ANALYSIS OF CHEMOKINE GENE EXPRESSION IN LUNG CELLS BY POLYMERASE CHAIN REACTION

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The article describes a polymerase chain reaction (PCR) based method for semiquantitative assessment of mRNA expression for chemotactic cytokines in the lower respiratory tract cells. Cytokine mRNA was detected using PCR preceded by reverse transcription of mRNA into cDNA. The procedures of RNA extraction and of reverse transcription-PCR assay are described in the first part of the article. The second part demonstrates optimalisation experiments performed to ensure specificity, accuracy and reproducibility of the RT-PCR assay and discuss the approach to mRNA quantification using normalisation of cytokine mRNA expression to the expression of β-actin mRNA.

INTRODUCTION

Chemokines, small molecular weight cytokines[1], act primarily as chemoattractants for several cellular types and also activate leukocytes and affect their effector functions[2]. The superfamily of human chemokines currently comprising more than 30 members has been further subdivided into four subclasses, namely C-X-C, C-C and C and CX_3C[3].

With regard to their chemoattractant properties, immunologists have been considering chemokines to be the prime candidates for mediators of leukocyte recruitment to the sites of inflammation. Providing evidence for participation of chemokines in pathophysiology of inflammatory disease has important implications for the development of novel anti-inflammatory therapies directed at blocking chemokine – induced cellular migration.

The "promigratory" function of chemokines has, therefore, been investigated in a number of clinical investigations which compared chemokine mRNA and protein expression in healthy subjects and in patients with inflammatory conditions. For example, increased chemokine expression has been shown in inflammatory diseases of the respiratory, gastrointestinal and cardiovascular system and it correlated with the extent of inflammatory infiltrate[2,4,5].

This article describes the method, which has been utilised in our studies directed at the assessment of mRNA expression for CC chemokines in the lower respiratory tract cells. The method (PCR in conjunction with reverse transcription) provided mRNA expression data suggesting importance of several CC chemokines for leukocyte recruitment to the lung of patients with inflammatory diseases of pulmonary interstitium[6,7,8]

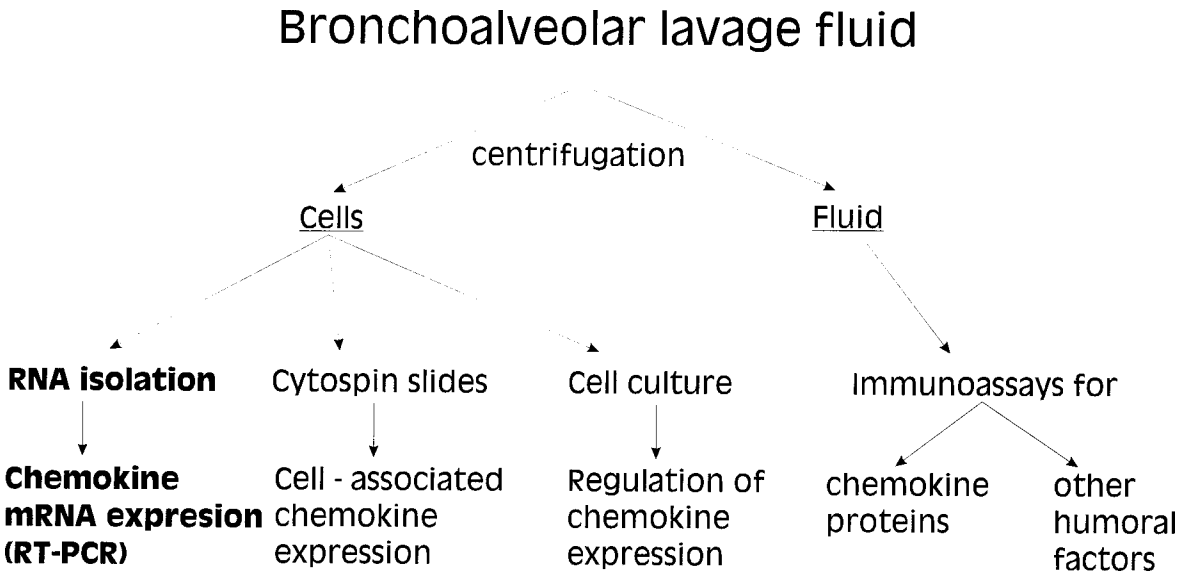
MATERIAL AND METHODS

Processing of the clinical material for the gene expression studies

The lower respiratory tract cells were recovered from the lung and the airways by bronchoalveolar lavage (BAL); BAL was performed according to the standard protocol[9]. The cells were separated from the BAL fluid (BALF) by centrifugation at 200 g, 4 °C for 7 minutes. The supernatant obtained after the centrifugation was collected, aliquoted and stored at –40 °C and at –80 °C for analysis of chemokine proteins and of other humoral factors. The cell pellet, left in the bottom of the tube after collection of the supernatant, was washed and the number of cells was determined. The cells were then divided into two parts. The first part was used to prepare cytospin slides stored in –20 °C for subsequent immunocytochemical localisation of cell-associated chemokines; usually total of $0.6x10^6$ cells was used to prepare 15 slides. RNA was extracted from the second part of the pelleted cells and used for the assessment of chemokine mRNA expression; usually 5–25 μg of RNA was extracted from $1.0x10^6$ – $5.0x10^6$ cells. The whole procedure of BALF processing is depicted in **Scheme 1**.

Determination of chemokine mRNA expression

Chemokine mRNA was detected using polymerase chain reaction (PCR)[10] preceded by reverse transcription of mRNA[11] to obtain cDNA for PCR amplification. At first, standard procedures of RNA extraction and of reverse transcription-PCR assay are described. Subsequently, the optimalisation experiments, which were necessary to perform to ensure specificity, accuracy and reproducibility of the RT-PCR assay, are demonstrated. Finally, the approach to mRNA quantification is discussed.



Scheme 1. Processing of the bronchoalveolar lavage fluid (BALF) for gene expression studies on mRNA and protein level. For details see methods section.

A. Basic methodical procedures

1. RNA extraction from bronchoalveolar cells

Total RNA was isolated from the bronchoalveolar cell pellets using either the single step acid-GITC method[12] or the procedure based on a RNA-isolation kit "RNA-blue" (Exbio Praha, Czech Rep.). The first method allows postponing RNA extraction until suitable time; after separation from the lavage fluid, the cells are lysed in GITC buffer and can be stored in –80 °C until further extraction. The second, "RNA-blue", procedure necessitates immediate processing of the cells until the final isolation of RNA; the procedure was carried out according to manufacturer's instructions. Once extracted, total RNA was stored in 50 µl of sterile deionized water with 40 U of human placental RNAse inhibitor (Promega) at –80 °C until further use.

2. Reverse transcription

The cDNA for PCR amplification was obtained by reverse transcription (RT) of mRNA using oligo(dT) primer[11]. Each reaction contained 1 µg total RNA, 0.4 µg oligo(dT)$_{15}$ primer, 50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM each dNTP, 20 U AMV reverse transcriptase HC (Promega) and 20 U placental RNAse inhibitor in a total reaction volume of 40 µl; a more economical version of the RT could be performed in total reaction volume of 20 µl

Table 1. The primers used for detection of chemokine and beta-actin mRNA by RT-PCR

Primer	Nucleotide sequence	Length	Size	Ref.
RANTES sense	5´TCATTGCTACTGCCCTCTGC3´	20	243 bp	13
RANTES antisense	5´CCTAGCTCATCTCCAAAGAG3´	20		
MIP-1 alpha sense	5´CTCTGCAACCAGTTCTCTGC3´	20	211 bp	14
MIP-1 alpha antisense	5´CGCTGACATATTTCTGGACC3´	20		
SCM-1 alpha sense	5´GGGAGTGAAGTCTCAGATAA3´	20	388 bp	15
SCM-1 alpha antisense	5´TAATTTTATTCATGCAGTGCTTTCATA3	27		
Beta-actin sense	5´TCCTGTGGCATCCACGAAACT3´	21	315 bp	16
Beta-actin antisense	5´GAAGCATTTGCGGTGGACGAT3´	21		

Note: The MCP-1 specific primers, which were designed from the cDNA sequence published in ref. 17, where kindly provided by Dr AM Southcott, the size of the product was 480 bp.

using 0.2 µg of the primer and 10 U of the both reagents. After incubation at 42 °C for 60 minutes the samples were heated to 80 °C for 10 minutes to terminate the reaction. The resulting cDNA was diluted 10-fold in sterile water and stored at 4 °C.

3. Polymerase chain reaction (PCR)

PCR reactions were performed with sequence-specific oligonucleotide primers. **Table 1** summarises the information about the primer sequences, the references to the published chemokine and β-actin cDNA sequences from which the primers were designed[13–17] and gives also the predicted sizes of the amplified PCR products.

The PCR reaction mix contained the forward and reverse primers (0.25 mM each), dNTPs (0.2 mM each), 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.6 U of Taq polymerase (Promega) and 10 µl cDNA in a total reaction volume of 25 µl. After initial denaturation (5 minutes, 95 °C), a range (28–38) of amplification cycles was performed using a thermocycler (MJR-Tetrad, MJR N.J., U.S.A). One cycle consisted of denaturation at 95 °C for 45 seconds, annealing at 55 °C for 45 seconds and extension at 72 °C for 90 seconds. After the last cycle, final extension (72 °C, 10 minutes) was carried out. A negative control reaction containing all components except template cDNA was set up with every PCR experiment.

4. Visualisation and measurements of PCR products

The amplification products were visualised by ethidium bromide fluorescence after 2.0% agarose gel electrophoresis.

For measurements of PCR products, the UV-transilluminated gels were photographed using Polaroid type 665 film. The negative images of the gels were scanned on a laser densitometer and the optical density (OD) values for the individual products were obtained using appropriate computer software (Image Master 1D, Pharmacia).

B. Special methodical procedures – optimalisation of RT-PCR assay

1. Specificity of PCR amplification

The primers were designed so that the amplified region spans one intron. This design enables to identify contamination of cDNA by genomic DNA. If the amplified sample was a mixture of cDNA and genomic DNA, the size of the amplicon would be greater than the predicted size, the difference being the size of the non-coding sequence.

Additionally, the quality of reverse-transcribed cDNA was tested before performing chemokine-specific PCR. For this purpose, an aliquot of the cDNA was used as a template for PCR amplification of a segment of the human histidyl-tRNA synthetase gene[18]. The presence of an intron of approx. 300 bp size in that region of the gene permits the identification of any genomic contamination in the cDNA sample; the negative result of the tests shows failure of cDNA synthesis.

To verify that the bands obtained after electrophoresis of the amplicons represented the specific RANTES, MIP-1α, MCP-1, SCM-1α and ß-actin products, the sizes of the bands were determined using a low molecular weight markers (pBR 322 DNA/Hae III, Boehringer Mannheim; PCR marker, Promega). The sizes were then compared with those predicted from the locations of the primers within chemokine or ß-actin cDNA sequences. In case of specific amplification, the band size was identical to the predicted size.

2. Definition of optimal PCR conditions

Optimal PCR conditions were defined in the experiments directed at 1) determination of optimal number of amplification cycles for each gene, and 2) evaluation of variability of the used PCR assay. These experiments aimed at ensuring accuracy and reproducibility of the amplification.

a) Kinetics of amplification of chemokine and ß-actin cDNA

The kinetics of PCR amplification of chemokine and ß-actin cDNA was determined as follows: Randomly selected cDNA samples were amplified in duplicate for increasing number of cycles. The kinetic curve was obtained by plotting the mean OD values of the amplification products against the number of cycles. The number of cycles located in the middle of exponential phase of the amplification curve (before it reached the plateau phase) was considered to be the optimum.

b) Assessment of inter-assay variability

Randomly selected cDNA samples were amplified on four different days under the same conditions. The optical densities of the products were determined and the variation coefficient was calculated from the values of the mean and standard deviation. The difference between the variances of PCR reactions with cDNA samples from different subjects was also assessed for significance.

c) Assessment of intra-assay variability

Intra-assay variability was tested by triplicate amplification of randomly selected cDNA samples on the same day. After measurements of OD values, the variation coefficients within the triplicates were calculated.

C. Quantification of chemokine expression.

Chemokine mRNA expression was semiquantified by normalising chemokine (e.g. RANTES or SCM-1α) expression to ß-actin, a gene expressed constitutively with little variability. This normalisation approach controls for variability in the amount of input RNA. The RANTES/ß-actin, MIP-1α/ß-actin, MCP-1/ß-actin and SCM-1α/ß-actin ratios (**o**ptical **d**ensity **r**atio, ODR) were derived for each subject by comparison of optical densi-

ties of the specific chemokine and ß-actin amplification products (ODR = OD chemokine/OD β-actin).

RESULTS

1. Specificity of PCR amplification: verification of identity of PCR products, quality of cDNA

As shown in **Fig. 1a** and **1b**, single bands of the predicted sizes were obtained from the amplification reactions with the primers specific for RANTES, MIP-1α and ß-actin, respectively; no products were detected

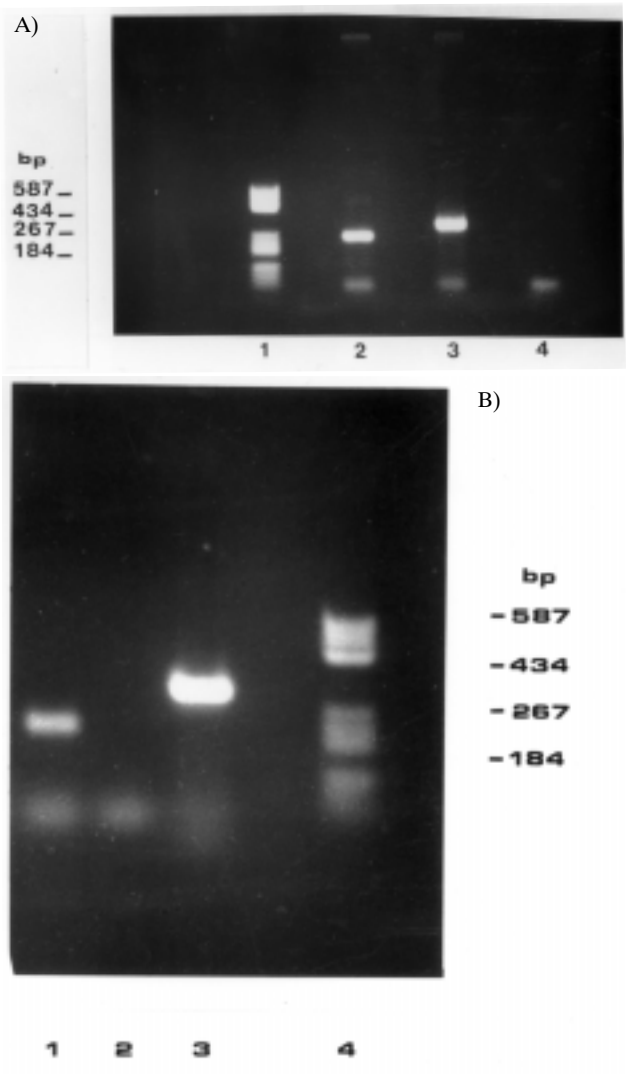


Fig. 1. Specificity of RANTES, MIP-1α and ß-actin PCR amplification reactions. RANTES-specific primers were designed as 20-mers, MIP-1α-specific primers as 20-mers and β-actin-specific primers as 21-mers from the published RANTES, MIP-1α and β–actin cDNA sequences. The sizes of the expected amplification products were 243 bp for RANTES, 211 bp for MIP-1α and 315 bp for β-actin.
A. Lane 1: DNA molecular weight marker V (pBR 322 DNA/ Hae III), lane 2: RANTES amplification product (243 bp), lane 3: ß-actin amplification product (315 bp), lane 4: negative control with RANTES-specific primers.
B. Lane 1: MIP-1α amplification product (211 bp), lane 2: negative control with primers specific for MIP-1α, lane 3: ß-actin amplification product (315 bp), lane 4: DNA molecular weight marker (pBR 322 DNA/ Hae III).

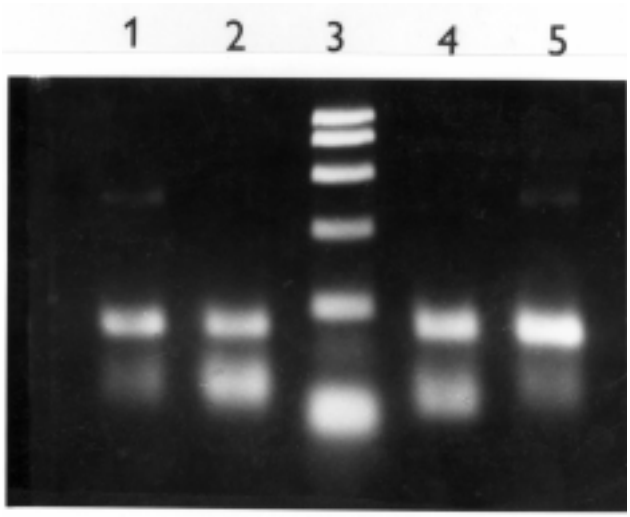


Fig. 2. Testing for contamination of cDNA by genomic DNA by PCR amplification of a segment of the human histidyl-tRNA synthetase gene.
PCR with histidyl-tRNA synthetase specific primers results in one product (128 bp) if the template was pure cDNA, lanes 2, 4. An additional product (approx. 400 bp) shows presence of contaminating genomic DNA, lanes 1,5. Lane 3 – DNA molecular weight marker (PCR marker, Promega); the bands, from the top, are 1000 bp, 750 bp, 500 bp, 300 bp, 150 bp and 50 bp.

in the negative control amplification reactions with the chemokine primers. The PCR reactions with primers specific for the remaining chemokines resulted in the products of 388 bp (SCM-1α) and 480 bp (MCP-1); the negative control reactions with these two chemokine primers yielded no products (data not shown).

PCR amplification of a segment of the human histidyl-tRNA synthetase gene resulted in one product of 128 bp if the template was pure cDNA. In case of contamination, presence of genomic DNA was shown by an additional product of approximately 400 bp. A representative result of the test is shown on **Fig. 2**.

2. Determination of optimal PCR conditions

a) Kinetics of amplification of chemokine and ß-actin cDNA

The determination of an optimal number of cycles for the PCR amplification of chemokine and ß-actin cDNA is illustrated on the example of RANTES and ß-actin – specific amplification.

The cDNA samples were amplified in duplicate for varying numbers of cycles. In case of RANTES, two cDNAs samples (one from a control subject and one from a patient) were amplified, in 4 cycle increments, from 28 to 44 cycles. **Fig. 3A,** demonstrating the kinetics of RANTES PCR, shows that the amplification process was not different in samples from the normal individual and the patient. In both cases the amplification reached the plateau after 40 cycles and was in the exponential phase between 32 and 40 cycles. Therefore, number of 36 cycles was selected as an optimum for further

RANTES amplification reactions. Similar kinetic studies determined 34 cycles to be an optimum for MIP-1α and MCP-1 cDNA amplification; SCM-1α amplification reaction was characterised by a 38-cycle optimum (data not shown).

The kinetics of ß-actin amplification is illustrated in **Fig. 3B**. The curve reaches the plateau phase after 32 cycles, 28 cycles lying within the exponential phase were, therefore, selected as an optimum for ß-actin amplification reactions.

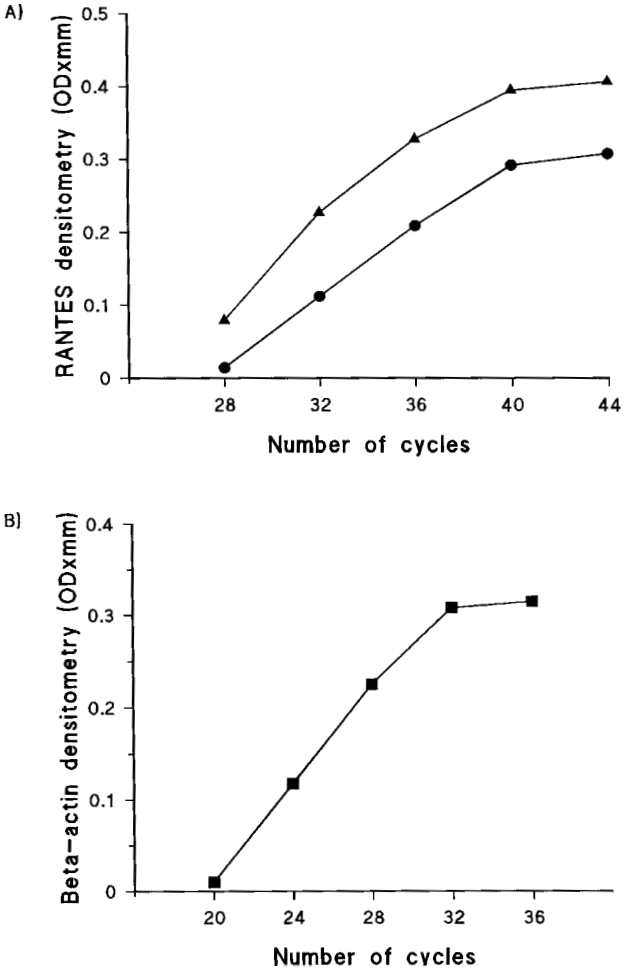


Fig. 3. A. Kinetic analysis of amplification of RANTES cDNA. Duplicate samples of reverse transcribed RNA from lower respiratory tract cells of a normal individual (circles) and of a patient with fibrosing alveolitis (triangles) were amplified using RANTES primers for increasing numbers of cycles. The mean RANTES optical density values were then plotted against the number of cycles.
B. Kinetic analysis of amplification of β-actin cDNA. Duplicate samples of reverse transcribed RNA from lower respiratory tract cells of a normal individual were amplified using β-actin primers for increasing numbers of cycles. The mean β-actin optical density values were then plotted against the number of cycles.

b) Assessment of inter-assay variability

To ensure the reproducibility of the used assay, cDNA samples from one control and one patient were amplified with chemokine-specific primers on four separate days and the OD values of the amplification products were determined. In case of RANTES amplification, which is demonstrated here as an example, the OD values were 1) control; 0.262, 0.180, 0.242, 0.252 (mean ± SD: 0.234 ± 0.037, variation coefficient 0.16), and 2) patient; 0.541, 0.445, 0.441, 0.518 (0.486 ± 0.051, 0.10). The difference between the variances of the four OD measurements of samples from control and patient was not significant. Experiments performed to test the reproducibility of other chemokine-specific PCR amplifications revealed similar degree of variability (data not shown).

c) Assessment of intra-assay variability

To control for intra-assay variability, chemokine-specific PCR amplifications were performed in triplicate samples within the same day: as an example, the uniform pattern of RANTES products obtained in multiple amplification of on one control and one patient cDNA sample is illustrated in **Fig. 4.** The coefficient of variation between OD values of the triplicate products was 0.04 (control cDNA) and 0.01 (patient cDNA), respectively.

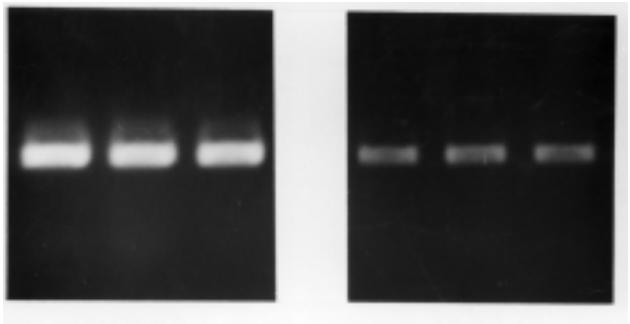


Fig. 4. Assessment of intra-assay variability in PCR amplification reactions with RANTES specific primers. Shown are the products of triplicate amplification reactions performed within the same experiment on samples randomly selected from the patient (left) and the control (right) groups.

3. Identification of chemokine and β-actin mRNA, quantification of chemokine expression.

Using the RT-PCR assay described above, chemokine mRNA was identified in BALF samples, which contained adequate number of cells for isolation of total RNA.

As assessed by their OD values, chemokine PCR products showed great degree of heterogeneity between cDNA from individual subjects. For example in a group of 63 probands, RANTES OD values ranged from 0.000 to 0.387 (mean ± SD: 0.184 ± 0.085, variation coefficient 0.46) and SCM-1α OD values ranged from 0.021 to 0.576 (mean ± SD: 0.302 ± 0.129, variation coeffi-

Table 2. Normalisation of chemokine (SCM-1 alpha) mRNA expression to expression of beta-actin mRNA.

Pair	Sample no.	OD SCM-1	OD Actin	OD ratio
1	46	0.397	0.167	2.38
	59	0.394	0.290	1.36
2	70	0.239	0.237	1.01
	73	0.240	0.075	3.20

Legend: Pair-two cDNA samples with similar chemokine, but different actin OD (refer to the text); OD – optical density; OD ratio = OD SCM-1/OD Actin.

cient 0.43). ß-actin PCR products showed lower degree of heterogeneity: mean OD (SD) values in the same group were 0.181 (52.3) with variation below 30%.

To provide semiquantitative assessments of chemokine mRNA expression, individual chemokine OD values were normalised to the corresponding OD values of ß-actin amplification products and OD ratios (ODR) were calculated to compare the expression in individual subjects. The principle of the normalisation procedure is schematically depicted in the **Table 2**.

Exemplified are two pairs of cDNA samples which have similar SCM-1α OD values but differ in ß-actin OD values. The distinct ß-actin values may either reflect different amount of input RNA used in RT reaction or reflect varying efficiency of RT. Normalisation using OD ratio can, therefore, reveal the difference in chemokine expression between the samples. If the normalisation to ß-actin OD was not performed, the two samples would be incorrectly considered to contain the same number of SCM-1α transcripts. The example provides evidence that this approach can distinguish between samples with different amount of chemokine mRNA.

DISCUSSION

Recently, there has been growing interest in studying expression of chemokines in patients with inflammatory diseases. Such studies should be properly designed and special care should be paid to applying adequate methodology. It will ensure that the results of these basic chemokine investigations may be later applied to immunopharmacological research or to clinical immunology diagnostics. Apart from the generally accepted rules of clinical research (careful selection of well-defined patients, adequate statistics, etc.), standardised and reproducible laboratory methods have to be used for determination and quantification of chemokine expression. This demand applies as well to the methods based on molecular genetic principles, which have recently become popular especially for the assessment of chemokine mRNA expression in human cells and tissues.

Several molecular genetic methods can be used for this purpose. Analysis of mRNA by Northern blotting with subsequent hybridisation to specific, labelled probes[19,20] is laborious and requires high amount of RNA. *In situ* hybridization[20,21] is suitable for mRNA identification at single cell level and requires specially stored clinical material. Solution hybridisation methods (e.g. nuclease protection assay) may fail to provide quantitative results and also require sufficient amount of RNA[20,22]. Polymerase chain reaction, which is sensitive, fast, less complicated than previous methods and relatively cheap[10], is therefore a method of choice for chemokine mRNA expression studies[20]. To detect mRNA, PCR must be performed in conjunction with reverse transcription of mRNA to cDNA, which then serves as a template for PCR (s.c. reverse transcription-PCR).

Despite the described advantages of RT-PCR, this method requires careful optimalisation in order to ensure specificity, reproducibility and also ability to discriminate between samples with varying levels of mRNA. We discuss these issues using examples obtained when optimising our RT-PCR assay, which we have been using for investigations of chemokine expression in lung cells. The basic principles of setting up PCR (e.g. titration of the reaction components, enzyme selection, primer design or creation of cycling protocol, etc.) can be referred to in comprehensive manuals[23].

Non-specific amplification either due to presence of genomic DNA or to carry-over of minute amounts of other amplicons may complicate the evaluation of RT-PCR results. We have attempted to prevent non-specific amplification by several measures related to different steps of our method. We limited chemokine mRNA detection only to those cDNA samples, which were not contaminated by genomic DNA. The method used to screen for contamination was PCR amplification of a segment of the human histidyl-tRNA synthetase gene[18] and we found that this was a simple and easily performed test to distinguish between high quality cDNA samples and cDNA contaminated by genomic DNA. Also, we derived our chemokine primers from separate exons of the gene sequence so that a possible amplification of genomic DNA may be distinguished from that of cDNA[20]. In addition, we tried to create our primer pairs in the way that the products for ß-actin and all four investigated chemokines are of different sizes to allow their easy, not dubious identification on the gel.

Reproducibility of PCR amplification can be influenced by various factors (variations in pipetting, cycler performance, differences between different batches of enzymes and other reagents, etc.). We have, therefore, tested the precision of our chemokine PCR assays in multiple (i.e. repetitive) amplifications both within one assay as well as between different assays. The data on intra-assay and inter-assay precision confirmed adequate reproducibility of the RT-PCR assay and substantiated its usage in long-term follow up of chemokine mRNA expression.

Another important issue concerning chemokine expression studies by PCR is **mRNA quantification**[20,24]. This may be approached in several ways, e.g. by includ-

ing an internal control template into PCR[24,25] or comparing the amplification of the mRNA of interest with another, more widely expressed gene[24,26]. In the latter case, it is important to ensure that the amplification does not continue until plateau phase because the differences between the samples with lower and higher number of specific mRNA transcripts could not be recognized[24,27]. Therefore we utilised for each chemokine PCR assay the optimal number of PCR cycles, i.e. the cycle number found in the preliminary kinetic experiments to be within the exponential phase of the amplification process. Thus our PCR assay could discriminate between different amounts of target sequences present in different samples[24,27]. To avoid the potential problems due to variations in input RNA or to varying efficiency of RT reaction, we normalised our data to the transcription product of the ß-actin gene. The use of this approach for semiquantification of chemokine mRNA was further substantiated by studies from our group and others, who have found significant differences between expression of other chemokines such as IL-8 in the cells from normal and fibrotic lungs using this normalisation method[26,28,29].

Finally, the indirect evidence that the results obtained with our RT-PCR assay are not over-interpreted and correspond to real situation *in vivo* came from our further experiments on protein level. Findings by RT-PCR of increased mRNA for chemokine RANTES in patients with sarcoidosis corresponded to the observations of increased RANTES protein expression in the lung cells of these patients[7]. RANTES gene expression is transcriptionally regulated[30] and, therefore, the relationship between mRNA and protein expression suggests that the methods provide adequate results. This conclusion was further substantiated by our recent findings with another chemokine: MCP-1 mRNA expression correlated with the level of immunoreactive MCP-1 protein in the bronchoalveolar fluid[8].

In conclusion, this study has shown that the described RT-PCR methodology is suitable for investigations of chemokine mRNA expression in clinical samples. Chemokines RANTES, MIP-1α, MCP-1 and SCM-1α could be identified in lower respiratory tract cells and their expression quantitated using normalisation of chemokine mRNA to mRNA for β-actin. This semiquantitative assessment of chemokine transcripts enables to compare levels of chemokine mRNA between the different individuals, or between different *in vitro* cell cultures. The described methodology is, therefore, generally applicable in studies of the role of chemokines in disease pathophysiology and therapy.

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