Short Communication

Differential Role for Competitive Reverse Transcriptase-Polymerase Chain Reaction and Intracellular Cytokine Staining as Diagnostic Tools for the Assessment of Intragraft Cytokine Profiles in Rejecting and Nonrejecting Heart Allografts

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The early and reliable diagnosis of allograft rejection is a difficult task and the assessment of cytokine expression in the grafts can be a helpful parameter. We have compared competitive reverse transcriptase-polymerase chain reaction (RT-PCR) with intracellular cytokine staining by flow cytometry as tools to measure cytokine expression in rejecting and nonrejecting murine cardiac allografts. Both techniques gave comparable results for cytokine expression in rejecting allografts and syngeneic controls. Grafts from mice pretreated with anti-CD4 antibody and donor-specific blood transfusion showed a marked reduction in cytokine expression, as assessed by competitive RT-PCR, even though a cellular infiltrate was present in the graft. In contrast, the cytokine production measured by intracellular cytokine staining of the isolated graft-infiltrating cells was high and exceeded even that of the rejecting allografts. We conclude that intracellular cytokine staining of graft-infiltrating leukocytes by flow cytometry does not necessarily reflect accurately the cytokine milieu in the graft. This technique might therefore have a limited clinical application in contrast to competitive RT-PCR for the differentiation between graft acceptance and graft rejection. (*Am J Pathol* 2000, 157:1453-1458)

Acute rejection is a major complication after organ transplantation and a risk factor for the development of chronic rejection and subsequent graft loss.¹ The early and reli-

able diagnosis of rejection episodes before irreversible damage to the graft has occurred, is still a challenge in clinical transplantation. Moreover, the ability to differentiate between rejection, infection, and graft acceptance is critical to avoid overimmunosuppression and inappropriate treatment. It is well known both clinically and experimentally, that the presence of graft-infiltrating cells does not necessarily correlate with rejection episodes.²⁻⁵ As T cells are the major effector cells in allograft rejection, analysis of the expression of T-cell immune activation genes, cytokines, and effector molecules has received major attention as a diagnostic tool.

One method that has provided useful insights into the mechanism of allograft rejection in experimental and clinical transplantation is reverse transcriptase-polymerase chain reaction (RT-PCR).⁶⁻⁸ More recently intracellular cytokine staining has been described as a method for determining cytokine synthesis at the single cell level using labeled anti-cytokine antibodies and subsequent flow cytometric analysis.^{9,10} The advantage of this latter method is that it allows the identification and quantification of the cellular source of the analyzed cytokine within a heterogeneous cell population.

Intracellular cytokine staining requires the isolation and subsequent *in vitro* stimulation of graft-infiltrating lymphocytes before flow cytometric analysis. The method has recently been exploited by Stinn and co-workers¹¹ to analyze interferon (IFN)- γ production by T cells in rejecting mouse cardiac grafts. However, the frequency of the cells actually producing cytokines in a rejecting organ

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might be rather low.¹² Furthermore, the *in vitro* stimulation of isolated graft-infiltrating cells required for subsequent intracellular cytokine analysis might lead either to an alteration in cytokine production compared to that actually occurring within the graft itself, or an increase in the frequency of the cells identified as cytokine producers. The aim of this study was to compare competitive RT-PCR, which determines the overall cytokine profile in a given sample of graft tissue, with intracellular cytokine staining of isolated graft-infiltrating cells as potential diagnostic tools in rejecting and nonrejecting heart allografts.

Materials and Methods

Heart Transplants and Recipient Pretreatment

CBA.Ca (H-2^k) mice were used as recipients or syngeneic heart donors and C57BL/10 (H-2^b) as fully allogeneic donors. Mice were bred and maintained in the Biomedical Services Unit at the John Radcliffe Hospital. All animals were treated in accordance with the Home Office Animals (Scientific Procedures) Act of 1986. Heterotopic heart transplants were performed broadly as described¹³ under general anesthesia with Hypnorm (Jansen Pharmaceutical, High Wycombe, UK) and Hypnoval (Roche Welwyn, Garden City, UK). Syngeneic grafts (CBA into CBA) were used to establish a baseline control for cellular infiltration and cytokine expression (group 1). Fully allogeneic (C57BL/10) grafts transplanted into CBA recipients without any pretreatment were acutely rejected with a median graft survival time of 8 days¹⁴ (group 2). CBA mice pretreated with a donor-specific blood transfusion (DST) and depleting anti-CD4 antibody YTA 3.1, (a kind gift from H. Waldmann, Dunn School of Pathology, Oxford, UK) 28 days before transplantation of a C57BL/10 heart, accepted the grafts indefinitely (median graft survival time >100 days) (group 3), as demonstrated previously.^{14,15} Heart grafts were removed from recipients in each group on day 5 after transplantation and flushed with sterile saline. One-third of the tissue was used for the preparation of RNA and two-thirds for isolation of graft-infiltrating cells for intracellular cytokine staining. For the experiments in which RNA was isolated from graft-infiltrating cells the whole graft was used for cell isolation. All groups consisted of three animals and results are given as mean per group $\pm$ SD.

Competitive RT-PCR

Total RNA was isolated either from heart tissue or graft-infiltrating leukocytes using RNeasy (Qiagen). Five μ g of RNA were subjected to cDNA synthesis using 2.5 μ g oligo(dT) (Pharmacia Biotech) and 200 U of Moloney leukemia virus RT (Life Technologies, Inc.). Twenty-five ng of cDNA was used in each PCR reaction. The multiple competitive construct for hypoxanthine-guanine phosphoribosyl-transferase, IFN- γ , interleukin (IL)-2, IL-4, and IL-10 was kindly provided by S. Reiner and S. Miller, Chicago, IL. The multiple competitive construct and oli-

gonucleotide-primers have been described earlier.¹⁶ A competitor construct for the constant region of the T-cell receptor β -chain (C- β) was constructed in a similar way using the following primer sequence: sense 5'-gaggatct-gagaaatgtgactcc-3', anti-sense 5'-tgctcgccccaggc-ctctgcactgat-3'.

Aliquots of each experimental cDNA were first amplified with oligonucleotides specific for the constitutively expressed gene hypoxanthine-guanine phosphoribosyl-transferase in the presence of equivalent amounts of the competitor construct. After electrophoresis on a 2% ethidium-bromide-stained agarose gel, the bands derived from the competitor construct and the wild-type cDNA were scanned using Alpmager software (Alpha Inotech Corp., San Leandro, CA). The ratio of the competitor construct to wild-type cDNA allowed standardization of hypoxanthine-guanine phosphoribosyl-transferase levels for all experimental cDNAs by adjusting the amount of cDNA amplified where necessary. For measuring cytokine gene expression equivalent amounts of the standardized cDNA were co-amplified with a suitable fixed amount of the competitor construct. Again the ratio of competitor construct to cytokine derived band was determined. All reactions were performed in triplicate and the mean used for further calculations. The final result is given as the ratio of (cytokine/competitor) divided by (hypoxanthine-guanine phosphoribosyl-transferase/competitor) in femtograms (fg) of the amount of competitor used for the amplification for the respective gene of interest.

Isolation of Graft-Infiltrating Cells and Intracellular Cytokine Staining

Heart tissue was diced coarsely and incubated in RPMI containing 1 mg/ml collagenase (Sigma) for 30 minutes at 37°C. After removing debris by passing through a cell strainer cells were centrifuged over Ficoll (Sigma), washed twice, resuspended in 500 μ l medium, and counted. Graft-infiltrating cells were stimulated with phorbol myristate acetate (50 ng/ml) plus ionomycin (500 ng/ml) for 4 hours at 37°C with brefeldin A (10 μ g/ml) added for the last 2 hours. Surface staining using anti CD4-PerCP and anti CD8-APC was performed in phosphate-buffered saline (PBS)/fetal calf serum plus brefeldin A for 30 minutes. The cells were then washed, fixed in 2% formaldehyde for 20 minutes, preincubated for 10 minutes in permeabilization buffer (PBS/1% fetal calf serum/0.5% saponin) (Sigma), fixed with 2% formaldehyde for 20 minutes, and incubated with either anti-IL-2 (JES6-5H4, 2.5 μ g/ml), anti-IFN- γ (XMG1.2, 5.0 μ g/ml), anti-IL-4 (11B11, 5.0 μ g/ml), anti-IL-10 (JES5-16E3, 5.0 μ g/ml), or rat IgG1 as isotope control (all PE-conjugated; Pharmingen). After two washes with permeabilization buffer, the cells were washed in PBS/1% fetal calf serum without saponin to allow membrane closure. Samples were analyzed on a FACSsort flow cytometer (Becton-Dickinson, Cowley, UK). Results were analyzed using Cellquest software (Becton-Dickinson).

Results

Competitive RT-PCR and Intracellular Cytokine Staining Detect Similar Cytokine Expression in Rejecting Heart Allografts

Five days after transplantation, untreated rejecting heart allografts showed a marked cellular infiltration. Infiltrating leukocytes ($204 \pm 38 \times 10^4$) were isolated from the grafts, 23.3% of which stained positive for either CD4 or CD8 on flow cytometric analysis (Figure 1, A and B). When stained for intracellular production of IFN- γ , IL-2, IL-4, or IL-10 a high number of cytokine producing T cells were identified. CD4⁺ T cells were the dominant source of IL-2 and IL-10. CD8⁺ T cells stained positive for IFN- γ with a slightly higher frequency compared to CD4⁺ T cells, and contributed markedly to the production of IL-4 (Figure 1, C–F). At the mRNA level the T cell infiltrate in the rejecting grafts was also readily detected by amplification of the T-cell receptor C- β chain (Figure 2A). In comparison to the flow cytometric analysis, a similar strong expression for all cytokines investigated was seen in the competitive RT-PCR assay (Figure 2, B–E).

In contrast, very low numbers of graft-infiltrating cells ($5.5 \pm 3.2 \times 10^4$) were isolated from syngeneic grafts at day 5 (Figure 1A), corresponding to 2.7% of the total cells recovered from rejecting hearts. Intracellular cytokine analysis was therefore not feasible because of the low cell number. Analysis of C- β expression by RT-PCR confirmed the marked reduction in the level of the T cell infiltrate (696 ± 357 fg compared to $5,165 \pm 154$ fg in rejecting hearts). Analysis of cytokine mRNA expression revealed very low expression of all cytokines analyzed in comparison to those present in untreated allogeneic hearts, 0.2% for IFN- γ (86 ± 103 fg *versus* $44,006 \pm 9,698$ fg), 0.48% for IL-2 (2.0 ± 1.2 fg *versus* 415 ± 22 fg), 1.9% for IL-4 (3.5 ± 2.9 fg *versus* 185 ± 88 fg), and 3% for IL-10 (85 ± 43 fg *versus* $2,828 \pm 762$ fg) (Figure 2, B–E).

Competitive RT-PCR and Intracellular Cytokine Staining Give Divergent Results in Nonrejecting Allografts

Pretreatment with depleting anti-CD4 antibody and DST leads to long-term graft survival (median graft survival time >100 days) and results in transferable donor-specific tolerance.¹⁴ A striking difference was seen in the cytokine profile in nonrejecting heart grafts when intracellular cytokine staining and competitive RT-PCR were used.

The total number of graft-infiltrating cells isolated from heart grafts transplanted into pretreated recipients was slightly higher compared to the number of cells isolated from hearts from untreated recipients ($266 \pm 135 \times 10^4$ *versus* $204 \pm 38 \times 10^4$). The proportion of T cells in the infiltrate was also greater, resulting in a marked increase in the absolute number of both CD4⁺ T cells ($68.7 \pm 36.8 \times 10^4$ *versus* $23.8 \pm 4.4 \times 10^4$) and CD8⁺ T cells ($46.6 \pm 24.1 \times 10^4$ *versus* $23.8 \pm 4.4 \times 10^4$) compared with untreated controls (Figure 1, A and B). Although the

percentage of T cells staining positive for intracellular cytokines was decreased in nonrejecting hearts, apart from IFN- γ -positive CD8⁺ T cells (Figure 1G), this decrease was counterbalanced by the enlarged population of infiltrating T cells in the nonrejecting hearts. Accordingly, the number of T cells staining positive for intracellular IFN- γ and IL-2 was also higher for both T cell subsets (Figure 1, C and D). The number of IL-4 and IL-10-positive T cells was also slightly higher in the CD4⁺ subset, with CD8⁺ T cells reaching the same level as in the untreated group (Figure 1, E and F).

In contrast to the intracellular cytokine staining, the amount of each of the cytokines measured by RT-PCR in nonrejecting heart allografts was only a fraction of that measured in untreated allografts (Figure 2). This gives a completely different picture of the activation state of the infiltrating T cells present in the nonrejecting grafts. The expression of IFN- γ as determined by competitive RT-PCR was reduced to 27% ($11,950 \pm 420$ fg), IL-2 to 5% (21 ± 5.8 fg), IL-4 to 2.5% (4.6 ± 1.1 fg), and IL-10 to 19% (544 ± 86 fg) compared to untreated allografts (Figure 2, B–E). The strong C- β expression reflected the degree of cellular infiltration in the nonrejecting heart grafts, but did not exceed that measured in the untreated allografts, as might have been expected from the increased number of T cells isolated from these grafts. The reason is most likely because of the lower expression of C- β by nonactivated T cells (Figure 3A).

Restimulation of Graft-Infiltrating Cells from Nonrejecting Heart Grafts with PMA and Ionomycin Results in Up-Regulation of Cytokine mRNA

One possible explanation for the discrepancy between the intracellular cytokine staining and RT-PCR data obtained from nonrejecting hearts is the *in vitro* stimulation required for the detection of cytokines by flow cytometry. Unmanipulated graft-infiltrating cells isolated from C57BL/10 hearts transplanted into CBA recipients pretreated with the tolerogenic protocol showed similar levels of mRNA expression for IL-2 (19 ± 11 fg), IL-4 (14 ± 17 fg), and IL-10 (355 ± 44 fg) compared to that amplified from whole heart tissue of nonrejecting grafts. The expression of IFN- γ in graft-infiltrating cells ($3,598 \pm 876$ fg) was only a third of that measured in whole heart tissue. In contrast, after stimulation with PMA and ionomycin under equivalent conditions to those used for intracellular cytokine staining, the levels of mRNA quantitated by RT-PCR for all cytokines increased dramatically. The expression of IFN- γ rose to $26,319 \pm 4,987$ fg (sevenfold) and IL-10 to $1,415 \pm 211$ fg (fourfold), nearly equivalent to the levels measured in untreated allografts. The increase in the expression of IL-2 was nearly 100-fold ($1,881 \pm 304$ fg) and for IL-4 was nearly 45-fold (639 ± 148 fg) (Figure 3). Thus the *ex vivo* restimulation of graft-infiltrating cells as required for intracellular cytokine staining leads to the *de novo* synthesis of mRNA for all of the cytokines analyzed in this study, resulting in an expression level comparable to that found in rejecting allografts.

Figure 1. Intracellular cytokine staining of graft-infiltrating cells isolated from syngeneic, untreated (rejecting), and YTA3.1+DST pretreated (nonrejecting) heart allografts 5 days after transplantation. Total number of graft-infiltrating cells (**A**) and graft-infiltrating T cells staining positive for CD4 (**black bar**) and CD8 (**white bar**) (**B**). Absolute number of CD4⁺ and CD8⁺ T cells staining positive for IFN- γ (**C**), IL-2 (**D**), IL-4 (**E**), and IL-10 (**F**). Numbers given are $\times 10^4$ and represent the mean of three experiments $\pm$ SD. n.a., not applicable. The flow-cytometry data of one set of experiments are shown in (**G**). Mean fluorescence intensity (MFI) for staining with anti-CD4 or anti-CD8 antibody is given on the x axis, and MFI for staining with anti-cytokine antibody or isotype control on the y axis, for which the percentages of positive cells are indicated.

Figure 2. Competitive RT-PCR analysis of cytokine gene expression in syngeneic, untreated (rejecting), and YTA3.1+DST pretreated (nonrejecting) heart allografts 5 days after transplantation. The expression of C- β , IFN- γ , IL-2, IL-4, and IL-10 (A-E) is given in femtogram (fg) of the amount of co-amplified competitor construct. The numbers represent the mean of three experiments $\pm$ SD.

Figure 3. Competitive RT-PCR for the expression of C- β , IFN- γ , IL-2, IL-4, and IL-10 (A-E) in graft-infiltrating cells isolated heart grafts transplanted into recipients pretreated with YTA3.1+DST, before (-, left bar) and after (+, right bar) restimulation with PMA/ionomycin. The result is given in femtogram (fg) of the amount of co-amplified competitor construct. The numbers represent the mean of three experiments $\pm$ SD.

The *in vitro* restimulation also increased the expression of C- β mRNA by the isolated graft-infiltrating cells that might influence the assessment of the T cell infiltrate using RT-PCR. This finding also limits the use of C- β expression, instead of a housekeeping gene as a T-cell-specific normalization factor for cytokine gene expression in tissue samples. The higher expression of C- β mRNA in activated T cells would result in cytokine expression to be underestimated after normalization.

Discussion

Our data confirm that the number of graft-infiltrating leukocytes present in heart allografts transplanted into recipients pretreated with anti-CD4 antibody and DST is the same as the number present in acutely rejecting allografts transplanted into untreated recipients in the early time period after transplantation (Figure 1).^{3,17} Thus, based on analysis of the degree of cellular infiltration alone, it would not be possible to distinguish between rejecting and nonrejecting grafts. Moreover, we have shown that graft-infiltrating cells from nonrejecting grafts retain their potential to produce cytokines after *in vitro* restimulation with polyclonal stimuli (Figure 3).

Intracellular cytokine analysis by flow cytometry is a powerful tool to investigate cytokine production at the single cell level.¹⁰ However, in the context of transplantation the method requires the isolation and restimulation of graft-infiltrating cells *in vitro*, because the direct staining of isolated T cells with anti-cytokine antibodies does not result in positive staining¹¹ (M. Hara, data not shown). The data obtained here clearly demonstrate that *ex vivo* restimulation with polyclonal stimuli can trigger cytokine production by cells isolated from nonrejecting grafts (Figure 3) that is not representative of the cytokines produced *in vivo* (Figure 2). Therefore, the use of intracellular cytokine staining in the assessment of allograft status is limited, because the restimulation of graft-infiltrating cells leads to cytokine production in T cells regardless of their actual activation state in the graft.

Competitive RT-PCR on the other hand, measures the level of cytokine transcripts reliably in small amounts of graft tissue without requiring cell isolation. The results obtained represent the overall cytokine milieu in the tissue sample including cytokines produced by nonimmune parenchymal cells, which may well influence the overall immune response in the microenvironment of the graft. The difference in the activation state of graft-infiltrating cells between rejecting and nonrejecting heart allografts was clearly detected by the analysis of cytokine transcripts using competitive RT-PCR in this study (Figure 2).

In contrast to the data reported by Stinn and co-workers¹¹ we were able to detect IL-4 in the rejecting heart allografts with RT-PCR and intracellular cytokine staining. The reason for this is most likely because of strain variations in the cytokine expression profile, as IL-4 has also been detected in other studies using C3H or CBA recipients (both H-2^k).^{18,19}

In summary, the assessment of cytokine production in the graft can be a useful addition to the diagnosis of

allograft rejection and acceptance. Our data clearly show that cytokine analysis, which requires polyclonal stimulation *in vitro*, can result in misleading conclusions. To our knowledge this is the first study to compare directly competitive RT-PCR and intracellular cytokine staining in rejecting and nonrejecting cardiac allografts.

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