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Review

The Clinical Usefulness of the Measurement of Cytokines

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The utilization of accurate and sensitive methods for the measurement of cytokines in body fluids is prerequisite for the proper use of these mediators in clinical practice. Many factors contribute to the complexity of cytokine quantitation. Bioassays historically preceded immunoassays, which are now very popular, but there is a need for standardization. Nevertheless, due to the local effects of cytokines, the study of their blood levels is of limited value for an understanding of the pathophysiology of these mediators. This explains the development of alternative approaches to assess the ability of cells to produce cytokines. These include the Enzyme-Linked Immuno Spot Assay (ELISPOT), the measurement of cell-associated cytokines by flow cytometry, and the study of cytokine secretion by isolated peripheral blood mononuclear cells or by whole blood test. All these techniques, associated with a local detection of cytokines by immunohistochemistry or *in situ* hybridization and reverse transcriptase polymerase chain reaction, appear to be complementary tools for a better understanding of the biology of cytokines. Selected examples of possible clinical applications related to infectious diseases, cancer, autoimmune diseases, allergy, transplantation and preclinical evaluation of drugs and biotechnology products are given.

Key-words: Cytokines; Cytokine receptors; Bioassays; Immunoassays; Clinical applications.

Abbreviations: CARS, Compensated Anti-inflammatory Response Syndrome; CRP, C-reactive protein; CSF, cerobrospinal fluid; CV, coefficient of variation; EGF, epidermal growth factor; ELISA, enzyme immunoassay; ELISPOT, Enzyme-Linked Immuno Spot Assay; GM-CSF, granulocyte macrophage-colony stimulating factor; HMG-1, high mobility group-1 protein; IDDM, insulin-dependent diabetis mellitus; IFN, interferon; IL, interleukin; IRMA, immunoradiometric assay; ISH, *in situ* hybridization; LBP, lipopolysaccharide binding protein; LPS, lipopolysaccharides; MGUS, monoclonal gammopathies of undetermined siginificance; MM, multiple myeloma; PAF, platelet activating factor; PE, phycoerythrin; PHA, phytohemagglutinin; PMA, phorbo-myristic acetate; RA, rheumatoid arthritis; RT-PCR, reverse transcriptase polymerase chain reaction; SAA, serum amyloid A protein; sCD23, soluble fragments of CD23; SIRS, Systemic Inflammatory Response Syndrome; SLE, systemic lupus erythematosus; TNF, tumor necrosis factor;

Introduction

Cytokines are low molecular weight proteic or glycoprotein mediators playing a major role in the communication between cells involved in the immune and acute phase responses. During the past 15 years considerable progress has been achieved in our knowledge of the functions of cytokines and their implication in the pathophysiology of a wide array of human diseases. This explains why a tremendous body of literature has been published describing various assays for the investigation of cytokines in biological fluids or tissues. Furthermore clinical trials using cytokines or cytokine inhibitors are underway making the measurement of these molecules thus necessary.

The aim of this review is to describe briefly the currently available tools for the investigation of cytokines in body fluids, to discuss the respective advantages and limitations of the different techniques and to give some examples of clinical situations in which cytokine determinations may be of practical value.

Properties of Cytokines Important for Their Investigation

Cytokines exert a pleiotropic activity as they act on numerous target cells. In this complex and highly regulated network it is difficult to individualize the precise role of a given molecule. The redundancy is a characteristics of cytokines and it represents a limitation of the use of bioassays. Due to their local mode of action involving to a paracrine and even autocrine processes, cytokines circulate at very low levels (< 10 pg/ml), often below the detection limit of the majority of assays. The existence of membrane-associated cytokines which possess important biological activities is now well documented. In body fluids and particularly within cells, cytokines can exist as multiple molecular forms: precursors, monomers/polymers, various degrees of glycosylation, degradation products. As examples the mature form of interleukin (IL)-1 (17 kDa) is generated after the proteolysis of a 31 kDa precursor, the native form of tumor necrosis factor (TNF) is a trimeric, non-glycosylated molecule, and IL-6 is a phosphoglycoprotein exhibiting 6 isoforms (23 to 30 kDa) when secreted

by fibroblasts (1). All these entities can behave very differently in the various assays: IL-1 precursors are biologically active whereas IL-1 ones are not and some protease-induced degradation products can be immunologically reactive, but are not detected in bioassays (2).

Many cytokine inhibitors have been described. Some of them are specific, such as IL-1ra (which binds to the specific IL-1 receptor without signal transduction) (3), soluble forms of cytokine receptors or autoantibodies. If the presence of IL1-ra does not interfere in immunoassays specifically designed for IL-1 measurement, the same is not true for bioassays. Soluble cytokine receptors are essentially generated after protease degradation of membrane receptors; usually soluble receptors inhibit cytokine bioactivity (TNF , IL-1), but soluble IL-6 receptor gp80 is an agonist of IL-6 due to the ability of the IL-6/IL-6 receptor complex to bind to gp130 on cell surface. All cytokine soluble receptors represent a potential source of interference in immunoassays if monoclonals/polyclonals used in the test recognize a cytokine epitope involved in the binding to the receptor. The existence of autoantibodies has been documented for TNF, IL-6 and IL-1 (4, 5). Autoantibodies against IL-1 are the best studied. Their prevalence is high with an affinity which can reach up to 10^{-11}M *i.e.* very similar to the affinity of antibodies developed for immunoassays. Non-specific inhibitors are essentially represented by plasma proteins, with ${}_2$-macroglobulin being the best example (6), but the affinity of this type of binding is rather low. The presence of cytokine inhibitors in biological samples can explain the poor results of recovery tests, as demonstrated after spiking synovial fluids with IL-1 (7); the interference of these inhibitors usually disappears after sample dilution.

The half-life of cytokines is very short (usually less than ten minutes), leading thus to brief peaks in blood. Circadian rhythms have been reported for IL-6 in patients with rheumatoid arthritis with a peak in the morning (8). The main factors influencing cytokine measurement in body fluids are summarized in Figure 1.

Cytokine Measurement in Body Fluids

The preanalytical phase

Whatever the assay used, special attention should be paid to the conditions of sampling and storage of biological fluids. The major risks are:

- the degradation of cytokines by proteases during storage,
- the cellular production or release of cytokines after sampling,
- the binding of cytokines to their membrane receptors.

For immunoassays, blood collection in sterile EDTA tubes is recommended since this chelating agent blocks any potential cytokine production after sampling. Heparin should be avoided as it is often contaminated by endotoxins which constitute a strong inducer of cytokine synthesis (9, 10). Some authors add pro-

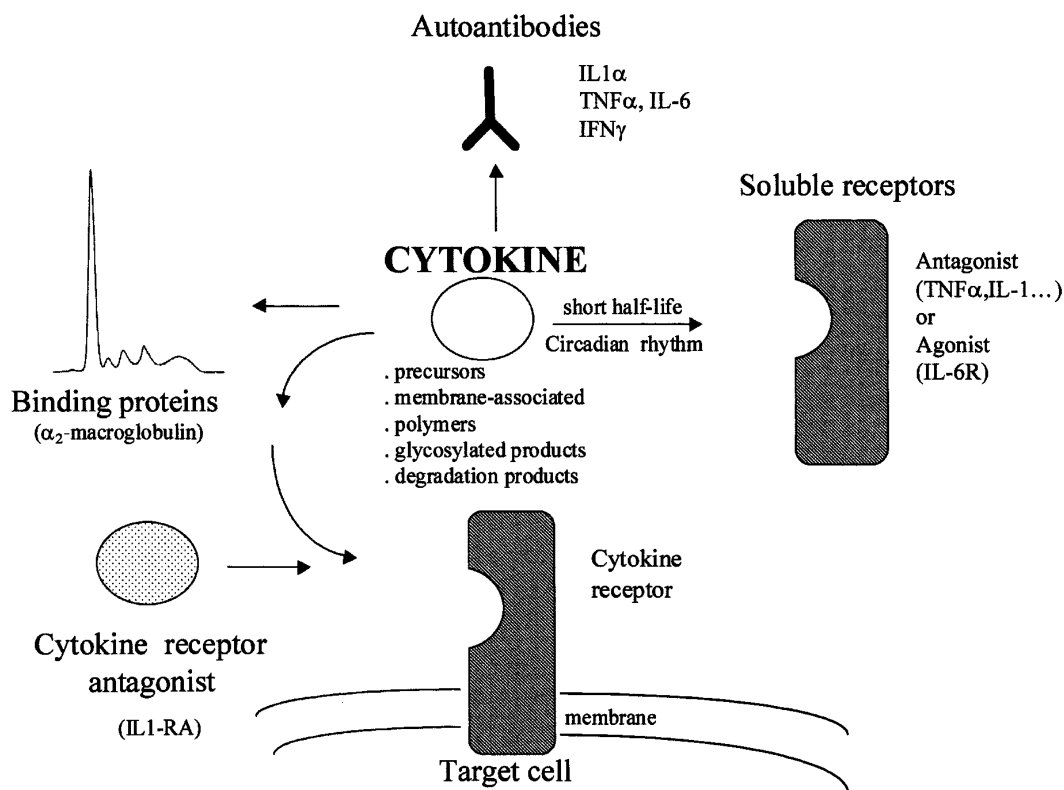

Fig. 1 Factors affecting cytokine measurement in body fluids.

tease inhibitors (trasylol) or inhibitors of platelet aggregation (indomethacin). For bioassays, the divalent ions blocking agent EDTA cannot be utilized, and serum is used, but it should be taken under strict sterile conditions.

Plasma or serum has to be decanted very rapidly to prevent any cytokine degradation or adsorption. Samples are stored deep-frozen (–80°C, if possible). The thawing-freezing cycles should be avoided, even though three cycles have no influence on IL-6 determination (11). When other samples than blood (cerebrospinal fluid, bronchoalveolar fluid, synovial fluid, etc.) are analyzed, a gentle centrifugation is performed and the absence of matrix effects should be controlled by recovery tests: one must keep in mind that these types of specimens are lower in protein content than serum and that the vast majority of kits have been developed for serum applications. In synovial fluids with high viscosity, hyaluronidase can be added.

Methods for cytokine measurement

The discovery of cytokines was based on their biological activity which explains that bioassays preceded immunoassays for the investigation of these molecules.

Bioassays

In these methods, the biological activity of a given cytokine present in the sample is tested on a sensitive cell line in comparison with a standard cytokine preparation. A dose-response curve is established and the concentration of the cytokine is calculated from the dilution giving the same biological response as the standard.

Bioassays are based on various biological responses (12):

- proliferation tests (murine thymocytes for IL-1, B9 hybridoma cell line for IL-6),
- cytotoxicity tests (WEHI 164 or L929 for TNF- and -),
- antiviral activity (reduction of virus induced cytopathic effect for interferon (IFN)),
- induction of cell surface molecules (major histocompatibility complex class II molecule expression induced by IFN-),
- chemotactic activity (Boyden chamber for IL-8),
- inhibition of cytokine secretion (inhibitory activity of IFN- on IL-10 production).

The major drawback of bioassays is their lack of specificity due to the fact that several cytokines can exert similar or synergistic effects. The possibility of adding antibodies directed against an interfering cytokine improves the specificity of the assay, but augments its cost. Furthermore, these methods are time-consuming (24–96 h) and their reproducibility is rather poor, with coefficients of variation (CV) ranging from 20 to 100%.

Immunoassays

Due to their excellent practicability, immunoassays have been rapidly introduced in clinical practice. Their specificity is high thanks to the use of monoclonal antibodies allowing distinction of molecules having the same biological activity (for example IL-1 and IL-1). Radioimmunoassays (essentially immunoradiometric assay (IRMA)) or ELISA have been developed using a first antibody (monoclonal) for the capture of the cytokine, and a second antibody (monoclonal or polyclonal) labeled for the revelation. The detection limit is highly dependent on the quality of the capture monoclonal antibody.

Many commercial kits are now available and are most often proposed according to an ELISA microplate format. Some adaptations on automated immunoassay analysers have been developed and allow the measurement of IL-1, IL6, IL-8, TNF and IL-2R as emergency tests within 90 minutes (13). For the user, it is often difficult to get relevant information from companies and, as a consequence, the analytical performance of a product in terms of detection limit, antibody specificity, standardization against international reference preparations, and potential interferences cannot be precisely evaluated. The detection limit of the majority of cytokine kits is around 5pg/ml, *i.e.* greater than the circulating concentrations of many cytokines not only in physiological, but also, sometimes, in pathological conditions. To improve the test sensitivity flow cytometric-based immunofluorescence assays (Flowmetrix™, Quantiflow™) using polystyrene microspheres of different sizes have been recently introduced (14). This new technology, which is completed within 60 minutes, is able to lower the detection limit to 100 fg/ml. Furthermore, it can be used in a multiplexed format thus allowing the simultaneous quantitation of up to 64 different cytokines in a sample volume of as little as 50 µl in some applications.

Standardization of immunoassays still remains a crucial issue. As shown in different studies (15, 16), there is an important need for standardization of the commercial calibrants used for measuring TNF, IL-6 or IL-2, despite the availability of internationally recognized reference preparations usually made of recombinant cytokines (NIBSC preparations, http://www.nibsc.ac.uk/catalog/index.html. Another source of discrepancy in the results lies in their mode of expression, which can be very confusing since data can be given in International Units, pg/ml, or pM for the same cytokine whereas only approximative correction factors are proposed to compare results. Some techniques are also strongly influenced by the presence of soluble receptors.

The major drawback of immunoassays lies in the fact that they measure a cytokine as an antigen, even though the molecule is devoid of any biological activity (17). This is particularly important for cytokines with precursors, such as IL-1, which should be detected according to Dinarello (18). The absence of interference of rheumatoid factor or heterophilic antibodies has to be controlled. The advantages of immunoassays are their excellent reproducibility (CV ranging 5 to 10%), and their practicability.

The major characteristics of bioassays and immunoassays are presented in Table 1.

Tab. 1 Comparative characteristics of bioassays and immunoassays for cytokine determination.

Bioassays	Immunoassays
– Highly sensitive (detection limit: < 1 pg/ml)	– Less sensitive (detection limit: a few pg/ml)
– Low specificity	– High specificity
– Detection of bioactive molecules	– Detection of antigenic molecules(potentially including biologically inactive degradation products)
– Narrow analytical range	– Wide analytical range
– Time consuming (24–96 h)	– Relatively rapid (hours)
– Low precision (CV = 20–100%)	– Excellent precision (CV = 5–10%)
– Drug interference (corticosteroids, cyclosporin)	– No drug interference
– High staff cost	– High reagent cost

Determination of the Capacity of Cells to Secrete Cytokines

The ability of cells to synthesize and to release cytokines in culture supernatants can be investigated by different techniques. It should be stressed that such assays are not adapted to the determination of circulating cells that actually secrete cytokines upon *in vivo* activation. They are rather designed for measuring the cytokine producing capacity of circulating leukocytes upon *in vitro* stimulation by bacterial lipopolysaccharides (LPS), vegetal lectins such as phytohemagglutinin (PHA) or a mixture of phorbol ester (PMA) and calcium ionophore (ionomycin). Thus, alterations assessed by these methods may only reflect major changes in cell subsets that may synthesize the cytokine of interest.

There is a growing interest in whole blood assays since this type of approach is very simple, does not necessitate a time-consuming separation step (potentially responsible for cellular activation) and leaves peripheral blood cells in their "natural" environment with the possible presence of binding proteins and inhibitors as well as synergistic or antagonistic cell to cell interactions. LPS and/or PHA are usually used as stimuli for cytokine synthesis and particularly for proinflammatory cytokines (IL-1, IL-6, IL-8, TNF) or Th1/Th2 cytokines (IFN , IL-10). Released cytokines are either measured in the cell supernatant by ELISA (19) or immunotrapped by specific antibodies coating culture wells or beads (20, 21). These last modifications add sensitivity to the tests. These models are well-adapted to the screening of potential immunomodulatory drugs (22). Nevertheless the major limitation of whole blood models lies in the fact that they explore a global cell population even if cytokine production measured in whole blood and mononuclear cell cultures are significantly correlated (23). The level of cytokine production appears to be characteristic of an individual, but there is a marked interindividual variability with 16-to 20-fold variation (23). This is essentially linked to the polymorphisms in the genes that control cytokine production, as will be discussed below.

ELISPOT (Enzyme-Linked Immuno Spot Assay) represents one of the first attempts to determine the frequency of cytokine producing cells among an heterogeneous population. In this technique, a stimulated cell suspension is laid on a microplate well coated with antibodies directed against the cytokine of interest (24). After incubation for 2–4 h at 37°C and washing, cytokine-producing cells are removed and the cytokine bound to the solid phase antibody is visualized by a second enzyme-labeled anti-cytokine antibody. A chromogenic substrate is then added and each cytokine-secreting cell can be visualized as a colored spot. ELISPOT is 10- to 100-fold more sensitive than classical ELISAs allowing the detection of as few as three to five cytokine secreting cells per 100000. The detection of cells simultaneously producing two cytokines is possible by this method (25). Because of its exquisite sensitivity, ELISPOT can be used to detect circulating cytokine producing cells without stimulation *in vitro*.

Determination of Cytokines at the Cellular Level

The local and paracrine activity of cytokines explains their low circulating levels and constitutes a strong limitation to the interpretation of their measurement in body fluids. This led to the introduction of cellular and tissue methods in order to investigate cytokines at a cellular level. The tissue distribution, the number and nature of cytokine-producing cells can be investigated using two different approaches :

- the detection of cell-associated cytokines as proteins after addition of specific antibodies (immunohistochemistry, flow cytometry),
- the detection of cytokine mRNA by *in situ* hybridization (ISH) or reverse transcriptase polymerase chain reaction (RT-PCR)

Detection of cytokines as proteins

Immunohistochemistry

This method allows both cytokine detection within tissues and the phenotyping of cytokine producing cells. Three successive steps are necessary: a cell-fixation, a cell-permeabilization and cytokine visualization using a specific anti-cytokine antibody. The selection of appropriate antibodies is very critical: some antibodies used in ELISA are not suitable for immunohistochemistry due to the existence of cytokine precursors within cells, a different molecular conformation or/and an alteration of some epitopes after fixation (26). Good results have been obtained using a paraformaldehyde-saponin procedure for fixation and permeabilization (27), and antibody concentration ranging from 0.5 to 5 µg/ml (28). The binding of antibodies to cytokines is visualized either by immunofluorescent or immunoenzymatic techniques. If peroxidase is used as label, the absence of endogenous peroxidase in specimens should be controlled or endogenous peroxidase should be neutralized. The detection limit of immunohistochemistry can be enhanced by "multilayer" procedures (peroxidase-antiperoxidase, alkaline phosphatase-antialkaline phosphatase, streptavidin-biotin). The possibility to combine double or triple labeling allows the identification of the phenotype of the cytokine-producing cells. The main limitation of immunochemistry is related to the low concentration of intracellular and membrane-bound cytokines. Besides, a significant background due to a non-specific antibody binding can be observed in some tissues, particularly in necrotic areas. The technique has been applied to quite diverse cell or tissue preparations: peripheral blood cells, bronchoalveolar lavage, cytopuncture or tissue biopsies.

Flow cytometry of cytokine-synthesizing cells

More recently the detection of intracellular cytokines by flow cytometry has been reported (29). This sophisticated technology has been made possible by the availability of fluorescent anti-cytokine antibodies and of agents able to block the intracellular transport of proteins and thus their secretion. Such molecules as monensin or brefeldin A induce an accumulation of cytokines within the Golgi apparatus which is sufficient to give a detectable fluorescent signal after adding the specific labeled antibody. Nevertheless, a cell stimulation is necessary, which is usually realized by the association of phorbol-myristic acetate (PMA) and ionomycin. More "physiological" stimuli such as a combination of anti CD2-anti CD28 antibodies have been proposed. Here also a fixation-permeabilization procedure (most often paraformaldehyde 4%-saponin 0.1%) is needed for the penetration of anti-cytokine antibodies into the cell and the respective antibodies have to be carefully selected. The potential clinical application of this flow cytometric assay is very promising (30) with a specific interest for the investigation of the Th1/Th2 balance by using anti IL-2 or anti IFN- for Th1 cells and anti IL-4 for Th2 cells (31). In reality, the detection of Th1 cytokines is easier than Th2 ones since the proportion of Th2 cells is low and the synthesis of IL-4 and IL-5 by these cells is weak. Nevertheless, flow cytometry offers the major advantage of an individual cell analysis performed on an important cell number (routinely 10^4 but with a possible extension to 10^5 cells) along with the phenotyping of the cytokine producing cells. There is still an important need for standardization of the procedure: stimulating agents, incubation time, reagents and control of permeabilization, Golgi apparatus blockers, anti-cytokine antibodies. Moreover, the clinical and physiological interpretation of the results remains difficult since the technology requires the addition of potent stimulating agents.

A new technology has been introduced for the detection and enrichment of live antigen-specific CD4 and CD8 T cells based on cytokine secretion (32, 33). Upon stimulation with antigen for 3–16 h, cells specific for this antigen secrete cytokines which will be captured by a specific reagent attached to the cell surface using an anti CD45 antibody. The bound cytokine is then detected by flow cytometry after adding a specific anti-cytokine antibody conjugated to phycoerythrin (PE). Antigen-specific T cells can be enriched after incubation with anti-PE magnetic microbeads which will be retained in the magnetic field of a separation column. This methodology is available for the study of IFN and IL-4 producing cells. The separated cells are viable and can thus be expanded and used for functional tests.

Flow cytometry assay of Th1 and Th2 cells using phenotypic markers

Potential phenotypic markers of Th1 and Th2 cells were recently reported as, for example, a distinct profile of the chain of IFN- R (34) and of the $_2$ chain of IL-12 R (35) on Th2 cells compared to Th1 cells. More interestingly, chemokine receptors seem to be useful to distinguish Th subsets. Both Th subsets express CCR1, CCR2 and CXCR4 but CCR5 and CXCR3 expression appears to be restricted to the Th1 subset (36, 37) whereas Th2 cells express CCR3, CCR4 and possibly CCR7 (38–40). The expression of chemokine receptors is in agreement with the differential regulation of the migration of these subpopulations during immune response and pathological conditions (41). Lastly, a cell surface molecule called ST2/T1 with a stable expression has been identified on mouse Th2 cells (42, 43). The cloning of the human equivalent of ST2 is under progress.

All these phenotypic markers look very promising due to the fact that they can be detected on cell membrane by flow cytometry without cell stimulation. Nevertheless, the major problem is their unstable expression with rapid down- or up-regulation during immune activation and after homing. Moreover, the frequency of cells expressing these markers is low and standardized reagents for their detection are not available yet.

Detection of cytokine mRNA

ISH

Cytokine mRNA detected on tissue fragments or cell suspensions allows the identification of cells directly involved in cytokine synthesis among a heterogeneous population. This method is complementary to cytokine detection by immunohistochemistry, since this latter technique cannot give precise information about the origin of the cytokine (direct production or internalization). The best results are obtained by using riboprobes (single strand RNA probes or oligonucleotides, which are labeled with a radio-isotope or digoxigenin (44). ISH is very sensitive, but time-consuming, expensive, and requires many internal controls.

RT-PCR

Among the recent tools developed for the molecular biology of cytokines, competitive RT-PCR is one of the most interesting (45). This technique uses an internal standard (often a plasmid) with the same primers as the cytokines. Increasing known amounts of this standard (S) are added to fixed amounts of total cellular RNA. After reverse transcription into cDNA followed by a PCR-coamplification, the signals for S and for the sample (E) are measured. A standard curve is established from the different S/E ratios, and the result is obtained from the ratio S/E = 1. Results are expressed as cytokine mRNA copies per gram of total cellular RNA. By using a multispecific standard, it is possible to study the messengers for several cytokines in the same experiment (46). Many applications of the procedure have been proposed in various clinical situations: central nervous system in inflammatory disorders (47), synovial fluid in rheumatoid arthritis (48), and bronchoalveolar fluid in allergic diseases (49).

Clinical Applications of Cytokine Measurement

Acute inflammation, acute infectious diseases and septic shock

Circulating acute phase proteins have been used for many years as markers of a cytokine-induced acute phase response. C-reactive protein (CRP) is the archetype of these proteins since its liver synthesis is highly IL-6 dependent. CRP is now routinely measured as a help to the diagnosis and more importantly to the follow-up of inflammatory and infectious diseases, and for assessing the efficacy of therapy (particularly antibiotherapy) (50).

Nevertheless, as shown by several studies in patients undergoing surgery, the respective kinetics of IL-6 and CRP are different, with IL-6 peaking at 4–6 h after incision compared to a delayed maximum at 24–48 h for CRP (51). These temporal discrepancies explain the inconstant correlation observed between IL-6 and CRP at a given time in acute circumstances. IL-6 appears to be a more rapid and sensitive marker of inflammatory reactions. The highly sensitive tests under development for CRP quantitation will lead to reconsideration of the present situation. The major advantages of CRP tests are their simplicity, excellent precision, low cost and 24 hour-availability in emergency laboratories.

Septic shock represents the most dramatic complication of acute infectious diseases. Among patients presenting with bacteremia approximately 20% will develop septic shock, which will be fatal in 50% of the cases (52). In spite of the use of new potent antimicrobial agents, the mortality rate from septic shock remains desperately stable. This clearly demonstrates that the lethality is not directly linked to the bacteria itself, but to a non-adapted host's response to the microbial aggression. In addition, septic shock belongs now to the so-called Systemic Inflammatory Response Syndrome (SIRS), which gathers various diseases characterized by an uncontrolled acute phase response occurring after severe injuries, some of them being of non-infectious origin (53).

Among the host's responses during sepsis, the generation of inflammatory mediators is of special importance. More specifically, there is considerable evidence on the role of cytokines in the pathogenesis of septic shock. TNF appears to be a key factor since high serum TNF levels are observed within 1 to 2 h after endotoxin injection to animals, recombinant TNF induces clinical manifestations very similar to septic shock and a pretreatment with anti-TNF antibodies protects animal from LPS challenge (54). However, the same type of results have also been obtained with IL-1, adding thus to the complexity of the mechanisms at the origin of septic shock (55). The sequence of events leading to septic shock has been partly delineated in the case of Gram-negative bacteria. These types of germs present in their outer membrane a substance common to all Gram-negative microorganisms and called LPS or endotoxin. In blood, LPS binds to a hepatic glycoprotein called lipopolysaccaride binding protein (LBP) (LPS binding protein). The LPS-LBP complex is a ligand for the CD14 receptor at the surface of monocytes and macrophages. The interaction of LPS-LBP with its receptor triggers the monocytic secretion of several proinflammatory cytokines, among which the early synthesized TNF and IL-1 represent the archetypes. TNF and IL-1 orchestrate the major alterations observed during septic shock, such as variations in body temperature, in vascular resistance and in permeability, elevation of white blood cells, and lactic acidosis (52). Some cytokine effects are mediated by prostaglandins, platelet activating factor (PAF) and leukotrienes. Rapidly, TNF and IL-1 induce the secretion of many other pro-inflammatory cytokines and also of cytokines or cytokine receptors modulating this inflammatory cascade. Among proinflammatory cytokines, IL-6 is the best systemic reflection of the activation of inflammatory cascade, while the local production of the chemokine IL-8 by various organs is responsible for the recruitment and stimulation of polymorphonuclear cells which cause tissue damage and organ dysfunction due to oxygen free radical production and enzymatic degradation. In addition, the complement, coag-

ulation and kinin systems are also activated by LPS. Finally, nitric oxide which constitutes a potent vasodilator is produced by macrophages and hepatocytes. Recently, a potential late mediator of endotoxin lethality called HMG-1 (high mobility group-1 protein) has been characterized in mice (56). This protein is released by cultured mouse macrophages more than 8 h after stimulation with LPS, TNF or IL-1. HMG-1 increases in serum from 8 to 32 h after mouse exposure to endotoxin. Administration of HMG-1 is lethal and antibodies to this protein protect animals from death. HMG-1 is undetectable in the serum of normal human subjects (n=8), but significant circulating concentrations are detected in septic patients (n=25) with higher levels in non-survivors (mean = 83.7 ng/ml) than in survivors (mean = 25.2 ng/ml) (56).

All these complex pathological cascades triggered and amplified by the cytokine network will result in multiorgan failure characterized by hypotension, renal failure, acute respiratory distress syndrome and heart failure. A schematic representation of the major events occurring during septic shock is given in Figure 2.

Gram-positive bacteria can also cause a SIRS, either by secreting a toxin acting as a superantigen in T cell activation or *via* a constituent of the cell wall similar to the lipid A of LPS (57) and using also CD14 as receptor.

Although the pathophysiology of septic shock is complex, TNF is a principal and early mediator in this disease. This explains why TNF has been a target for clinical trials using murine monoclonal antibodies to human TNF (TNF Mab). However, all these attempts at immunotherapy have been dissappointing as illustrated by the key paper of Abraham *et al.* (58). These authors did not find any improvement in survival (mortality at day 28) after septic shock with the administration of TNF Mab in a randomized multicenter, double blind, placebo-controlled study where 1879 randomly assigned patients received a single infusion of 7.5 mg/kg TNF Mab (949 patients) or placebo (0.25% human serum albumin, 930 patients). Similar negative results have been obtained in all clinical trials using immunotherapy as recently reviewed by Zeni *et al.* (59) (Table 2). Several factors may explain these disappointing data:

- patients entering these clinical trials are non-homogeneous with regard to the etiology of septic shock, the severity of disease, the type of germs, the site of the initial infection, the delay in hospital admission, the underlying disease and immune status,
- there is no specific marker for sepsis and the exact chronology of immunoinflammatory events occurring during sepsis is not precisely documented.

In sepsis, the initial pro-inflammatory response allows the detection of inflammatory cytokines in body fluids. Within 1 h after LPS administration in healthy subjects, TNF is released in blood rapidly followed by IL-

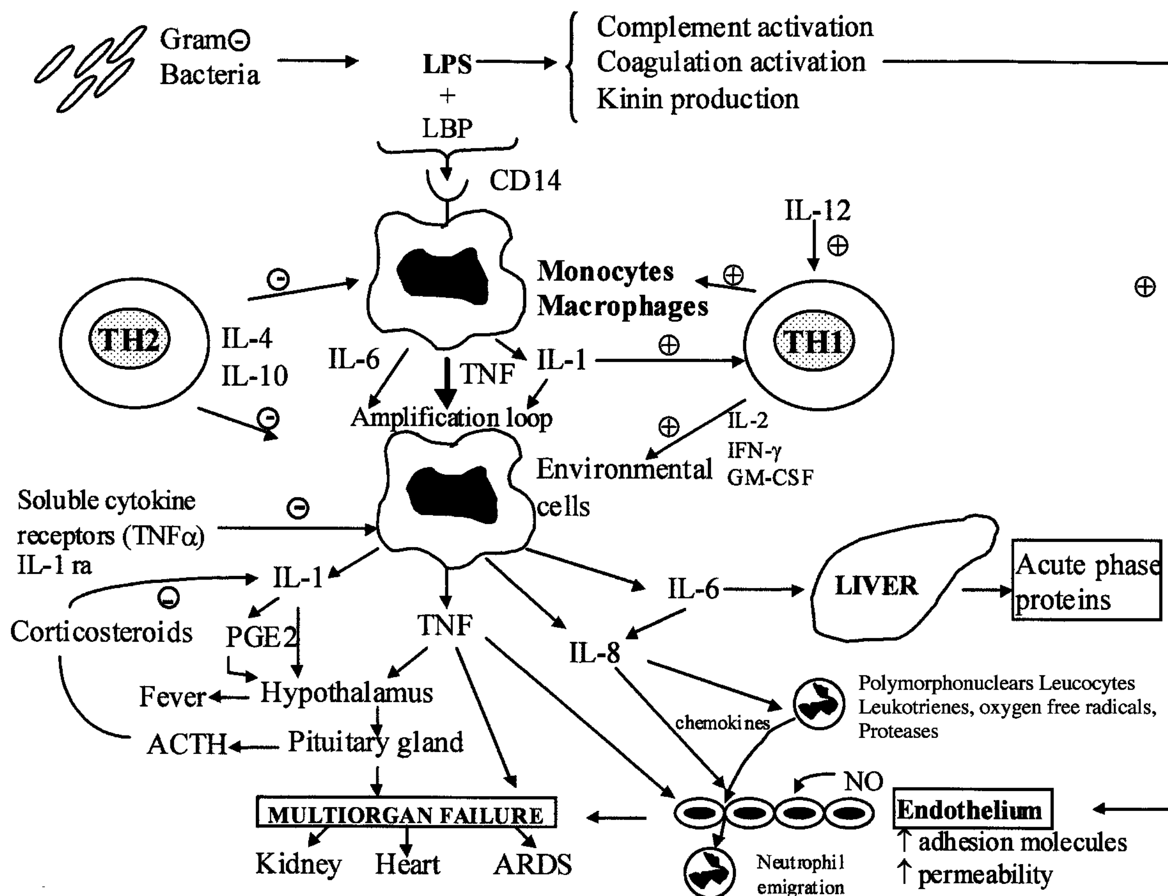

Fig. 2 The pathogenesis of septic shock. ACTH = adronocorticoluopic hormone; ARDS = acute respiratory distress syndrome; PGEZ = prostaglandin EZ

Tab. 2 Randomised controlled trials of immunotherapy in sepsis and septic shock.

Type of trial	Number of trials	Total number of patients	Mortality	
			Placebo (%)	Treatment (%)
Antiendotoxin	4	2010	35	35
Antibody to interleukin-1-receptor	3	1898	35	31
Antibradykinin	2	755	36	39
AntiPAF	2	870	50	45
AntiTNF	8	4132	41	40
Soluble TNF receptor	2	688	38	40
NSAIDs	3	514	40	37
Steroids	9	1267	35	39
All studies	33	12034	38	38

PAF = platelet-activating factor, NSAIDs=non-steroidal anti-inflammatory drugs.
Adapted from Zeni *et al.* (59)

1, IL-6 and IL-8 (60). Many clinical studies have documented the augmentation of circulating cytokines in patients suffering from severe bacterial infections. Elevations of serum TNF have been reported during severe Gram-negative infections and a correlation between TNF increase and mortality has been found in meningococcal diseases (61), particularly when associated with *purpura fulminans* (62); in fatal cases, TNF levels over 1ng/ml can be observed (62). While inconstant, marked elevations of blood TNF in sepsis have repeatedly been confirmed as being of poor prognosis (63–65). But, as discussed by Heney *et al.* (66), it is not possible in clinical practice to precisely define a TNF threshold above which mortality increases rapidly; in fact, if TNF value may constitute a good prognostic index for a group as a whole, when individual patients are considered the situation is very different due to a large interpatient variability. Often classical clinical indicators are more useful and Calandra *et al.* (67) conclude that clinical severity, the patient's age, blood culture, urine output and arterial pH are better predictors of outcome than TNF levels. Persistent low TNF levels could also be of poor prognosis (65). In cerebrospinal fluid, elevated TNF concentrations have been measured during meningitis with higher values when infection was of bacterial origin (68). Actually, although IL-6 is not directly the causative mediator of septic shock, it constitutes a more reliable and sensitive marker (about 100 times higher) than TNF since that cytokine is the final result of the amplification loop triggered by the pro-inflammatory cytokine cascade. In bacterial sepsis, Hack *et al.* (69) reported high serum IL-6 measured by the B9 hybridoma bioassay, with values below 40 U/ml in survivors, whereas 89% of patients with concentrations more than 7900 U/ml died. In HIV infections, IL-6 is elevated in blood and a correlation with the severity of the acquired immunodeficiency syndrome is observed (70, 71). Amniotic fluid IL-6 determination is of particular diagnostic utility for the early detection of microbial invasion of the amniotic cavity in women in preterm labor (72). A major interest in IL-6 determination lies in the early diagnosis of neonatal infections, with a very high sensitivity until the twelfth hour of life (73, 74) at day 0. Twenty-four hours after admission, cytokine sensitivity decreases, while CRP appears to become a more sensitive indicator of sepsis. Thus, for Buck *et al.* (73) the "combination of CRP and IL-6 seems to be the ideal tool for early diagnosis of neonatal infection". This has been confirmed by Doellner *et al.* (75) who found that a combined parameter of IL-6 (> 50 pg/ml) and CRP (> 10 mg/l) yielded a sensitivity of 96%, a specificity of 74%, a positive predictive value of 49% and a negative predictive value of 99%. Recently, Kuster *et al.* (76) have reported in neonates at day 0 a higher diagnostic sensitivity for IL-1-ra as compared to IL-6 and CRP with values of 93%, 86% and 43% respectively. The specificities of IL-1ra, IL-6 and CRP were 92%, 83% and 93% respectively. IL-1ra rise can predict sepsis one or more days before clinical diagnosis.

After the initial acute phase response of sepsis characterized by the above-mentioned systemic elevation of pro-inflammatory cytokines, it is now clearly documented that a rapid and potent anti-inflammatory response develops. This response is accompanied by the release of anti-inflammatory cytokines, among which IL-10, IL-1ra along with soluble TNF receptor are the most important (77). These substances induce an alteration of immune function which leads to a period of immune depression defined by Bone *et al.*(78) as a "Compensated Anti-inflammatory Response Syndrome" (CARS). The balance between the pro- and anti-inflammatory responses is very delicate and it represents the key to the survival of the patients. As illustrated by Van Dissel *et al.* (79), an anti-inflammatory cytokine profile with a high IL-10/TNF ratio on admission is associated with fatal outcome in patients who presented to hospital with fever above 38.2°C: the median of this ratio is 6.9 (range : 3.0–21) in non-survivors and 3.9 (range 2.0–7.0) in survivors (p = 0.04). The determination of this pro-inflammatory/anti-inflammatory balance appears now to be essential in the monitoring of sepsis.

Thus, in some patients an “immune paralysis” may occur as objectivized by a profound deactivation of immune responses with a diminution of phagocytosis, a defect in pro-inflammatory cytokine production, a shift toward a Th2 profile and an alteration of HLA-DR expression on monocytes. Thus, these patients have a high risk of contracting nosocomial infections. Consequently, the administration of an immunosuppressive therapy during this phase of the disease is deleterious. On the contrary, the potential benefit to these patients of the administration of “priming” pro-inflammatory therapies such as IFN has been shown by Docke *et al.* (80). In agreement with this immuno-depression, it has been demonstrated that TNF, IL-1 and IL-6 synthesis by whole blood of polytraumatized and septic patients stimulated by endotoxin *ex-vivo* is considerably suppressed compared to healthy volunteers (81). The same type of results has been obtained for IL-8 production by neutrophils isolated from patients suffering from sepsis or non-septic SIRS and stimulated *ex-vivo* by LPS or heat-killed streptococci (82). Interestingly, Haupt *et al.* (83) observed a diminution in TNF and IL-10 production (38% and 36% respectively) by whole blood cells of healthy donors when the plasma of septic patients was added.

The flow-cytometric study of the expression of TNF receptors on peripheral leukocytes of patients with SIRS brings interesting and provocative complementary information. TNF-R I (p55) is upregulated, and does not correlate with bad prognosis, but strongly with body temperature, whereas TNF-RII (p75) is down-regulated and correlates with severity and prognosis. TNF-RI up-regulation and TNF-RII down-regulation are not correlated (84). Soluble TNF receptor concentrations of both receptors were increased in the serum of the patients studied. These results are in disagreement with LPS challenge experiments in healthy volunteers, where a down-regulation of type I and type II receptors was found at the surface monocytes and granulocytes (85). This discrepancy is probably linked to the time of observation, since in human volunteers a rapid decrease of surface TNF receptors is observed within 2 h with an up-regulation at 24 h. These data tend to demonstrate that different factors regulate TNF-RI and TNF-RII expression on the cell surface.

In viral infections, the determination of serum and/or cerebrospinal fluid (CSF) IFN has been proposed as a help to the diagnosis. In a study involving 31 neonates hospitalized for suspected sepsis, serum and/or CSF IFN has been measured (86). Among the 13 neonates with viral infection (enterovirus and coxsackie virus) CSF and/or serum IFN was elevated except in one case in which serum IFN result was not available. Conversely, IFN was not detected in CSF and/or serum of patients with bacterial sepsis (n=15) or without infection (n=3).

In parasitic diseases, the implication of cytokines has also been documented, particularly in leishmaniasis and malaria. In this latter infection elevations of TNF in blood have been described and correlate with parasitemia; the highest TNF levels are observed in cerebral malaria (87). There is still no clear indication as to the actual clinical help provided by TNF measurement for the patient monitoring.

Cancer

The role of cytokines in cancer was first reported in 1891 by William Coley at the Sloan-Kettering Cancer Institute, where patients with sarcomas were treated with mixtures of killed preparations of Gram-negative bacteria (88). Some of these patients exhibited dramatic although transient regressions of their tumors. The explanation for this clinical observation was given 80 years later in the same Institute by Carswell *et al.*: a factor induced by bacterial endotoxins caused the hemorrhagic necrosis of transplantable murine tumors, and they called it Tumor Necrosis Factor (89).

Cancers represent a complex and heterogeneous group of diseases in which the mechanisms of malignant transformation are very diverse. Cytokines are involved in many aspects of this malignant process. Some proto-oncogenes and oncogenes code for normal or abnormal components of cytokine receptors or signal transduction pathways (90). Under physiological conditions, cytokine expression is only transient, whereas, in cancer, malignant cells are able to produce large amounts of cytokines, resulting thus in significant and stable local elevations of these mediators (91). Cytokines may possess growth inhibitory activities directly on cancer cells such as TNF. The IFNs down-regulate the expression of cellular proto-oncogenes such as *myc*, the enzymes associated with DNA replication, and the receptors for growth factors such as (EGF) (90). On the contrary, some cytokines produced by certain tumor cells may stimulate cell growth directly (autocrine stimulation) or cytokines synthesized by stromal cells in the tumor environment induce cell proliferation (paracrine stimulation) (91). Sometimes, the same cytokine can lead either to cell activation/proliferation or to cell death: the best illustration is given by the TNF-receptor family where, for example, the trimerisation of TNF-RI after TNF binding can induce cell activation via NF- B or can trigger the apoptotic machinery after activation of a cascade of intracellular proteins with death domains (92).

Cytokines can modulate immune response to tumor. They can augment antitumor immunity. In this context, the interferons up-regulate the expression of MHC class I antigens at the surface of normal and malignant cells, stimulating thus the killing by cytotoxic T cells (90). IL-2 activates cytotoxicity against tumor cells, by stimulating a subpopulation called lymphokine-activated killer cells (LAK cells); this has been the basis for the use of IL-2 as a therapy in renal carcinoma and melanoma (93). IL-12 appears to be a potent antitumoral factor by stimulating Th1 cells and IFN production, and by enhancing cytotoxicity and NK cell proliferation (94). Alternatively, cytokines can diminish antitumor immunity of the host; TGF inhibits T cell responses *in vitro* and TGF producing tumors escape immune destruction (91). Cytokines may also play a

role in tumor metastasis and more specifically in angiogenesis; as an example, TGF- constitutes an important angiogenic factor and it stimulates the production of extracellular matrix and other cytokines by fibroblasts and endothelial cells (91).

Several paraneoplastic effects are mediated by cytokines: fever (IL-6, IL-1, TNF), anemia (TNF), thrombocytosis (IL-6), cachexia (TNF- , IFN-), bone resorption and hypercalcemia (IL-1, IL-6).

As IL-6 has been historically described as a plasmocytoma and myeloma growth factor, several studies were focused on IL-6 production in multiple myeloma (MM). Initially IL-6 was considered as an autocrine factor for myeloma cells since freshly isolated human myeloma cells produced IL-6 in culture (95). However, it is more likely that IL-6 exerts a paracrine action after production by stromal cells in the bone-marrow environment (96). Other cytokines can also stimulate myeloma cell growth by synergizing with IL-6 (hematopoietic cytokines such as granulocyte macrophage-colony stimulating factor (GM-CSF), IL-3, IL-5) or by inducing an autocrine production of IL-6 (IFN or TNF) (97). Conversely, IFN- is a potent inhibitor of myeloma cell proliferation by down-regulating the IL-6 receptor (97). IL-1 and CSF-1 (M-CSF) are able to stimulate the tumor environment. The role of the cytokine network involved in human MM is summarized in Figure 3. Various studies tried to use serum IL-6 concentrations as a help to the staging and the prognosis of MM and to the distinction of MM from monoclonal gammopathies of undetermined significance (MGUS) (98–102). The results are very contradictory, due the methods of measurement used (bioassays or immunoassays), the multiple molecular forms of circulatory IL-6, and the detection limits of the assays. As a consequence, the value of serum IL-6 as a tumor marker in myeloma remains unproved (103). The same is true for IL-6 receptor (gp80) which is raised in the serum of patients with myeloma, but no correlation with tumor mass or well-established prognostic factors has been demonstrated (104).

In solid tumors, circulating IL-6 concentrations are significantly increased in patients with untreated renal cell carcinoma and in case of marked elevation, a relationship with fever or weight loss or metastatic disease has been documented (105). An interesting finding is the correlation between baseline IL-6 or CRP concentration and the response to IL-2 therapy in patients with renal adenocarcinoma, colorectal cancer or melanoma: IL-2 responders have significantly lower levels than non-responders prior to therapy (106).

The soluble IL-2 receptor (s IL-2R) has been studied in various malignant diseases. In children suffering from acute lymphoid leukemia, elevation of serum s IL-2R correlates with survival and predicts relapse (107, 108). In solid tumors, raised levels of s IL-2R have been shown to correlate with prognosis in lung cancer with a diminution after response to therapy (109).

The use of M-CSF (also called CSF-1) as a tumor marker has been recently discussed by Whicher and Banks (103). M-CSF is elevated in a high proportion of patients with hematological malignant diseases and M-CSF serum levels are related to disease type, status and response to therapy (110, 111). But, the major clinical interest of M-CSF is the diagnosis and follow-up of ovarian cancer (112, 113). The association of M-CSF and CA125 determinations is recommended as illustrated by Susuki *et al.* (114): in a study including 69 patients with ovarian cancer 61% and 90% of these patients were diagnosed on the basis of an abnormal M-CSF or CA125 concentration while the combination of the two markers detected 96% of the patients.

TNF has been studied in various malignancies and it is elevated in many tumors such as oat cell carci-

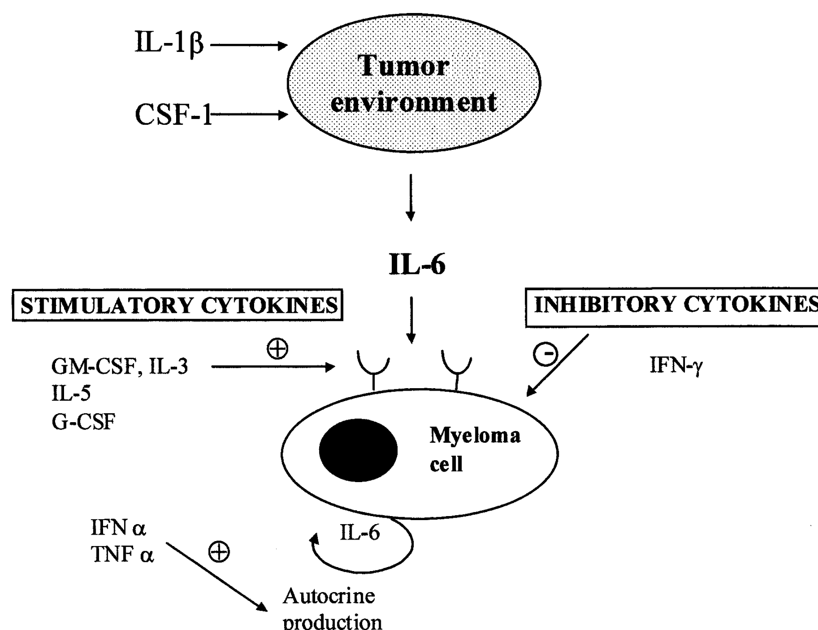

Fig. 3 The cytokine network in multiple myeloma.

noma, renal cell carcinoma or ovarian cancer. Recently, plasma levels of TNF and its soluble receptors (p55 and p75) have been shown to be higher in Hodgkin's disease patients than in healthy controls and these concentrations were associated with several prognostic factors related either to the host (age, performance status) or to the tumor (disease stage, extranodal site involvement, histology and $_{1}$-microglobulin (115). These examples illustrate the use of cytokine measurement for the identification of prognostic factors that will influence outcome of patients and response to therapy. This is a fundamental issue for selecting and staging patients for clinical trials. Nevertheless, there is no documented evaluation on the best cytokine(s) and for cytokine receptor(s) to be determined in the different malignant diseases.

Transplantation

Graft rejection is clinically characterized by the alteration of the function of the transplanted organ. Unfortunately, this criterion is not specific, since many factors other than rejection can induce the same clinical picture. The availability of markers of the immune mechanisms involved in the process would be of major interest, but the existence of confounding factors such as concomitant infections and cyclosporin toxicity is a strong limitation. In the late 80's an abundant literature has described the potential interest of measuring cytokines in serum, urine or bile for the prediction of rejection episodes. Effectively kidney, liver, or heart rejection and acute graft *vs.* host disease after hematopoietic stem cell allografts are generally associated with an elevation of circulating IL-1, IL-6 or TNF , but, due to the modest cytokine increase, the inter-patient and inter-assay variability and the overlapping results with those found in infections, the practical clinical value remains low. The possible value of serum soluble fragments of CD 23 (sCD23) determination in chronic graft *vs.* host disease, which represents a typical Th2 condition remains to be investigated. Many studies have been focused on IL-2 and sIL-2R in relation to the immunosuppressive activity of cyclosporin A. Thus, a rise in IL-2 or sIL-2R could be a sensitive and early marker of escape to T cell activation blockade by cyclosporin A. Both plasma and urinary elevations of this cytokine and its soluble receptor chain have been reported in acute renal rejection (116),with a better specificity for IL-2 than for sIL-2R. Sequential measurement is recommended. *In situ* techniques (PCR, immunohistochemistry) for detecting IL-2 and IFN within the graft have been proposed (117). The ELISPOT technique has been used for analyzing cells producing IFN , IL-6 and IL-10 in transplanted kidney removed after graft failure (118), but these are pathophysiological studies not applicable to routine monitoring. It should be stressed that the delicate balance between acceptance and rejection of organ allografts under immunosuppressive treatment may be drastically altered within a few hours. Therefore, early diagnosis of acute rejection prior to conventional clinical or biological alterations would require at least a daily monitoring in all patients. Moreover, excessive sensitivity or poor specificity of any early biological marker of rejection would result in unnecessary high dose steroid treatments or other anti-rejection therapies with a major risk of iatrogenic pathology and over-immunosuppression. In renal transplantation, serum CRP levels were shown to be an early and highly sensitive marker of acute rejection, correlated with rejection grade, and a reliable indicator of the response to anti-rejection therapy. The only confounding factors were the development of an IgM and IgG antibody response to equine anti-lymphocyte globulins and ureteral fistulae. Of note is that ischemic renal failure was associated with a rapid decrease of CRP, whereas sustained high CRP levels indicated an acute rejection associated with ischemic renal insufficiency, a condition that could be diagnosed only by renal biopsy and immunohistochemistry. Retrospectively, it seems possible that serum IL-6 determination may be an earlier marker of rejection than CRP. It is noteworthy that the rapid rise in CRP levels induced by rejection was not inhibited by corticosteroid therapy (prednisolone 0.5 mg/kg/d). Another acute phase protein, serum amyloid A protein (SAA), appears to be a more sensitive marker of acute renal allograft rejection than CRP (119). Further clinical studies of serum CRP, SAA and IL-6 as indicators of rejection seem to be warranted.

Autoimmune disease

The role of cytokines in autoimmune disorders is now clearly established. Thus, the implication of Th1 cells in the pathogenesis of autoimmune diabetes, multiple sclerosis and rheumatoid arthritis (RA) is documented (120). Furthermore, TNF is a key mediator in RA since its production is uncontrolled due to an insufficient IL-10 synthesis (121). This observation has been the basis of the rationale for a therapeutical use of humanized anti TNF antibodies in this disease. In RA synovium, a Th1 cytokine profile has been reported (122). In contrast to this finding, a Th2- over Th1-balance profile has been observed in blood of RA patients using a one step culture-immunoassay (123). The data tend to demonstrate that joint and blood compartments are differently regulated. This difference could lie in the respective migratory pattern of T cell subsets, with a limited access of blood Th2 cells to the joint space as evidenced by the expression of chemokine receptors (37).

In systemic lupus erythematosus (SLE), there is no clear polarization towards a Th1 or a Th2 profile, but IL-6 and IL-10 play a major role. IL-6 synthesis is highly augmented, and there is a ten-fold increase in mRNA for IL-6 compared to healthy individuals (124), but this alteration is not related to disease activity. IL-10 modifications are more characteristic of SLE since circulating monocytes and B cells produce high quantities of this cytokine. IL-10 overproduction could explain the increased synthesis of both immunoglobulins and antibodies and the defect in cell immunity *via* the deactivating role of IL-10 on macrophages (125). If these

cytokine abnormalities are very important in the pathophysiology of the disease, there is still no real application of their determination in clinical practice.

Allergic disease

The essential mechanism of type I allergy is the induction of IgE synthesis. At least two signals are necessary for immunoglobulin isotype switching towards IgE. The first is antigen dependent and represented by IL-4 (and, to a lesser extent, by IL-13). The second is provided by direct contact between B cells and activated T cells. The molecular basis of this contact is CD 40 (on B cells) and CD 40 ligand (on T cells) interaction (126). IgE generation is potentiated by other surface adhesion molecule interactions among which the binding of CD 23 on T cells with CD 21 on B cells is clearly documented (127). Membrane CD 23 can be cleaved, thus giving rise to a soluble form of CD 23 which after binding to IgE can potentiate IL-4 activity. A schematic representation of IgE synthesis and its molecular control is given in Figure 4.

A wide body of literature has documented an altered Th1/Th2 balance in allergic diseases leading to an elevation of the Th2 response. This is particularly true for allergic asthma, rhinitis and conjunctivitis associated with increased levels of serum IgE and allergen-specific IgE antibodies (128). In atopic individuals, the persistence of the fetal Th2 responses during early childhood seems to be a characteristic feature of the inductive phase of the allergic disease (129). Although the assessment of an increased Th2 response has not yet been validated as a diagnostic tool for allergic diseases, one may foresee possible clinical applications of such investigations. Extrinsic asthma due to IgE hyperresponse to environmental allergens is frequent in children and often vanishes after puberty, whereas instrinsic asthma associated with bronchial hyperactivity, but not with IgE mediated allergic reaction, is frequent in adults. Determination of Th2 cells in blood and bronchoalveolar lavage by their chemokine receptor phenotype may prove to be an additional marker of allergic asthma. Furthermore, evolution of the Th2 phenotype under immunotherapy by injection or sublingual administration of specific allergens could provide in the future a useful indicator of response to treatments.

The low affinity Fc receptor for IgE (Fc RII/CD-23) can be used as an indirect assessment of a Th2 response in allergic diseases. It is expressed on a large variety of cells including lymphocytes, monocytes and eosinophils. sCD23 generated following sequential cleavage of the membrane form are involved in B cell activation, control of IgE synthesis and IgE binding. CD 23 and sCD23 are up-regulated by IL-4 and generally down-regulated by IFN- . As circulating levels of IL-4 are most often undetectable even in pathological conditions, and as this cytokine is a potent inducer of sCD23 release, the measurement of sCD23 has been proposed as a marker of IL-4 activity in clinical situations in which this cytokine is involved such as hyper IgE and hypereosinophilic syndromes, parasitic infections (130) and rheumatoid arthritis (131). In fact, sCD23 is not used on a routine basis in these situations. More interestingly, hyper-eosinophilia can be considered as a good index of IL-5 production, since this cytokine represents a key factor in the differentiation of eosinophils.

Preclinical evaluation of monoclonal antibodies and biotechnology products for therapeutical use

Infusion of CD3 (OKT3) or CDw52 (CAMPATH-1) monoclonal antibodies results in a "first dose syndrome" characterised by high fever, chills, headache and sometimes diarrhea, and pulmonary and cerebral oedema.

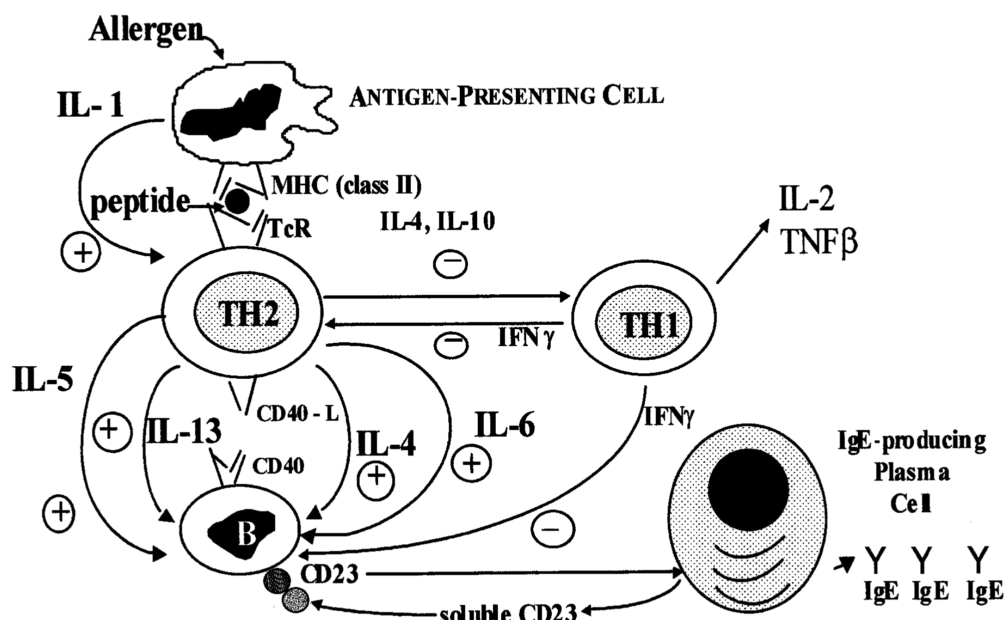

Fig. 4 The role of cytokines in the control of IgE synthesis.

Subsequent infusions are symptomless, because of down-modulation of antigen surface expression or other deactivating processes of intracellular signaling. Such activation syndromes may be decreased by concomitant administration of high dose corticosteroids or antibodies to TNF (132). Therefore any biotechnology molecule that is developed for clinical application should be tested for its capacity to induce a first dose syndrome. The whole blood assay for TNF and IFN production proved to be correlated with the reported clinical manifestations for a wide variety of monoclonal antibodies (133, 134). TNF may be induced either by T cell activation (OKT3, CAMPATH-1) or by LPS or LPS-like contaminants. With CDw52 antibodies, it was shown that cytokine release was isotype-dependent, with the rat IgG2b and human IgG1 isotype inducing the highest levels of cytokine release. It was also reported that cytokine release resulted from ligation of CD16 on the NK cells rather than Fc-receptor-dependent cross-linking of CD52 on the targeted cell (135). Cytokine-release syndrome is also documented in patients with B cell chronic lymphocytic leukemia or B cell non-Hodgkin's lymphoma after treatment with anti-CD20 monoclonal antibody (Rituximab); 90 minutes after onset of infusion, serum levels of TNF and IL-6-peaked in all patients and this cytokine elevation was associated with clinical symptoms including fever, chills, nausea, hypotension and dyspnea (136). During clinical trials a precise documentation of cytokine production and kinetics is important to select the best infusion schedule for new immunotherapies.

Diagnosis of genetic defects/predisposition of cytokines and cytokine receptors

The level of cytokine production upon *in vitro* or *in vivo* activation varies markedly among individuals. Genetic deficiencies of cytokine or cytokine receptor genes have been recently characterized and in depth observation of the defective phenotypes has been instrumental for assessing the role of IL-2, IL-2R chain, CD132 (common c chain), CD131 (chain), and CD119 (IFN RI chain). In addition to complete defects attributed to various mutations, an allelic polymorphism has been reported in some cytokine or cytokine receptor genes and, in a few instances, allelic variants could be correlated with the overall biological efficiency of the cytokine/receptor pair. The mechanisms accounting for such correlations are often unknown. They may involve transcriptional regulation (polymorphism in promotor elements), post-transcriptional control, transport, and proteolysis of cytokines, as well as interaction of receptor intracellular regions with adaptator proteins involved in signal transduction.

Two alleles of the TNF gene have been described : TNF 1 and TNF 2 bearing Asp and Thr respectively in position 26 (137, 138). These variants are correlated with a polymorphism in the first intron and different levels of TNF and TNF production. Whole blood cells from TNF 2 allele carriers produce more TNF upon LPS stimulation than TNF 1 cells (139). The role of this polymorphism has been suggested in cerebral malaria (140), lepromatous leprosy (141), meningococcal disease (142), non-Hodgkin's lymphoma (143) and septic shock (144). Recently, six different missense mutations of the 55 kDa TNF receptor (TNF-RI) have been reported in seven families suffering from autosomal dominant periodic fever syndromes (145). In these periodic syndromes, it is proposed that the auto-inflammatory phenotype results from impaired down-regulation of membrane TNF-RI and diminished shedding of potentially antagonistic soluble receptor.

Five polymorphisms have been identified in the TGF-1 gene: two in the promoter region at positions –800 and –509, one at position +72 in a non-translated region, and two in the signal sequence at positions +869 and +915. The polymorphism at position +915 in the signal sequence, which changes codon 25 (arginine proline) is associated with inter-individual variation in levels of TGF 1 production. Cells from homozygous (arg/arg) individuals produce more TGF 1 than those from heterozygous individuals. The homozygous condition is frequent in diseases associated with lung fibrosis and it is accompanied by an increased risk of fibrosis after lung transplantation (146).

Three dimorphic polymorphisms have recently been described in the IL-10 promoter. The 1082*A allele has been associated with low and the 1082*G allele with high *in vitro* IL-10 production. In rheumatoid arthritis, the presence of IgA rheumatoid factor, usually found in aggressive forms of the disease, is associated with the 1082*A allele (147).

The methylation of the IFN- promoter has been shown to be negatively correlated with the transcription of the gene. As a consequence, in Th1 clones this site is completely or partially hypomethylated whereas in Th2 clones this site is more than 98% methylated (148). Transfection experiments have indicated that hypomethylation *per se* was not sufficient for inducing IFN- gene expression. The implication of an IFN- gene polymorphism in the pathogenesis of certain human diseases has been studied. Thus, the existence of a polymorphism within the first intron of the IFN- gene has been documented in Japanese diabetic patients, the alleles "3" and "6" being increased in these patients compared to controls (149). This association has not been confirmed in a Danish population (150); in Finnish insulin-dependent diabetes mellitus (IDDM) patients, a modest link was observed in a case-control study, but in this group it was impossible to reproduce it by transmission disequilibrium test, which provides little support for the association of IFN- gene microsatellite with IDDM (149). As for IL-1 ra, relationship between IL-1 production and an 86-bp variable repeat polymorphism in intron 2 of the IL-1 ra gene has been looked at; a less common allele (allele 2) is associated with an increased IL-1 ra protein production and a reduced synthesis of IL-1 protein (151). IL-1ra allele 2 may contribute to susceptibility to sepsis, but this allele is not associated with patients outcome (152).

Polymorphism of microsatellites in the 5q31./1 region which contains genes encoding IL-3, IL-4, IL-5 and

Tab. 3 Major clinical indications for cytokine measurement in body fluids.

Cytokine/Cytokine receptor	Clinical condition	Clinical usefulness
• IL-6		
EDTA- plasma/serum	Bacterial infection	Early detection of neonatal infection (73, 74) Efficacy of antibiotherapy
	Sepsis/septic shock	Prognostic marker (69)
	HIV infection	Marker of the severity of AIDS (70, 71)
	Rheumatoid arthritis	Marker of disease activity
	Crohn's disease	Marker of disease activity
	Lymphoma	Prognostic marker
	Renal cancer	Indicator of response to IL-2 therapy (106)
	Melanoma	Indicator of response to IL-2 therapy (106)
	Kidney transplantation	Marker of rejection /infection
Urine	Kidney transplantation	Marker of rejection /infection
Amniotic fluid	Infection of amniotic cavity	Early detection of microbial invasion during preterm labor (72)
Bronchoalveolar fluid	Lung inflammation/infection	Marker of ARDS
Synovial fluid	Rheumatoid arthritis	Marker of disease activity
• IL-8		
EDTA-plasma/serum	Bacterial infection	Early detection of neonatal infection
	Sepsis/septic shock	Prognostic marker
Broncho-alveolar fluid	Lung inflammation/infection	Early diagnosis of ARDS
• IL-10		
EDTA-plasma/serum	Multiple trauma, infection, SIRS	Quantification of immunosuppression (79) Use of IL-10/TNF ratio as a prognostic marker
• IL-1ra		
EDTA-plasma/serum	Bacterial infection	Early detection of neonatal infection (76)
• TNF		
EDTA-plasma/serum	Bacterial infection, sepsis	Prognostic marker particularly in meningococcal diseases with *purpura fulminans* (61–65)
	Leishmaniasis, malaria	Marker of disease activity particularly for cerebral malaria (87)
	Rheumatoid arthritis	Marker of disease activity (121)
	Non-Hodgkin's lymphoma	Prognostic marker (115)
	Transplantation, graft	Marker of GVH
	Immunotherapy (OKT3 ...)	Detection of the "first dose syndrome" (136)
Cerebrospinal fluid	Meningitis	Help to the differential diagnosis of bacterial/viral infection (68)
	Multiple sclerosis	Marker of disease activity
• Soluble TNF receptors (p55, p 75)		
EDTA-plasma/serum	Sepsis, septic shock	Prognostic marker (84)
	Non-Hodgkin's lymphoma	Prognostic marker (115)
Synovial fluid	Rheumatoid arthritis	Marker of disease activity
• Soluble IL-2 receptor		
EDTA-plasma	Acute lymphoid leukemia	Prognostic marker, detection of relapse (107, 108)
	Lung cancer	Prognostic marker (109)
	Sarcoidosis	Marker of disease activity
	Transplantation	Early detection of rejection/infection (116)
Urine	Transplantation	Early detection of rejection/infection (116)
• IFN		
EDTA-plasma/serum	Viral infection	Help to the diagnosis of viral infection (86)
Cerebrospinal fluid	Viral infection	Help to the diagnosis of viral infection (86)
• M-CSF		
EDTA-plasma/serum	Ovarian cancer	Help to the diagnosis and follow up (in conjunction with CA-125) (114)

(AIDS: acquired immunodeficiency syndrome, ARDS: acute respiratory distress syndrome,
GVH: graft *vs.* host disease)

IL-13 in humans has been correlated with serum IgE levels. Moreover, certain allelic variants of IL-4R may be correlated with elevated serum IgE and atopy, but conflicting results have been published as regards the 150V and Q57R.

Practical Clinical Usefulness of Cytokine Measurement

As previously illustrated, the major limitation of cytokine clinical usefulness lies in the lack of specificity of these molecules for a given disease; due to their pleiotropic and redundant activity, cytokines can exhibit a very similar pattern in different clinical circumstances. Because of their very low circulating levels, only elevations of these mediators can be detected in biological fluids; this is the case for systemic diseases such as SLE or sepsis which are characterized by a dramatic activation of the immune system and the acute phase response. In fact, even if very important, the practical help of cytokine determination in the diagnosis is limited to very specific situations such as the early detection of neonatal sepsis by measuring IL-6 (73) or IL-1ra (76) in the first hours after birth. Conversely, cytokine investigation is more interesting for assessing the activity of a disease, and its prognosis. In this context, measurement of cytokines appears to be very useful as a means to select the most suitable therapeutical agents and to monitor the efficiency and tolerance of immunotherapy. Cytokine determination will be also used in the future as a help to stratify patients who will enter clinical trials. With the introduction of the abovementioned sensitive new technologies the humoral and cellular investigation of the local production and biological activity of cytokines will be possible in specific body fluids (such as broncho-alveolar fluid or CSF) or tissues; this approach is particularly clinically relevant since it allows the exploration of an immune or an inflammatory reaction where it takes place.

Among the numerous known cytokines only a limited number of them is commonly measured in clinical practice. A list of these cytokines and the main clinical indications of their determination are given in Table 3.

Conclusions

The major progress achieved during the last 25 years in exploring the cytokine network in many pathological conditions has generated a large series of analytical tools which could be applied to routine clinical laboratories. However, significant alterations in cytokine profiles revealed by clinical studies may not necessarily indicate that cytokine determination could be applied to the diagnosis or the monitoring of the disease. Limitation of large scale applications are both technical and biological, owing to the short half life and rapidly changing levels, the heterogeneity of cell sources and the possible discrepancies between circulating leukocytes and cells in the lesion. The field of clinical application of cytokine investigation will only be progressively defined by carefully designed clinical studies that assess the relative impact of cytokine determination on clinical decisions.

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