Brief Definitive Report

Cytokine Gene Transcription in Vascularised Organ Grafts: Analysis Using Semiquantitative Polymerase Chain Reaction

By Margaret J. Dallman, Christian P. Larsen, and Peter J. Morris

From the Nuffield Department of Surgery, John Radcliffe Hospital, Headington, Oxford OX3 9DU, United Kingdom

Summary

Cytokine gene transcription has been analyzed by direct analysis of RNA obtained from mouse heterotopic cardiac transplants. The level of expression of the cytokine genes was assessed using semiquantitative polymerase chain reaction (PCR). Expression of the cytokines investigated fell into three groups. The first group included interleukin 1β (IL-1β), IL-5, IL-6, and interferon γ (IFNγ). These genes were expressed in normal heart tissue at low level and were upregulated following both syngeneic and allogeneic transplantation. Genes in the second group (IL-1α, IL-3) were not expressed at detectable levels in normal heart but were induced following either syngeneic or allogeneic heart grafting. IL-2, IL-4, and tumor necrosis factor β (IFNβ) comprised the third group and these cytokines were expressed only in allogeneic grafts after transplantation.

Cytokines play a central role in the regulation of immune responses. The main role of cytokines in the immune system has been described as inducing differentiation and proliferation of leucocytes (1, 2), but it is becoming increasingly clear that they may also regulate the production (3, 4) or function (5, 6) of one another.

Much of our understanding of cytokine interactions in the immune response has relied on the use of cell culture systems. We were particularly interested in trying to localize the production of cytokines in the whole animal. Previously, cytokines have been measured by bioassays, which although very sensitive, were notoriously nonspecific due to the highly overlapping functional activities of cytokines. Although the availability of antibodies that inhibit specific cytokines has made this less of a problem, it is still difficult to envisage how the bioassay may be used to directly analyze cytokine production at the site of an immune response in the whole animal. Antibodies have also been used to measure the levels of certain cytokines in serum or other body fluids by either ELISA or radioimmune assay (7, 8). However, the relationship of systemic levels of cytokines with localized immune reactions, is not known. The analysis of gene transcription lends itself to localized detection of cytokine expression. We (9) and others (10) have previously attempted to examine cytokine gene transcription in solid organ transplants using northern blotting or nuclease protection analysis. In our hands however, northern blotting was not sensitive enough to detect many genes of interest and the amounts of RNA often required for nuclease protection analysis were prohibitive in many instances. Therefore, a semiquantitive PCR that we have previously described (11, 12) was used to compare cytokine gene transcription in normal mouse heart tissue with that in allogeneic or syngeneic heart grafts.

Materials and Methods

Animals. Inbred BALB/c (H-2^d) and CBA (H-2^k) male mice were obtained from Harlan Olac UK, Ltd. (Bicester, UK) and maintained in the Biomedical Services Unit at the John Radcliffe Hospital, Oxford.

Transplantation. Heterotopic vascularised heart transplantation was performed as previously described (13, 14). Grafts were monitored daily for signs of rejection by palpation and/or electrocardiograph (ECG).

RNA Preparation and Polymerase Chain Reaction. Organ grafts were removed at the times indicated, diced coarsely, rinsed in ice cold RNAse free saline and immediately frozen in liquid N_2. Tissues were then homogenized in guanidine thiocyanate solution using a Janke and Kunkel Ultraturrax T25 tissue homogenizer (Oxford Laboratories, High Wycombe, UK). Total RNA was prepared, cDNA synthesis and PCR were performed as previously described (12). Briefly, 10 μg total RNA was used in cDNA synthesis. 1/20th or 1/200th of the resulting cDNA was used for PCR. The primers, $MgCl_2$ concentration and annealing conditions used to yield single specific products in PCR have been previously described (15). Primers were used at a final concentration of 1 μM except for IL-1αA (0.2 μM); IL-1αB (0.2 μM); IL-1βA (0.5 μM); IL-1βB (0.5 μM); IL-2A (0.2 μM); IL-2B (0.2 μM); IL-3A (0.1 μM); IL-3B (0.1 μM); IL-4A (0.5 μM); IL-4B (1.5 μM). Primer sequences were chosen from separate exons of the genes so that product from cDNA could be distinguished from product derived from any contaminating genomic DNA. PCR products (15 μl) were analyzed by gel electrophoresis,

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/91/08/0493/04 $2.00
Volume 174 August 1991 493-496

Southern or dot blot. A third oligonucleotide endlabeled with (γ^{32}-P) ATP was used to probe blots (internal oligo). The position of internal oligo sequences in cytokine cDNAs is internal to that of the original two primers. Oligonucleotides were synthesized on a 380B DNA synthesizer (Applied Biosystems, Warrington, UK). Blots were washed in 6 × SSC (20 × SSC is 3 M NaCl, 0.3 M $Na_3C_6H_5O_7.2H_2O$), 0.1% SDS at the melting temperature of the internal oligo.

Results and Discussion

Expression of Cytokine Genes in Rejecting Heart Allografts. RNA prepared from either syngeneic (CBA>CBA) or allogeneic (BALB/c>CBA) transplanted hearts was used for cDNA synthesis and PCR. RNA prepared from the whole organ was used for two reasons. First, it is known that cells other than leucocytes are able to produce cytokines which may influence an immune response (16, 17, 18) and therefore we did not wish to exclude any cells from our analysis. Second many cytokine RNAs contain in their 3′ untranslated regions AU-rich sequences which confer a high level of instability

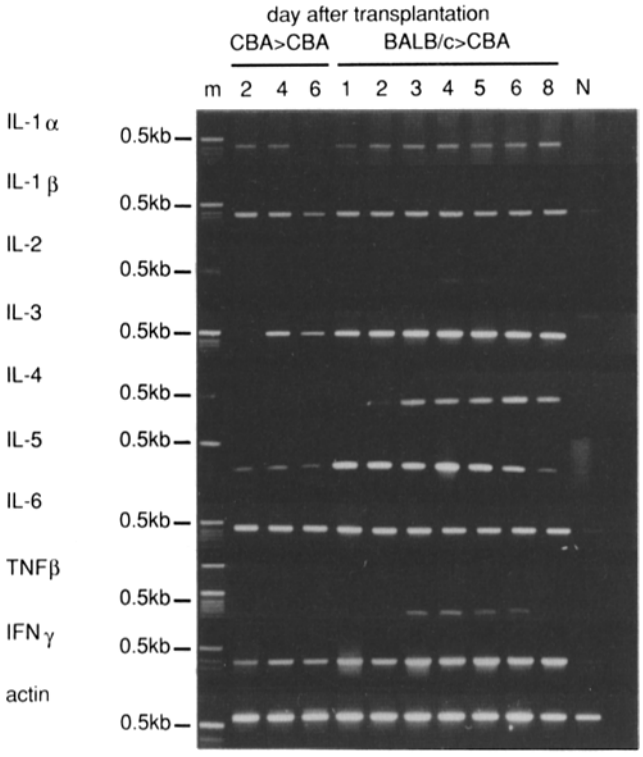

Figure 1. Cytokine gene transcription in normal heart and syngeneic or allogeneic heart. RNA was isolated from normal (N, CBA or BALB/c) heart tissue or syngeneic (CBA>CBA) and allogeneic (BALB/c>CBA) heterotopic heart grafts at the times indicated. cDNA was synthesized from 10 μg total RNA isolated from transplants at the times indicated after grafting using oligo dT as a primer. 1/20th of the cDNA was used in PCR with the primers and under the conditions described in materials and methods. After 60 cycles of amplification, 15 μl of the reaction mix was removed and analyzed by gel electrophoresis through 1% agarose gels containing ethidium bromide. (−) no RNA in cDNA synthesis; (m) marker track (kb ladder). This analysis was repeated at least twice for all cytokines using RNA from two groups of animals.

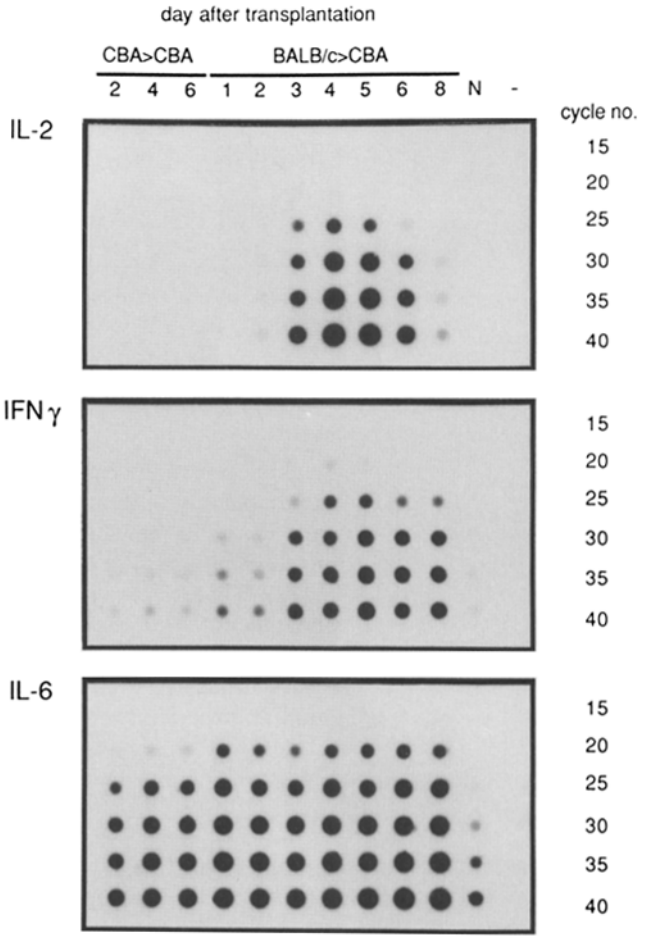

Figure 2. Semiquantitative analysis of cytokine gene expression in heart grafts. RNA was isolated from transplants as described Fig. 1. cDNA synthesis and PCR was carried out as described in Fig. 1 with primers specific for IL-2, IFN-γ, or IL-6, except that 1/200th of the cDNA was used in PCR and 15 μl of the amplification mix was removed every 5 cycles from 15–40 cycles in the PCR. The samples were dot-blotted to nitrocellulose membrane and probed using ^{32}P-end-labeled internal oligos. (N) normal heart; (−) no RNA in cDNA synthesis. The analysis was repeated using RNA from two groups of animals with essentially the same results.

on the RNA (19, 20). Therefore, once the inducing signal for cytokine transcription is removed by isolating cells from their microenvironment, the level of cytokine transcript rapidly declines. Thus, analysis of cytokine RNA in isolated cell populations becomes difficult to interpret.

In the initial series of experiments, shown in Fig. 1, cDNA samples were cycled through 60 cycles of PCR. We felt it important to use this number of cycles initially to assess if there was any transcription of these genes within the tissues. Some cytokines were expressed in normal heart tissue, syngeneic, and allogeneic grafts (IL-1β, IL-5, IL-6, IFN-γ), others in syngeneic and allogeneic grafts but not in normal tissue (IL-1α, IL-3) while IL-2, IL-4, TNFβ were expressed exclusively in allogeneic grafts. Experiments using BALB/c>C3H mouse cardiac allografts indicated that the induction of cytokine transcription during cardiac allograft rejection described here is not unique to the BALB/c>CBA strain combination (not shown). Further, our more recent experiments

Table 1. *Kinetic Analysis of Cytokine Gene Expression During Mouse Heart Graft Rejection Using Semiquantitative Polymerase Chain Reaction*

Cytokine	Day after transplantation										
	Syngeneic			Allogeneic							Normal heart
	2	4	6	1	2	3	4	5	6	8	
IL-1α	(+)	+	(+)	(+)	+ +	+ +	+ +	+ +	+ +	+ +	−
IL-1β	+	+	+	+ +	+ +	+ +	+ +	+ +	+ +	+ +	(+)
IL-2	−	−	−	(+)	+	+ +	+ +	+ +	+	(+)	−
IL-3	(+)	+	(+)	+	+ +	+ +	+ +	+ +	+ +	+ +	−
IL-4	−	−	−	−	(+)	+	+ +	+ +	+ +	+	−
IL-5	+	+	+	+ +	+ +	+ +	+ +	+ +	+ +	+	(+)
IL-6	+	+	+	+ +	+ +	+ +	+ +	+ +	+ +	+ +	(+)
TNFβ	−	−	−	−	−	+	+	+	+	−	−
IFN-γ	+	+	+	+	+	+ +	+ +	+ +	+ +	+ +	(+)
ACTIN	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +

(−) no detectable product. (+), +, + +: Increasing amount of product detectable. This system is only designed to compare the amount of product between samples using the same primer pair.

have shown that similar patterns of cytokine transcription are seen following transplantation of syngeneic and allogeneic rat renal grafts (reference 12 and our unpublished observations).

Semiquantitative Polymerase Chain Reaction for Analysis of Cytokine Transcription. Even when samples were taken through very high numbers of PCR cycles as shown in Fig. 1, it was apparent that there was some difference in the amount of product obtained from different cDNAs e.g., compare normal and transplanted tissue with IL-1β primers. We have recently described the use of semiquantitative PCR to compare the level of transcription of cytokine genes in different samples (11, 12). Cytokine gene expression was assessed in the transplanted tissues using the semiquantitative PCR. Aliquots of the PCR reaction (15 μl) were removed after increasing numbers of cycles (15–40) and analyzed by dot blot. Examples of the data are shown in Fig. 2 using primers for IL-2, IL-6, and IFN-γ and the data for all primer pairs summarized in Table 1. As may be seen it was now possible to discriminate between the level of expression of the cytokines in normal tissue, syngeneic and allogeneic transplants. IFN-γ illustrates this well. In our initial analysis (Fig. 1) it was difficult to see clearly different levels of expression of IFN-γ RNA in syngeneic and allogeneic grafts. It seemed very likely that there would be a difference in the level of transcription of this gene in syngeneic and allogeneic grafts since the degree of MHC class II induction in allogeneic grafts is much greater than that in syngeneic grafts (21). The semiquantitative analysis (Fig. 2) shows that the level of expression of the IFN-γ gene is lowest in normal heart tissue and is higher in allogeneic than in syngeneic transplants. It further shows that IFN-γ transcripts reach their highest level in allogeneic transplants at 3–8 d post transplantation.

Of further interest from this type of analysis is the different kinetics of transcription of the various cytokines. For instance IL-2, IL-4, and TNFβ are all transcribed transiently within rejecting grafts, their expression being downregulated before the macroscopic signs of acute rejection (i.e., reduced strength of palpation and decreased rate on ECG) are obvious. Expression of the cytokines was again clearly divided into three types. In the first group (IL-1β, IL-5, IL-6, IFN-γ) cytokine expression was seen in normal tissue, syngeneic and allogeneic tissue although the semi-quantitative analysis shows a clear upregulation of gene expression following transplantation and a higher level of induction in allogeneic than in syngeneic transplants. Genes of the second group (IL-1α, IL-3) were expressed only following transplantation and again were induced to higher levels in allogeneic than in syngeneic grafts. Presumably, transcription of cytokines in these first two groups was induced following the nonspecific insult of the transplant procedure itself. IL-2, IL-4 and TNFβ were expressed only following allogeneic transplantation, that is during an immune response to alloantigen. Thus, monitoring these latter cytokines may be of particular interest in the diagnosis of clinical transplant rejection. Our most recent experiments have shown that PCR may be easily performed with starting amounts of total RNA as low as 0.5–1.0 μg thus making the analysis of clinical material feasible. Indeed, our initial work has shown that expression of the IL-2 gene in fine needle aspiration samples from renal transplant patients may be a useful early marker of rejection (Dallman, M.J., J. Roake, D. Hughes, G. Toogood, and P.J. Morris, manuscript submitted for publication).

This approach should prove to be very useful in a variety of experimental and clinical situations where one wishes to analyze cytokine gene transcription in the grafted tissues of the intact animal.

We thank Nick Aarons for excellent technical assistance.

This work was supported by the Medical Research Council (MRC), UK, and the National Kidney Research Fund, UK. M. J. Dallman is an MRC Senior Research Fellow.

Address correspondence to Margaret J. Dallman, Nuffield Department of Surgery, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK.

Received for publication 9 January 1991 and in revised form 6 May 1991.

References

1. O'Garra, A. 1989. Interleukins and the immune system 1. *Lancet.* I:943.
2. O'Garra, A. 1989. Interleukins and the immune system. 2. *Lancet.* I:1003.
3. Fiorentino, D.F., M.W. Bond, and T.R. Mossmann. 1989. Two types of mouse T helper cell iv. Th2 cells secrete a factor that inhibits cytokine production by Th1 clones. *J. Exp. Med.* 170:2081.
4. Moore, K.W., P. Vieira, D.F. Fiorentino, M.L. Trounstine, T.A. Khan, and T.R. Mosmann. 1990. Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein Barr Virus gene BCRF1. *Science (Wash. DC).* 248:1230.
5. Llorente, L., M.-C. Crevon, S. Karrey, T. deFrance, J. Banchereau, and P. Galanaud. 1989. Interleukin (IL) 4 counteracts the helper effect of IL-2 on antigen-activated human B cells. *Eur. J. Immunol.* 19:765.
6. Spits, H., H. Yssel, X. Paliard, R. Kasteleih, C. Figdor, and R.J. deVries. 1988. IL-4 inhibits IL-2 mediated induction of human lymphokine activated killer cells, but not the generation of antigen-specific cytotoxic lymphocytes in mixed leucocyte cultures. *J. Immunol.* 141:29.
7. Simpson, M.A., P.N. Madras, A.J. Cornaby, T. Etienne, R.A. Dempsey, G.H.A. Clowes, and A.P. Monaco. 1989. Sequential determinations of urinary cytology and plasma and urinary lymphokines in the management of renal allograft recipients. *Transplantation (Baltimore).* 47:218.
8. Johnson, C.P., A. Chaharmohal, E. Buchmann, A.M. Roza, and M.B. Adams. 1990. Plasma IL-2 levels and diagnosis of renal transplant rejection. *Transplant. Proc.* 22:1849.
9. Dallman, M.J., A.C.G. Porter, C.P. Larsen, and P.J. Morris. 1989. Lymphokine production in allografts: Analysis of northern blotting. *Transplant. Proc.* 21:296.
10. Lowry, R.P., K. Wang, B. Venooij, and D. Harcus. 1989. Lymphokine transcription in vascularized mouse heart grafts: effects of "tolerance" induction. *Transplant. Proc.* 21:72.
11. Dallman, M.J., and A.C.G. Porter. 1991. Semi-quantitative PCR for the analysis of gene expression. *In* PCR: A Practical Approach. McPherson, M.J., P. Quirke, and G.R. Taylor, editors. (In press).
12. Dallman, M.J., O. Shiho, T.H. Page, K.J. Wood, and P.J. Morris. 1991. Peripheral tolerance to alloantigen results from altered regulation of the interleukin 2 pathway. *J. Exp. Med.* 173:79.
13. Corry, R.J., H.J. Winn, and P.S. Russell. 1973. Primarily vascularized allografts of hearts in mice: The role of H-2D, H-2K, and non H-2 antigens in rejection. *Transplantation (Baltimore).* 16:343.
14. Superina, R.A., W.N. Peugh, K.J. Wood, and P.J. Morris. 1986. Assessment of Primarily Vascularized Cardiac Allografts in Mice. *Transplantation (Baltimore).* 42:226.
15. Montgomery, R.A., and M.J. Dallman. Analysis of cytokine gene expression during fetal thymic ontogeny using the polymerase chain reaction (PCR). *J. Immunol.* In press.
16. O'Garra, A., S. Umland, T. DeFrance, and J. Christiansen. 1988. The pleiotropic effects of B cell factors. *Immunol. Today.* 9:45.
17. Kupper, T. 1990. The role of epidermal cytokines in the Immunophysiology of cells and cytokines. *In* J.J. Oppenheim, and E. Shevach, editors. Oxford University Press, New York. pp. 285.
18. Kock, A., T. Schwarz, R. Kirnbauer, A. Urbanski, P. Perry, J.C. Ansel, and T.A. Luger. 1990. Human keratinocytes are a source for tumor necrosis factor α: Evidence for synthesis and release upon stimulation with endotoxin or ultraviolet light. *J. Exp. Med.* 172:1609.
19. Caput, D., B. Beutler, K. Hartog, R. Thayer, S. Brown-Shimer, and A. Cerami. 1986. Identification of a common nucleotide sequence in the 3′ untranslated region of mRNA molecules specifying inflammatory mediators. *Proc. Natl. Acad. Sci. USA.* 83:1670.
20. Shaw, G., and R.A. Kamen. 1986. Conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell.* 46:659.
21. Milton, A.D., and J.W. Fabre. 1985. Massive induction of donor-type class I and II Major histocompatibility complex antigens in rejecting cardiac allografts in the rat. *J. Exp. Med.* 161:98.