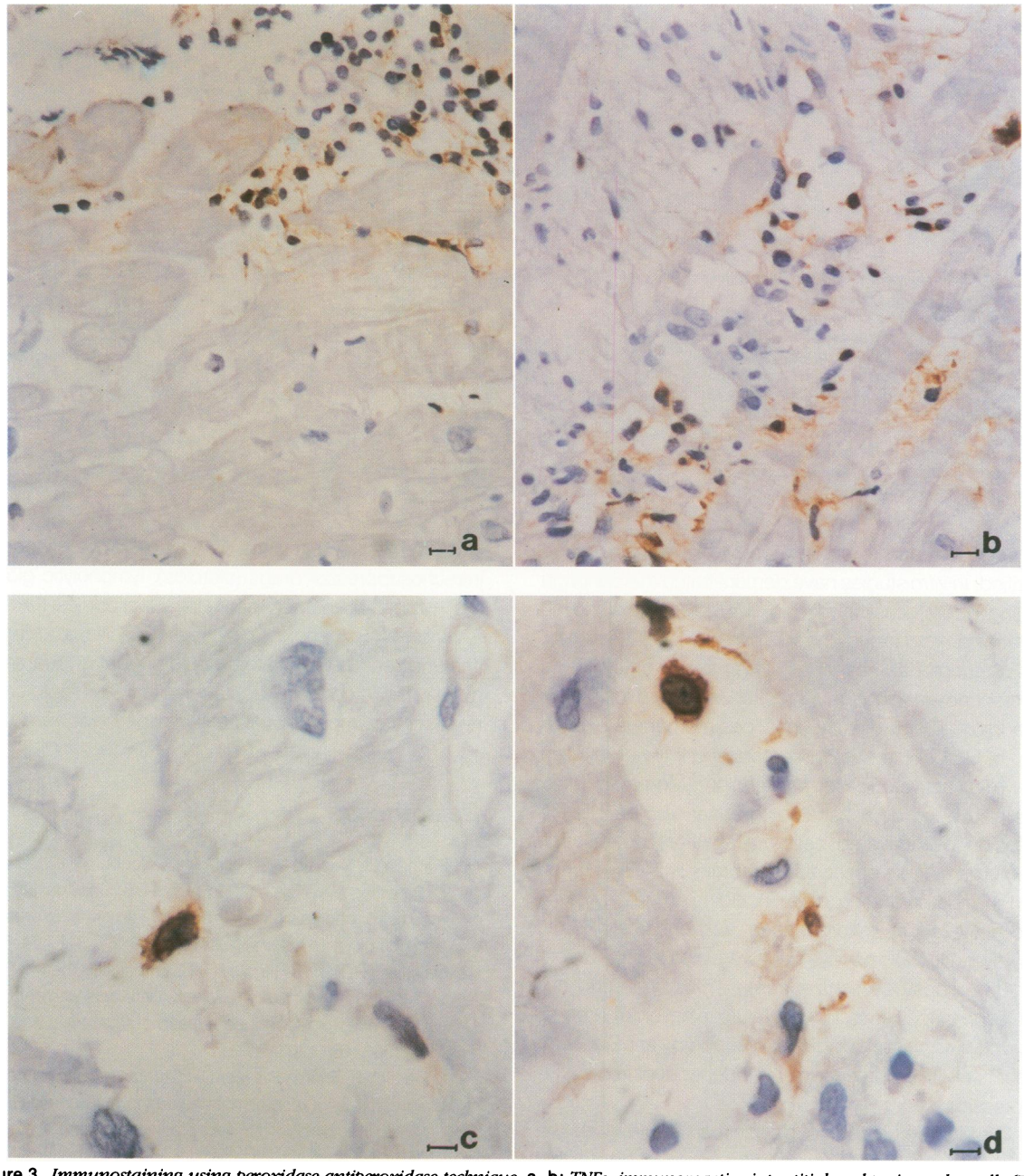

Figure 3. *Immunostaining using peroxidase-antiperoxidase technique.* **a, b:** *TNFα immunoreactive interstitial and perivascular cells (Bar = 8 μm).* **c, d:** *Positive cells are close to damaged myocytes (Bar = 2,5 μm).*

sponding to TNFα dimeric form was also observed. Normal myocardium samples gave negative results.

Discussion

In the present study, we demonstrated that cell-mediated immunologic reactions occurring in human acute cardiac rejection induce TNFα expression by immunocompetent cells and that TNFα expression increases with worsening rejection. The increased TNFα serum levels during allograft rejection[13] and benefits of anti-TNFα antibodies in management of transplanted grafts in experimental animals[15] also indicate that TNFα mediates major roles in acute rejection mechanisms.

TNFα can either participate in immune-cell activation or damage graft tissue mechanisms. Whether TNFα expression by T lymphocytes observed in our series is en-

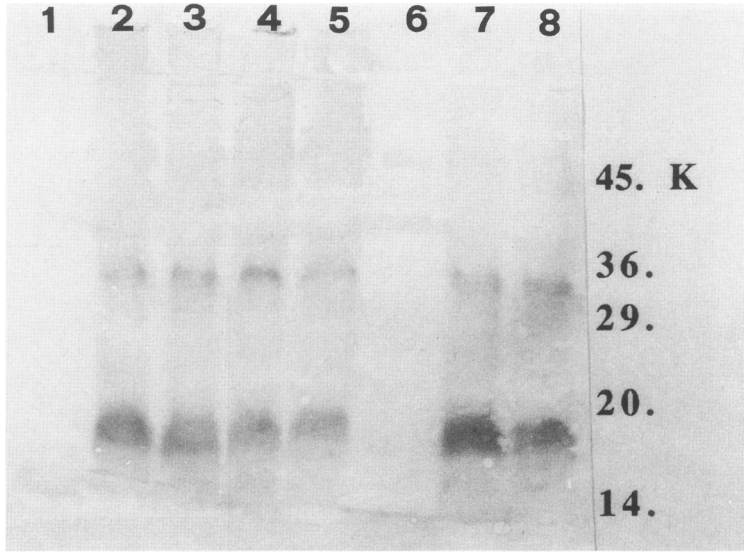

Figure 4. *Immunoblot of TNFα from heart allografts with acute cardiac rejection. Antibodies raised against recombinant human TNFα recognize this antigen (17 KDa) as well as another antigen, likely its dimeric form, on Western blots of proteins derived from myocardial samples from heart allografts with acute rejection. 1, 6: Atrial and ventricular samples from normal heart; 2, 3, 4, 5, 7, 8: Right and left atrial and ventricular samples from heart allografts explanted for persistent severe acute rejection.*

dogenous or due to exogenous uptake remains to be clarified. *In vitro* studies have demonstrated that although resting T cells constitutively lack TNFα receptors, the latter are *de novo* induced upon primary T-cell activation. TNFα receptor expression is similar to that of IL2 with a different peak timing (day 3 for IL2 and day 6 for TNFα). In addition, TNFα directly enhances membrane expression of HLA DR and IL2 receptors and IFNγ production as well as activated T-cell proliferation.[3] Moreover, TNFα has also been demonstrated to enhance HLA DR expression by activated cells via an IFNγ potentiation mechanism.[12] We observed membrane HLA DR expression in most infiltrating cells, including lymphocytes (B and activated T), macrophages, and endothelial cells. However, HLA DR positive cells were also found in rejection-free biopsy sections, whereas TNFα was only observed in biopsy sections with acute rejection. Therefore, *in vivo* MHC class II antigen expression by immunocompetent cells likely proceeds or is independent from TNFα expression.

The high percentage of biopsy results with moderate rejection displaying TNFα immunoreactivity suggests that TNFα may also contribute to myocyte damage. We observed that myocytes adjacent to positive inflammatory cells often present lysed contractile filaments and membrane irregularities. This damage, however, could result from multiple insulting stimuli. Therefore TNFα-mediated myocyte damage deserves further investigation. Mild rejection with positive reaction could indicate either early damage or increased aggressiveness of infiltrating cells. This hypothesis is suggested by the high incidence of TNFα-positive mild rejections evolving to moderate grades and vice versa by the significantly lower percentage of TNFα-negative mild rejections progressing to higher degrees.

Currently, acute rejection is controlled by nonspecific immunosuppressor drugs (steroids), lympholytic globulins (tymoglobuline), or monoclonal antibodies (OKT3). As shown in experimental settings, specific blocking of immune reaction mediators could probably guarantee more specific control of acute rejection. This topic could represent an important field of investigation in the future.

References

1. Pennica D, Nedwin GE, Hayflick JS, Seeburg PH, Derynck R, Palladino MA, Kohr WJ, Aggarwal BB, Goeddel DV: Human tumor necrosis factor: Precursor, structure, expression and homology to lymphotoxin. Nature 1984, 312:724–9
2. Nawroth PP, Bank I, Handley D, Cassimeris J, Chess I, Stern D: Tumors necrosis factor/cachectin interacts with endothelial cell receptors to induce release of Interleukin 1. J Exp Med 1986, 163:1363–75
3. Scheurich P, Thoma B, Ucer U, Pfizenmaier K: Immunoregulatory activity of recombinant human tumor necrosis factor (TNF)-α: induction of TNF receptors on human T cells and TNF-α-mediated enhancement of T cells responses. J Immunol 1987, 138:1786–90
4. Shalaby MR, Espevik T, Rice GC, Ammann AJ, Figari IS, Ranges GE, Palladino MA: The involvement of human tumor necrosis factor-α and -β in the mixed lymphocyte reaction. J Immunol 1988, 141:499–503
5. Havell EA: Evidence that tumor necrosis factor has an important role in antibacterial resistance. J Immunol 1989, 143:2894–9
6. Fong Y, Tracey KJ, Moldawer LL, Hesse DG, Hanogue KB, Kenney JS, Lee AT, Kuo GC, Allison AC, Lowry SF, Cerami A: Antibodies to cachectin tumor necrosis factor reduce Interleukin 1β and Interleukin 6 appearance during lethal bacteremia. J Exp Med 1989, 170:1627–33
7. Piguet PF, Grau GE, Allet B, Vassalli P: tumor necrosis fac-

tor/cachectin is an effector of skin and gut lesions of the acute phase of graft-vs.-host disease. J Exp Med 1987, 166:1280–9

8. Bermudez LEM, Stevens P, Kolonoski P, Wu M, Young LS: Treatment of experimental disseminated *Mycobacterium avium* complex infection in mice with recombinant IL-2 and tumor necrosis factor. J Immunol 1989, 143:2996–3000
9. Dinarello CA, Cannon JG, Wolff SM, Bernheim HA, Beutler B, Cerami A, Figari IS, Palladino MA, O'Connor JV: Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of Interleukin 1. J Exp Med 1986, 163:1433–1450
10. Waage A, Halstensen A, Shalaby R, Brandtz AEG, Kierulf P, Espevik T: Local production of tumor necrosis factor α, Interleukin 1, and Interleukin 6 in meningococcal meningitis. Relation to the inflammatory response. J Exp Med 1989, 170:1859–67
11. Chavin KD, Bromberg JS, Kunkel SL, Naji A, Barker CF: Effects of a policlonal anti TNF antibody on cell mediated immunity *in vivo*. Transplant Proc 1991, 23:847–8
12. Leszczynsky D: Interleukin-1 alpha inhibits the effects of gamma-interferon and tumor necrosis factor alpha on the expression of the major histocompatibility antigens by the rat endothelium. Am J Pathol 1990, 136:229–37
13. Maury CPJ, Teppo AM: Raised serum levels of cachectin/tumor necrosis factor α in renal allograft rejection. J Exp Med 1987, 166:1132–7
14. Chatenoud L, Ferran C, Legendre C, Thouard I, Merite S, Reuter A, Gevaert Y, Kreis H, Franchimont P, Bach JF: *In vivo* cell activation following OKT3 administration. Transplantation 1990, 49:697–702
15. Sheringa M, de Bruin RWF, Jeekel H, Marquet RL: Antitumor necrosis factor alpha serum prolongs heart allograft survival in rats transplantation. Transplant Proc 1991, 23:547–8
16. Shalaby MR, Fendly B, Sheehan KC, Schreiber RD, Ammann AJ: Prevention of the graft-versus-host reaction in newborn mice by antibodies to tumor necrosis factor-alpha. Transplantation 1989, 47:1057–1061
17. Ferran C, Sheeham K, Schreiber R, Bach JF, Chatenoud L: Anti-TNF abrogates the cytokine-related anti-CD3 induced syndrome. Transplant Proc 1991, 23:849–50
18. Caves PK, Stinson EB, Billingham ME, Shumway NE: Percutaneous transvenous endomyocardial biopsy in human heart recipients. Ann Thorac Surg 1973, 16:325–36
19. Billingham ME: Diagnosis of cardiac rejection by endomyocardial biopsy. Heart Transplant 1981, 1:25–9
20. Billingham ME, Cary NRB, Hammond ME, Kemnitz J, Marboe C, McAllister HA, Snovar DC, Winters GL, Zerbe A: A working formulation for the standardization of nomenclature in the diagnosis of heart and lung rejection: heart rejection study group. J Heart Transplant 1990, 9:587–93
21. Sternberger LA: The unlabelled antibody enzyme peroxidase-antiperoxidase (PAP) method. Immunocytochemistry. Edited by LA Sternberger. New York, John Wiley, 1979, pp 104–69
22. Hsu SM, Rainel L, Fanger H: Use of avidin–biotin peroxidase complex (ABC) in immunoperoxidase techniques. J Histochem Cytochem 1981, 29:577–80
23. Arbustini E, Pucci A, Grasso M, Diegoli M, Pozzi R, Gavazzi A, Graziano G, Campana C, Goggi C, Martinelli L, Silini E, Specchia G, Viganò M, Solcia E: Expression of natriuretic peptide in ventricular myocardium of failing human hearts and its correlation with the severity of clinical and hemodynamic impairment. Am J Cardiol 1990, 66:973–80
24. Laemmly UK: Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature 1970, 227:680–5
25. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 1979, 76:4350–4