

Microarray Profiling of Lymphocytes in Internal Diseases With an Altered Immune Response: Potential and Methodology

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Recently it has become possible to investigate expression of all human genes with microarray technique. The authors provide arguments to consider peripheral white blood cells and in particular lymphocytes as a model for the investigation of pathophysiology of asthma, RA, and SLE diseases in which inflammation is a major component. Lymphocytes are an alternative to tissue biopsies that are most often difficult to collect systematically. Lymphocytes express more than 75% of the human genome, and, being an important part of the immune system, they play a central role in the pathogenesis of asthma, RA, and SLE. Here we review alterations of gene expression in lymphocytes and methodological aspects of the microarray technique in these diseases. Lymphocytic genes may become activated because of a general nonspecific versus disease-specific mechanism. The authors suppose that in these diseases microarray profiles of gene expression in lymphocytes can be disease specific, rather than inflammation specific. Some potentials and pitfalls of the array technologies are discussed. Optimal clinical designs aimed to identify disease-specific genes are proposed. Lymphocytes can be explored for research, diagnostic, and possible treatment purposes in these diseases, but their precise value should be clarified in future investigation.

INTRODUCTION

During the last 5 years, microarrays have been developed for large-scale clinical research and routine to identify genes involved in disease states. At present, microarrays comprising the whole human genome (all 30 000 genes) have become commercially available and their potential to identify abnormal gene activity in disease is now well recognized. The microarray approach is particularly suited to identify the activity of genes that are differently expressed during a disease state and that may become normalized following recovery of the patient. So, perhaps more than with the conventional gene-identification approach, the success of the microarrays technique depends heavily on proper clinical designs. The most promising

gene expression profiles have been derived from cancer research [1, 2, 3, 4] using tumor tissue and peripheral blood mononuclear cells (PBMCs). In internal diseases such as pulmonary diseases [5], including asthma [6], autoimmune disorders, for example, rheumatoid arthritis (RA) [7], systemic lupus erythematosus (SLE) [8], cardiovascular diseases [9], psychiatric disorders [10, 11], and others as nicely reviewed [12] have used mainly conventional mRNA-assay techniques.

PBMCs—and in particular lymphocytes—are particularly convenient for medical research and diagnostic applications, because they can easily and repeatedly be collected in sufficient quantities in the course of the disease. The involvement of T cells and B cells in the pathogenesis of asthma is well recognized; particularly that of Th2 cells both in atopic allergic asthma and nonatopic and occupational asthma [6, 13]. In SLE immune abnormalities in a wide variety of cell populations to include B and T lymphocytes, monocytes, and natural killer (NK) cells have been noticed [8]. Defects of phenotypic and functional T cells were found in RA, including abnormal clonal expansions and suppressed proliferative responses, which suggest a defect in T-cell differentiation [14]. Excessive Th1-type cytokines have been associated with tissue destruction as found in autoimmune disease as RA, whereas overabundant Th2-type cytokines have been

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implicated in atopy and allergic asthma. Altered function of lymphocytes in diseases is a result of abnormal expression of genes for numerous cytokines, receptor components, signal transduction pathways, and modulators of transcription and translation. In addition, polymorphism of the genes affects their functional properties. Despite intensive studies, the pathophysiological and pathogenetic mechanisms behind these diseases are still poorly understood.

PBMCs comprise a very heterogeneous population and the question may arise whether meaningful information on gene expression on particular processes can indeed be deduced from these cells. Could it be that—for instance—in the diseased state the relative composition of the population just changes without alterations in the gene-expression of the individual cell per se? In that case the changes seen may merely reflect changes in population of cells. But even if this is the case, it should be realized that such changes do not occur without changes in intracellular transduction and gene expression. Activation of sympathetic associated beta-adrenergic receptors causes the release of lymphocytes from the spleen. Changes in cortisol suppress proliferation of certain subpopulations of lymphocytes. Anyway, such receptor-mediated effects are concomitant with altered gene expression, which is considered indicative of the disease state.

Interest of gene expression of PBMCs concerns with identifying both pathogenetic and pathophysiological processes. Pathogenetic processes are primarily associated with the cause of a disease, so microarrays may lead to the identification of abnormal genes and gene activities that may not be only limited to PBMCs, but may occur in cells of pathological tissue as well, or—alternatively—that the immune reaction by itself is abnormal. In contrast, pathophysiological changes in lymphocytic gene expression are considered as an essentially normal reaction of the immune system to an abnormal, that is, pathological, stimulus. So, at least theoretically, pathophysiological gene profiles may be shared in a variety of diseases, whereas pathogenetic gene expression is expected to be disease specific. To discuss this issue in more detail, we will compare lymphocytic gene expression profiles in asthma, SLE, and RA. Because of presumably different pathogenesis of these diseases, common gene-expression profiles point to a general response of the immune system, whereas differential profiles are likely to be related to disease-specific processes.

The latter is important for diagnostic applications, for research purposes less stringent criteria may also be, and a variety of strategies have been proposed, so that the focus may be on a limited number or profiles of genes related to a particular gene. Accordingly, insight in gene activities in the diseased state is obtained, but the chance is relatively small to identify novel pathogenetic or diagnostic genes. Often, the so-called supervised or unsupervised analyses are applied. In the supervised approach all the expressed genes are correlated with that of the expression of a known

aberrant gene, so clusters of genes involved in a particular disease are identified. In the unsupervised approach clusters of gene expression the structure (clustering) of genes is determined, without a priori assumptions about gene expression. Both strategies have recently been applied in acute myeloid leukemia [3, 4].

In this review we consider the potential of the microarray gene expression in lymphocytes as a proper and convenient approach to investigate in detail pathophysiological processes at the molecular level. We review the molecular mechanisms and gene-expression patterns involved in asthma, SLE, and RA, taken the lymphocyte as pivot.

ASTHMA

Molecular mechanisms

Allergic asthma is a chronic pulmonary disease associated with bronchoconstriction and inflammation, in which Th2 cells play a central role. It is well known that allergen-specific IgE synthesis is T-cell dependent on cognate activation of B lymphocytes and Th2-derived cytokines, such as IL (interleukin)-4 and IL-13 [15]. T lymphocytes of the T-helper-2 subtype, producing IL-4, IL-5 and IL-13, have been shown to be prominent in airways of the asthmatic patients [13, 16]. These cytokines play pivotal roles in asthmatic disorder, since IL-4 induces IgE production by B lymphocytes and IL-5 is the main factor regulating eosinophilia. Elevated percentages of CD4 T cells expressing mRNA encoding IL-4 and IL-5, and CD8 T lymphocytes expressing IL-5, were found in asthmatics as compared with the controls [17]. IL-13 is responsible for inducing most of the phenotypic features of allergic asthma (ie, airway responsiveness, mucus metaplasia, and eosinophilic inflammation). The cellular targets of IL-13 responsible for each of the phenotypic features of asthma and the specific genes whose expression is regulated in these target cells remain, however, unknown. Since the main mode of action of IL-13 is regulating gene expression of airway tissue cells, these questions seem ideally suited to expression array experiments [18]. Moreover, it has recently been hypothesized that IL-13 may participate in the pathogenesis of asthma by activating a set of “proasthmatic” genes in airway smooth muscle cells [19]. These investigators further confirm the hypothesis that gene modulation by IL-13 in airway smooth cells is essential in the development of allergic asthma [19]. Both IL-13 and IL-4 are capable of inducing isotype class switching of B cell to produce IgE after allergen exposure [20].

Many more cytokines with potential relevance to asthma have been described. So, IL-25 acts in Th2 differentiation; IL-9, IL-11, IL-13, IL-16, and IL-17 have been linked to asthma; and IL-12, IL-18, IL-23, IL-27 are involved in Th1 development and IFN- γ production, which might be deficient in patients with asthma [13]. In addition, IL-12 and IL-18 have the potential to reduce airway inflammation to inhaled challenge after Th2

sensitization [21]. Data of Kodama et al point to a role for IL-18 in allergic inflammation in which IL-18 limits the development of the local inflammatory response to antigen [22]. It has been reported that atopic asthma is associated with a downregulation of IL-12 mRNA and also that IL-12 suppresses the expression of Th2 cytokines and their associated responses, including eosinophilia, serum IgE, and mucosal mastocytosis [21]. It has been shown that IL-3 and GM-CSF also may influence the inflammatory process in asthma through their regulatory role on eosinophil survival, differentiation, and effector function [23]. Furthermore Ferreira showed that after allergic challenge in atopic patients IL-4, IL-5, and IL-13, as well as the pro-inflammatory cytokines GM-CSF, TNF- α , and IL-6 are consistently increased when compared with the respective control value [24]. IL-10 modulates IgE production and induces apoptosis of eosinophils [25]. IL-8, TNF- α , and leukotriene B4 can be used as markers of neutrophilic inflammation and to evaluate the response to inhaled steroid therapy in asthma patients [26].

The transcription factors T-bet and GATA3 may affect airway immunopathology in asthma [27]. T-bet induces an IFN- γ producing Th1 phenotype, and also represses IL-4 and IL-5 production from differentiated Th2 cells [28]. Finotto et al [29] provided the evidence for decreased number of CD4+ T cells expressing T-bet in the airway of human asthma patients relative to control subjects. Moreover, they showed deletion of the T-bet gene in mice resulted in airway eosinophilia, Th2 cytokine production, airway hyperresponsiveness, and changes of airway remodeling in the absence of allergen sensitization and inhaled allergen challenge. GATA3 could repress IFN- γ production, induces IL-4 and IL-5 [30], and is an important controller of the IL-5 gene locus [31]. T cells from asthmatic patients expressed 5 times the level of GATA3; so increased expression of GATA3 may underlie augmented Th2-like cytokines in this disease. The transcription factors c-Maf, NIP45, and JunB that increase IL-4 activation appear to be expressed solely in Th2-cells and not Th1 cells [32]. Gene deletion or overexpression of these factors correspondingly affects IL-4 production [32]. Other transcription factors might be crucially involved in gene expression of IL-5 such as NFAT, AP-1, IL-4, IL-13, and the signal transducer and activator of transcription (STAT6) [33, 34].

Various G-protein-coupled receptors are expressed on lymphocytes. Muscarinic receptor subtypes (M1–M5) are involved in the control of airway function. Dysfunction of these receptors contributes to the development of airway hyperresponsiveness and bronchomotor responses associated with asthma [35]. Human lymphocytes express M2–M5 receptors and corresponding mRNA [36, 37]. Ricci et al [38] reported that lymphocytic M2 and lesser amount of M5 receptor subtypes are increased in bronchial asthma and that these changes are related to bronchial hyperresponsiveness. They suggest that peripheral blood cholinergic receptors reflect the status of

cholinergic dysfunction and involvement of lymphocyte cholinergic system in allergic inflammation.

A reduced β -adrenergic responsiveness plays an important role in the increased airway reactivity of asthmatic patients. This hypothesis has been supported showing a reduced β -adrenergic responsiveness in lymphocytes of asthmatic patients, predominantly during the occurrence of active and severe symptoms [39]. The results of Motojima et al [40] suggest that beta-blockade and bronchial hypersensitivity in asthmatic patients may in part be due to a decreased number of β -adrenoreceptors.

An increasing body of evidence shows that nerve growth factor (NGF) exerts biological activity not only on the central and peripheral nervous system but also on the immune system, thereby influencing allergic diseases and asthma. NGF is produced by cells of the nervous system, and also by T and B lymphocytes, which display functional NGF receptors [41, 42]. Bonini et al [43] reported that NGF circulating levels of NGF are increased in patients with allergic diseases and asthma. Moreover, NGF increases airway hyperreactivity to histamine in an animal model of asthma, while anti-NGF treatment reduces airway hyperreactivity in sensitized mouse induced by topical challenge of ovalbumin.

Gene expression

There are a number of candidate genes thought to play a role in the development of asthma. The region of chromosome 5q31–33 contains several genes that modulate atopic responses, including IL-4, IL-5, IL-13, and GM-CSF, as well as the GR and β -AR. Polymorphism identified in the IL-13 gene has convincingly been associated with a variation in IgE levels and with various features of the asthmatic phenotype [44, 45, 46]. The IL-4 gene is of a great interest because it causes B-cell isotype switching from IgM to IgE and stimulates IgE production in allergic sensitization. It has been shown that polymorphism of the IL-4 receptor gene is associated with atopy and asthma [47]. Moreover, gene-gene interaction between IL-4 receptor and IL-13 was associated with asthma [20]. The gene of the inflammatory marker TNF- α has also been tested as candidate gene leading to asthma [48]. The recent investigation of Karjalainen et al [25] showed that also IL-10 gene polymorphism is associated with eosinophil count and circulating IgE in asthma.

The polymorphism of the β 2-AR promotor at positions 16 (arginine to glycine) and 27 (glutamine to glutamic acid) is known to be functionally relevant and has been associated with more severe forms of asthma, nocturnal asthma, and decreased airway responsiveness in asthmatic subjects [49].

It has been shown that corticosteroid-resistant bronchial asthma is associated with increased c-fos expression in T lymphocytes [50]. Glucocorticoids (GCs) are involved in the regulation of numerous physiological processes and, as drugs, represent the cornerstone of anti-inflammatory treatment in asthma. Their

anti-inflammatory effects are mediated by the GR- α which represses expression of various genes encoding inflammatory mediators [51]. Alternative splicing of the human GR gene produces a splice variant, GR- β , termed the silent receptor. Increased expression of GR- β is found in GC insensitive patients with asthma [52]. It seems that overexpression of GR- β may play a role in GC-resistant asthmatics, whereas in GCs-dependent asthmatics, a predominant GR- α expression was found. Suppression of the expression IL-5 and GM-CSF genes by GCs has been shown in asthmatics recovering from acute exacerbation of disease [23].

CTLA-4 is a second costimulatory molecule that is expressed only on activated T cells. It is considered to be important in the development of many of the immunological and physiological features of asthma [53]. Polymorphisms of the CTLA-4 gene have effects on immune response in asthma and may serve as a clinically useful marker of severe asthma [53].

Microarray technology offers the new opportunity to gain insight into global gene-expression profiles in asthma, allowing the identification of novel asthma-associated and inflammatory genes.

Differentially expressed genes in a monkey model of allergic asthma showed that of the approximately 40 000 cDNAs represented on the microarray, expression levels of 169 changed by more than 2.5-fold in at least one of the pairwise probe comparisons; these cDNA encoded 149 genes, of which 52 were novel and 97 were known genes for which a role in asthma pathogenesis had been implicated before, such as chemokines and other inflammation-associated genes, matrix proteins, and matrix metalloproteases, involved in airway remodeling [54]. Gene expression in a mice model of asthma revealed among the hundreds of differentially expressed genes the unexpected observation of the increased expression of arginase [55].

Gene-expression profiling airway inflammation in mice showed that of the 1176 genes on the array, the expression patterns of 280 genes were consistently altered. Of these genes, the steady-state levels of 93 genes were up-regulated and 29 were downregulated [56].

The effect of inhaled corticosteroid therapy on gene expression was followed using bronchial mucosa biopsies from healthy controls and subjects with allergic asthma, it appeared that corticoid therapy normalizes partly or completely the expression of 26 of the 79 genes of known function identified as differentially expressed in asthmatics [57].

To conclude, there are several abnormalities of function and metabolism of lymphocytes in which an altered gene expression plays a main role. In particular, differently expressed genes involved in inflammation remodeling and epithelium activation, genes encoding cytokines, transcription factors, costimulatory molecules, lymphocytic receptors, and other genes with unknown functions were detected. But, till present, there are no reports of

microarray gene expression in human lymphocytes in asthma.

RHEUMATOID ARTHRITIS

Molecular mechanisms

RA is a chronic, autoimmune inflammatory disease of the synovium with progressive destruction of affected joints. The Th1 cells are thought to contribute to the inflammation by inducing high levels of the proinflammatory cytokines TNF α , IL-1, IL-6, and IL-17 in the synovium [58], which leads to cartilage destruction and bone erosions [59]. IL-6 prompts B cells to differentiate and mature into antibody-secreting cells. Increased levels of IL-6 correlate with increased levels of rheumatoid factor. Moreover, IL-6 enhances bone resorption and may play a role in the periarticular osteoporosis characteristic of early RA [60]. Also it stimulates the production of C-reactive protein in RA, nonspecific indicator of disease activity [60]. IL-4 mRNA is almost absent in RA synovium and IL-4-producing Th2 clones can rarely be detected [61]. IL-2 is thought to play an important role in the pathogenesis of RA. Serum IL-2 levels and sIL-2R were elevated in patients with RA as compared to controls [62, 63].

TNF- α and IL-8 mediate ongoing destruction of cartilage, subchondral bone, and other joint-related tissues [64]. Cytokine IL-18 has been cloned that exhibits powerful Th1-promoting activities in synergy with IL-12. IL-18 induces production by Th1 clones [65]. IL-18 receptor expression was detected on synovial proliferation, upregulates IL-2R expression, and promotes IFN- γ , TNF- α , and GM-CSF lymphocytes and macrophages [66]. Small but physiologically relevant amounts of IFN- γ and the Th1 cytokine IL-17 are expressed in RA and could contribute to immune responses, fibroblast activation, and bone destruction [67].

B cells have been shown to participate in chronic rheumatoid synovitis. They undergo antigen-dependent clonal expansion, affinity maturation, and differentiation into plasma cells, and produce rheumatoid factor, a well-recognized prognostic factor for aggressive RA [68].

Activation of c-fos may be involved in cartilage metabolism and might play a crucial role in the pathogenesis of arthritic destruction [69]. TNF- α and IL-6 augmented c-fos gene expression of rheumatoid synovial cells, but transactivation of c-fos gene became resistant against cytokine stimulation under prolonged expression of c-fos gene [70].

GCs are the most powerful anti-inflammatory drugs used in the treatment of RA. In particular, most immunosuppressive and anti-inflammatory effects are exerted by an interaction of GRs with AP-1 and NF-kappaB [71]. GCs inhibit also IL-1 and TNF- α forming a cytokine-HPA axis feedback circuit [71]. The GR number in lymphocytes might be helpful to predict which patients with RA will respond to low- or medium-dose prednisone

and therefore do not or will not require higher doses [72].

An increased nitric oxide (NO) level as a result of activity of the enzyme NO synthase has been shown in the serum and in synovial macrophages of patients with RA. NO causes chondrocytes apoptosis and plays a role in various other inflammatory and destructive processes [73, 74].

An inducible form of this enzyme is present in macrophages, polymorphonuclear leukocytes, and NK cells. In murine models, blockade of NO production prevents and treats autoimmune disease [75].

Several studies have demonstrated a significant rise and fall in the expression levels of μ - and κ -opioid receptors, respectively, in rat with polyarthritis [76, 77]. In RA patients, the κ -opioid receptor mRNA was expressed on T and B cells and NK cells [78]. They also reported that the levels of expression of κ -opioid receptor mRNA in lymphocytes were decreased in RA patients in comparison with healthy volunteers; and it was significantly related to the inflammatory activity or chronic pain in the RA patients.

Gene expression

In RA, inflammation of the joint is caused by the gene products of lymphocytes and other cell types from the circulating blood presenting in the synovium and cartilage tissues. To get insights into pathophysiological pathways, Justen et al [79] used the suppression subtractive hybridization method to identify differentially expressed genes in synovial tissue from RA patients. DNA sequencing identified 12 gene products including cytoskeletal γ -actin, fibronectin, superficial zone protein, IFN- γ inducible genes such as a novel thiol reductase, and two genes of unknown function (HSIFNIN4, RING3). Compared to osteoarthritis patients, 9 of the 12 genes were overexpressed.

Using microarray technology in human RA monocytes, chondrocytes, and synoviocytes, Heller et al [7] showed that prominent upregulated genes are IL-6, the MMPs Strom-1, Col-1, Ge1A, HME, and (in certain samples) PUMP, TIMPs, and the adhesion molecule VCAM. With the 1046-element array of randomly selected cDNAs from peripheral blood library probes, RA samples showed hybridizations to large number of genes. Of these three genes were upregulated and they are TIMP-1, apoferritin light chain, and manganese superoxide dismutase (Mn-SOD), while others were differentially expressed.

In the rheumatoid nodule there was a prominent expression of IL-1 β , and TNF- α together with IL-12, IL-18, IL-15, and IL-10 represents a cytokine profile similar to that of the synovial lesion of RA, which is generally accepted as being due to Th1-mediated inflammation [80].

A recent study of the rheumatoid synovium using cDNA microarray has demonstrated that a total of 121 genes were significantly higher expressed in the RA-I tissues, whereas 39 genes were overexpressed in the RA-II

tissues [81]. In this study, an attempt was made to subclassify RA patients based on the global expression of genes in affected synovial tissue. The RA-II group showed expression of genes suggestive for fibroblast dedifferentiation. Within the RA-I group, two groups were distinguished; the RA-Ia group showed predominantly immune-related gene activity, while the RA-Ib group showed an additional higher activity of genes indicative of the classical pathway of complement activation. The differences in expression profiles provide opportunities to stratify patients based on molecular criteria that may require different treatment strategies. Thus, altered expression of numerous genes in a different tissue was observed, including lymphocytes. We suppose that using a microarray approach in lymphocytes could be an attractive approach toward our understanding of the molecular mechanisms of pathogenesis of RA and will allow to identify potential targets for diagnostic procedures and therapeutic intervention.

SYSTEMIC LUPUS ERYTHEMATOSUS

Molecular mechanisms

SLE involves immune abnormalities in a wide variety of cell populations including B and T lymphocytes, monocytes, and NK cells. In murine models of SLE, an altered production of both Th1 (such as IFN- γ and IL-2) and Th2 (such as IL-4 and IL-10) cytokines has been reported [82]. The disease activity in animals significantly improved and/or the production of antibodies decreased by treatment with anti-IFN- γ receptor [83]. A similar effect was obtained with anti-IL-4 [84] or IL-10 antibodies [85]. The entire cytokine profile produced by circulating lymphocytes has as yet not been clearly elucidated in human SLE. Production of intracellular IL-10 was higher in B cells (predominantly in the CD5⁺ cell subset) of SLE patients as compared to controls, indicating its implication in the immune dysregulation in SLE [86]. Serum IL-10 values seem to reflect SLE disease activity and it has been suggested that overexpression of IL-10 might play a pathogenic role [87] and that modulation of the level of IL-10 may be of potential therapeutic benefit [88]. It has been shown by Sturfelt et al [89] that relative absence of IL-Ra response appears to be a feature characteristic of kidney involvement in SLE patients.

Imbalance between Th1 and Th2 cytokines appears also to be a hallmark for SLE. Patients had increased levels of serum IL-4, IL-10, IL-12, and IL-18. Moreover, IL-18 has been found to be disease-activity related. A recent study has demonstrated that IL-12 inhibits in vitro immunoglobulin G production in SLE patients and reduces anti-dsDNA-secreting cells [90].

Several defects of T-cell signal transduction pathways have been discovered over the last decade [91, 92]. They include diminished T-cell receptor ζ -chain expression in T cells in a majority of SLE patients with defective cAMP-dependent protein phosphorylation due to deficient activities of type I and type II isozymes of protein kinase

A (PKA) [93]. Moreover, deficient PKA activity in SLE T cells may contribute in part to impaired Ca^{2+} homeostasis resulting in calcineurin-catalyzed dephosphorylation of the transcription factor NF-AT. Genes whose transcription is regulated by NF-AT, such as CD154, Fas ligand, and c-myc, are overexpressed in SLE T cells [94]. Also Georgescu et al [95] showed increased spontaneous apoptosis of lymphocytes that has been linked to increased IL-10 production, release of FasL, and overexpression of the FasR in SLE. Abnormalities of two transcription factors have recently been identified in SLE T cells: (1) reduced/absent p65-RelA subunit of NF- κ B and (2) increased phosphorylated cAMP response modulator (p-CREM) binding to the IL-12 promoter [96].

Changes in the NGF levels were found in plasma of adult patients with SLE [97]. Already in childhood NGF levels can be correlated with disease activity [98]. These results suggest that NGF may play a role in the pathogenesis of SLE and that NGF levels may be of prognostic value in evaluating the course of the disease and outlining the medication.

Gene expression

Gene expression in lymphocytes showed high expression of IL-4, IL-6, IL-10, and TNF- α in SLE patients as compared to control [99]. The resulting high level of cytokines with strong effect on proliferation and differentiation of B lymphocytes could be responsible for characteristic B-cell hyperactivity and autoantibody production seen in SLE. Results of other investigations [100] demonstrated impaired production of IL-12 in SLE lymphocytes and deficient IL-12 p40 gene expression. Downregulation of IL-12 p40 gene expression appears to be the cause of IL-12 p70 deficiency in SLE. IL-12 and IFN- γ inhibit IL-10 expression and reduce IL-10-secreting cells. This indicates that correction of the deficiency of IL-12 and IFN- γ in SLE may normalize pathogenically excessive IL-10 and help remit the disease.

Rus et al [8] using cDNA microarray in PBMCs of 21 SLE patients and 12 controls demonstrated that of 375 potentially relevant genes 50 genes exhibited more than 2.5-fold difference in expression level compared to healthy control and twenty genes were significantly different. Most of these genes have not previously been associated with SLE and belong to a variety of families such as TNF/death receptor, IL-1 cytokine family, and IL-8 and its receptors. For five of the differentially expressed genes found in this study, changes at the protein-expression level have been previously reported in SLE. These investigators have recently published expression pattern of 375 genes in PBMCs from 12 patients with active and 14 patients with inactive diseases; 29 genes were found to best discriminate. Among these genes, 14 were upregulated and 15 were downregulated in patients with active diseases compared to those with inactive diseases. Most of these genes have not been previously associated with disease activity and belong to a variety of families such as ad-

hesion molecules, proteases, TNF superfamily, and neurotrophic factors [101]. Very interesting results that immunosuppressive therapy modulates T lymphocyte gene expression in SLE patient were presented by Pereira et al [102]. They observed that untreated patients have 38 repressed genes as compared to healthy control. When untreated patients were compared to treated ones, 154 genes were upregulated.

Serum soluble receptor (srIL2f, p55 srTNFf, p75 srTNFf) levels were higher in SLE patients with nephritis before treatment and decreased significantly 6 month after treatment, suggesting that soluble receptors of cytokines are sensitive markers of diseases activity [103, 104]. SLE B cells have bone-resorbing activity which corresponds to IL- α , and IL- α produced by B cells might be one of the causes of bone destruction in SLE patients which has also been reported [105].

In T cells from patients with SLE, activity of type 1 protein kinase A isozymes is greatly reduced because of decreased expression of the α and β regulatory subunits (RI α and RI β). Mutations of the RI α subunit were observed in T cells of patients with SLE, caused by overexpression of an IFN- α -inducible transcript editing gene, ADAR1 [106].

Gene-expression profiling with a microarray using PBMCs from SLE patients and controls comprised of monocytes/macrophages, B and T lymphocytes, and NK cells demonstrated expression of 4566 genes represented on the chip. This analysis identified 161 unique genes that were differentially expressed by the following criteria: changes in expression of least 1.5-fold, and difference in expression of at least 100 expression units when comparing the means of two groups. Most of the genes that best distinguished SLE from control PBMCs were more expressed in SLE (124 of 161, 77%). Many SLE patients were found to overexpress mRNA for the cell surface markers: TNFR6 (Fas/CD95), a death receptor, intercellular adhesion molecule-1 (CD54), a lymphocyte activation antigen, and complement receptor. Other notable overexpressed genes included the signaling molecules MAP3K-8, RAB27, and the interleukin-6 signal transducer, and the transcription factors v-ets 2, and others [107].

Increased IFN- γ , IL-10 and decreased IL-4 mRNA expression in PMBCs from patients with SLE have been reported [108]. The results of several investigations showed that IFN-regulated genes are among the most significantly overexpressed in SLE mononuclear cells [109].

The changes in gene expression after IFN treatment have recently been investigated. This analysis identified 286 genes that demonstrated > 2-fold change in expression from baseline, and absolute mean difference in the level of expression > 500 units. The induction of many IFN-regulated genes, such as STAT1, myxovirus resistance 1 (Mx-1), and ISGF-3, validated the approach. Linear regression analysis showed that the IFN score was significantly correlated with the number of SLE criteria, so an elevated IFN score is strongly associated with the most

TABLE 1. Examples of general gene-expression profiles and disease-specific profiles in asthma, RA, and SLE. (* nonspecific gene expression, ** disease-specific gene expression, — no information.)

Genes	Asthma	RA	SLE
IL-2	*	*	*
IL-4	*	*	*
IL-5	*	*	*
IL-10	*	*	*
IL-12 p40	—	—	**
TNF α	*	*	*
IFN γ	*	*	*
c-fos	**	**	—
TNFR6 (Fas/CD95)	—	—	**
CD54	—	—	**
MAP3K-8	—	—	**
RAB27	—	—	**
RAG genes	—	—	**
ADAR1	—	—	**
STAT1	—	—	**
T-bet	**	—	—
GATA3	**	—	—
CTLA4	**	—	—
β 2-AR gene	**	—	—
GR- α/β	**	—	—
MR2/5	**	—	—
NGF	*	—	*
Cytoskeletal γ -actin	—	**	—
Fibronectin	—	**	—
MMPs Strom	—	**	—
Col-1	—	**	—
HME	—	**	—
PUMP	—	**	—
VCAM	—	**	—

severe manifestation of SLE [107]. In addition, enhanced mutational activity of immunoglobulin genes has been implicated in the pathogenesis SLE [110, 111]. Although the causes of autoantibody production have not been completely delineated, it has been proposed that a failure of editing or revision of autoreactive B-cell receptors contributes [112]. The rearrangement of immune-receptor genes depends on recombination activating gene (RAG) enzymes [113]. A recent study of Girschick et al [114] has showed that RAG expression is upregulated in peripheral IgD+ and VpreB+ B cells of patients with active SLE. These cells may contribute to the immunoregulatory abnormalities in patients with SLE. Thus, there are several well-documented disturbances of gene expression in lymphocytes in SLE. Therefore, these cells have the potential for identification of genes responsible for development and progression of SLE, prognosis, and treatment strategies.

Comparison of the expression profiles

In Table 1, we summarize the expression profiles of PMBCs during the diseased state of asthma, RA, and SLE. It can be concluded that there is a significant overlap concerning direct inflammatory markers, whereas of the others no systematic investigations are known to conclude disease specificity. Such data show that inflammation-related gene expression is likely to be of little use for diagnostic purposes, but may be well suitable to follow the time course of the 3 disorders.

Methodological considerations

Any method of isolation of white blood cells stimulates lymphocytes and mRNA expression. Therefore, the method of collection lymphocytes should be well standardized. In addition, a lymphocyte activation procedure may be considered. Accordingly, the expression of particular mRNA may thus become increased

several-fold allowing precise determination. We have experience using Ca^{2+} -ionophore to increase the expression of transduction-associated genes several up to 100-fold (unpublished data). It is therefore important to standardize collection of PBMCs carefully, which may pose a logistic burden in interinstitutional clinical investigations. In some cases, a specific activation technique or circumstances may be necessary to optimize gene expression. Gene expression requires specific laboratory facilities, but in essence PBMCs can be recovered at an extra-laboratory site and shipped afterwards to laboratory facilities for activation and mRNA extraction. In this scenario, there must be equipment available to isolate vital cells from blood on location, including a cooled centrifuge. Then storage and transport of vital cells become mandatory. In the last decade, techniques have developed to keep cells vital for longer periods (months) by controlled freezing (eg, -1°C per min) and then storing them at -80°C . Although these logistic problems can be overcome, most experiments will be designed in such a manner that blood can be taken at a laboratory facility.

It is here not the place to discuss strong and weak points of the microarray technique in detail. Excellent reviews on technical aspects have appeared and sources are available online at <http://www.gene-chips.com/>. So we are not discussing the variations of the technique itself, but rather whether some of the shortcomings of the techniques may be avoided or at least the consequences of them reduced. The basis of the microarray technique is those cDNA oligonucleotides spotted on the array that are complementary to the mRNA of the biological sample. But because of a nonspecificity of the binding, there is a relatively large noise and consequently a low signal-to-noise ratio. In particular, when the levels of expression of the mRNA are low, the general background fluorescence and nonspecific binding of the labels mRNAs at the microarray may overshadow the biologically significant signals. Amplification of the mRNA, to reach higher levels of mRNA, is then considered. Such amplification may, however, not always be linear and may artificially increase or decrease the relative expression. Pooling of mRNA extracts of various blood samples may indeed lead to higher biological signals, but may also dilute the relevant differences, unless done carefully. We will propose a procedure for optimal pooling (see below).

Finally, we would emphasize the potential of the microarray technique to allow direct comparison of 2 samples with each other on a single microarray. In that case, the biomedical samples are stained with different fluorescent labels (Cy3 and Cy5), so the ratio of the labels indicates the relative expression of a gene. In such analysis, an ideal competition between the differentially labeled mRNAs is assumed to occur. This may, however, not be the case, as labeling may influence binding properties. Such complication may well be avoided by repeating the assay, but with changing the labeling. If its results are consistent, it is obvious that labeling did not influ-

ence the profiles. Another concern may be the technique of averaging the signal. In most procedures, the average fluorescence of the chip is taken as an indication of the mean background. Such signal may, however, be unevenly distributed over the array and in some cases mean background of an area surrounding the genes of interest is taken, which may comprise less than 10% of the all detected gene products.

Dedicated clinical designs

Peripheral white blood cells (and lymphocytes) are easy to obtain and express a substantial part of the human genome. Here we will discuss some of the potentials and also pitfalls of the current use of microarrays in clinical studies. It may be emphasized here again that mRNA differs from gene analysis as it shows state-dependent variations, for example, it can be used to assess differences of gene expression in the diseased and the recovered state of the same subject. In the currently published studies, this unique possibility has not well been exploited. State-of-the-art microarrays allow detecting the whole genome (30 000 genes) and may easily lead to several thousands of false positives that are changes seen in cases as compared to the reference samples. In the case with largely different expression, such as in tumor cells, comparison with nonproliferating cell may suffice to discern differences in expression. In the here considered use of microarrays in clinical studies, a far more sophisticated approach is required to obtain maximal benefit of the array technology.

Another relevant question is whether differential expressions should be studied in pairwise or against a reference sample (or standard). As described below, we prefer to compare the most relevant samples directly, thus avoiding the use of a reference mixture or sample. In current microarrays, the relative differences in a single microarray can be shown directly, for example, as one sample is tagged with green fluorescence and the other (eg, control) is provided with a red fluorescent label. It should be noted here that the efficacy of the labeling may differ, so that there is no guaranteed linearity between the green and red labels. Moreover, each labeling is not always linear and the linear range of Cy3 and Cy5 may differ. Current practice is to label the mRNA extracts to be compared on a single array in different proportions and with alternating label.

Increase of the amount of mRNA increases the signal-to-noise ratio of the array. Moreover, genes that are very well expressed give—obviously—a better signal-to-noise ratio as well. The relative differences in expression may well differ more than 1 000 000 for the genes. Pooling of samples may well increase the sensitivity of the measurement, but still combining 10 blood samples the expression level may still remain low and marginal. Pooling has, however, in addition to the possible increased signal-to-noise ratio also the advantage that it may eliminate some of the biological variance. For instance, consider a noise of 100 and a net biologically relevant signal of 30, so to conclude to a significant difference one must distinguish 100 from

130, which falls below the current technical possibilities. If however one pools 5 samples, then the difference between (relevant) signal and noise is now 100 and 250, which is well detectable. But also in an attempt to detect the differences between state 1 and state 2 of a subject, pooling of the extracts obtained at state 1 versus those at state 2 may well be helpful. If, for example, the relative alterations in gene expression are variable and small, but the direction of change is similar, already pools of 5 samples and 10 arrays may well allow the identification of a small number of genes, characteristic for the disease state investigated.

CONCLUSION

In this review, we summarize relevant investigations of the gene expression in asthma, RA, and SLE, internal diseases with altered immune response in which lymphocytes play a central role. In our opinion, microarray studies in human lymphocytes would allow us to monitor alteration in gene expression relevant to asthma, RA and SLE and to possibilities of future therapeutically intervention. Further characterization of the gene expression in lymphocytes using cDNA microarray could help identify more precise pathophysiological mechanisms in these disorders, and could find beneficial therapeutic approaches. This suggestion has recently been supported demonstrating coordinate overexpression of interferon- α -induced genes using PMBCs as model in SLE [115]. SLE is characterized by various alterations in gene (and gene products) expression leading to diverse dysfunctions of T cells, B cells, and NK cells and as result development of clinical symptoms. Our suggestions have also been supported by the very recent report of Qing and Putterman [116] from the 4th International Congress of Autoimmunity.

Most of the genes involved in the pathogenesis of these diseases are belonging to sets of signal transduction molecules, inflammation-related cytokines (and chemokines), apoptosis-inducing molecules, cell-cycle proteins, or transcription factors. Most of these subsets of genes are now commercially available to be used on various microarray systems. The application of these systems has become more user-friendly and less expensive lately, and approaches the point to be a common tool available at most medicine departments. Alizadeh et al [117, 118] have developed a "lymphochip," which is a microarray of merely 10 000 individual human cDNA, representing genes of known and unknown function expressed on lymphocytes.

Such microarray technique gives the clues for identifying a novel responsible genes which underlie the process of the disease, and also could help identify appropriate targets for therapeutic intervention. Beside this, the microarrays using a "lymphochip" could be potential tools for investigating the mechanism of drug action. The use of the microarrays does not allow only comparison of the expression profile in different subjects (for intersubject designs), but also in individuals before, during,

and after the disease (for intrasubjects designs). Lymphocytes are an easily accessible model to be investigated with microarray techniques. This approach is minimally invasive, brings us valuable information at the cellular and molecular level of the disease, and can be universally applied in clinical medicine. There are, however, many potential pitfalls in the use of microarrays that result in false leads and erroneous conclusion [119]. In order to control the many sources of variation and the many opportunities for misanalysis, DNA microarray studies require careful planning, experimental design, statistical analysis, and interpretation. Different studies have different objectives, and important aspects of design and analysis strategy differ for different types of studies. Studies of disease with cDNA microarray technology can be split into two main categories with interrelated goals: identification of key molecular changes in diseases and identification of biomarkers or molecular fingerprints that will aid in patient diagnosis and classification. Studies that identify molecular changes in disease will advance our understanding of disease pathophysiology, whereas studies that identify biomarkers will improve diagnostic accuracy and targeting of specific therapeutic interventions [120]. We therefore suggest that exploring of the cDNA microarray gene expression in blood lymphocytes could be an advantageous and powerful tool for research, diagnostic, and treatment purposes in internal medicine.

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