INTRODUCTION

This review describes not how the scientific community in general, but rather how the Laboratory of Neuroimmunology at the Mondino Foundation in Pavia, has approached the problem of cytokine measurement. This report, with regard to both methodologies and studied cytokines, is therefore necessarily unbalanced and inherently incomplete. But it has been conducted in the spirit of this congress, which is devoted to ten years of research in experimental neurobiology at the Mondino Foundation.

The term “cytokines” comprises a group of regulatory peptides involved in immune responses, as well as in hematopoiesis and repair processes. Virtually every nucleated cell can produce cytokines. Just as humans communicate with words, so immune-inflammatory cells communicate by means of cytokines. The initial concept of “one producer cell – one cytokine – one target cell” has been disproved for almost every cytokine. More than one cell type can produce a cytokine that has more than one cell type as a target. Therefore, the cytokine network is highly redundant. In turn, this implies that a classification of cytokines on the basis of their producer or target cells is problematic. Even more problemat-
ic is the attempt to define unique biological activities for each cytokine. Almost every cytokine shares more than one function with more than one other cytokine. Ultimately, cytokines work within an intricate and complex network.

CYTOKINE MEASUREMENT FROM THE LABORATORY POINT OF VIEW

Regrettably, data are emerging that show that the context in which cytokines are involved makes a single cytokine act beneficial in some conditions, but harmful in others. To summarise, we know that cytokines play a pivotal role within the immuno-inflammatory response, and act through a complex network.

There are two questions to ask: 1) in the near future, will we pathologists be measuring cytokines in biological fluids, as we now routinely measure other immuno-inflammatory molecules, such as C reactive protein, complement factors, etc.? 2) Does the measurement of single, or even a few, cytokines in biological fluids make sense? In other words, even if we had reliable methods for cytokine measurement, could we get reliable information about a pathological process by exploring the modifications of a few, selected cytokines without exploring the whole cytokine network? It is evident that a holistic approach should be adopted. More practically, as the huge body of literature on the topic indicates, there is a trend towards gathering pieces of information, and trying to surmise clinico-laboratory associations and pathogenetic scenarios.

Accurate and sensitive methods for the measurement and detection of circulating cytokines are a prerequisite for understanding the biology of cytokines, and for determining both their clinical usefulness and their pathogenetic role in various diseases.

Above all, the biological activities exerted by cytokines have been considered as the basis of their biological detection and thus of assays of their molecules (for example, protection from cell killing by cytocidal viruses in the case of interferons). However, bioassays present drawbacks in terms of sensitivity, specificity, reliability, and simplicity. Cell-line based assays are sufficiently precise. Their main problem is that they are not specific to a single cytokine. As a result, antagonistic or synergistic molecules may respectively lead to an underestimation or overestimation of a single cytokine content in a given fluid. Moreover, they are not sensitive enough to detect very low concentrations of circulating cytokines. In short, bioassays are not suitable for routine laboratory purposes.

Immunoassays provide a convenient method for cytokine measurement. They are easier and quicker to perform than bioassays. Based on a "two site" principle, the most useful immunoassays are immunometric assays. These assays can be immunometric radioimmunoassays (IRMAs), or enzyme-linked immunoassays (ELISA), with respectively radiolabelled or enzyme-labelled detector antibodies. In immunometry, one antibody is used to capture cytokine antigen in the sample, and another one, which is labelled, to detect bound antigen. The main problem of immunoassays is that they detect biologically inactive or partially active cytokine molecules too. Furthermore, the antibodies used cannot recognise various, differing subspecies of a cytokine. The "matrix effect", due to the characteristics of biological fluids, may also interfere with measurements. Nevertheless, immunometric assays have prerequisites, in terms of sensitivity, specificity and feasibility, for use in routine laboratory practice.

Over the past decade, our main field of study has been pro-inflammatory cytokines in multiple sclerosis. The primary goal has been to identify reliable peripheral markers of disease (disease trait) and of disease activity (state trait). Multiple sclerosis is the most common, disabling neurological disease in young adults. It affects about 1 in 1,000 members of the European populations. Its aetiology is unknown, but we do know that the disease is T-cell mediated (probably autoimmune), chronic inflammatory, demyelinating, and
that it is confined to the central nervous system. The inflammatory process in multiple sclerosis is promoted mostly by pro-inflammatory cytokines. Within the large family of cytokines, we have focused on a primary inflammatory cytokine, tumor necrosis factor (TNF-α), and on interferon (IFN)-γ and interleukin (IL)-4, which are, respectively, cytokines that characterise Th1 and Th2 cells. Demonstrated first in mice by Mosmann and Coffman (1,2), and then in humans by Romagnani (3), the cytokine-induced polarization of T lymphocytes can be considered as one of the most outstanding discoveries in immunology in the past decade. Naïve CD4+ T cells, under the effect of specific cytokines (mainly IL-12 and IL-4), differentiate into Th1 and Th2 cells. Th1 cells produce cytokines involved in cell-mediated immunity and autoimmunity. Th2 cells produce cytokines that promote humoral immunity and allergic/atopic phenomena. Cytokines from each subset are inhibitory towards those of the other subset.

TNF-α seems to be particularly involved in multiple sclerosis. It has toxic effects on organotypic oligodendrocyte cultures (4), and has been identified in demyelinating lesions, typical of multiple sclerosis (5). A huge amount of data has been produced on circulating TNF-α levels in serum and cerebrospinal fluid of multiple sclerosis patients. Opinions are divided over the use of measurements of circulating TNF-α (either alone or together with its soluble receptors) as a laboratory support for active phases of disease in multiple sclerosis (6-15). Table I shows some of the most important papers in the literature that are for or against this use. Our data indicate that TNF-α and its receptors cannot be used as reliable markers of disease activity in multiple sclerosis (7,15). A rationale, which supports these data, and can be extended to any other cytokine, derives from the following evidence: a) cytokines have a short half-life, b) they mainly act in an autocrine-paracrine fashion, and c) they are often “buffered” by soluble receptors in the peripheral blood. These soluble receptors can also help to prevent cytokines from being detected with immunometric methods. Another issue is the possibility of finding different cytokine profiles in the target organ and in the periphery. For example, we measured cerebrospinal fluid levels of the chemokine monocyte chemoattractant protein (MCP)-1 in multiple sclerosis (chemokines are particular members of the cytokine family that play a key role in leukocyte migration). Cerebrospinal fluid MCP-1 levels were lower in active than in stable multiple sclerosis (16), whereas McManus et al. demonstrated that MCP-1 is highly overexpressed in astrocytes from acute multiple sclerosis lesions, but not at all in inactive lesions (17). To date, this phenomenon has not been fully explained, and were cerebrospinal fluid MCP-1 levels considered as isolated data, it could result in misinterpretation of MCP-1 involvement in the development of pathological lesions in multiple sclerosis.

Table I - Some of the most important papers in the literature that are for or against the use of measurements of circulating TNF-α, either alone or together with its soluble receptors, as a laboratory support for the demonstration of active phases of disease in multiple sclerosis.

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<th>TNF-α measurements are useful</th>
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<td>• Beck et al. (Acta Neurol Scand, 1988)</td>
<td>• Franciotta et al. (Ann Neurol, 1989)</td>
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<td>• Gallo et al. (J Neuroimmunol, 1989)</td>
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<td>• Sharief et al. (N Engl J Med, 1991)</td>
<td>• Peter et al. (Neurology, 1991)</td>
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<td>• Chofflon et al. (Eur Cytokine Netw, 1992)</td>
<td>• Martino et al. (J Neurol Sci, 1997)</td>
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<td>• Drulovic et al. (J Neurol Sci, 1997)</td>
<td>• Franciotta et al. (Eur Cytokine Netw, 1999)</td>
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To summarise, the detection of circulating cytokines with immunometric assays does not seem to give reliable information about the immune-inflammatory processes underlying their production in multiple sclerosis. We were thus prompted to look for alternative cytokine measurement strategies. Specific procedures have been developed that allow the detection of cytokines in the cells that produce them. Basically, it is possible to detect a) cellular cytokine mRNA, and b) the intracellular presence of translated cytokine products, together with the phenotype of the producer cell.

When appropriate primers are used, cytokine mRNA can be amplified with a reverse transcriptase-polymerase chain reaction (RT-PCR), and quantified in comparison with mRNA from a housekeeping gene. The technique is relatively difficult and cumbersome, especially if a precise quantitation of transcripts using nested-PCR is required. New technologies have recently delivered instruments with a high level of automation, e.g., the so-called Taqman®. It is assumed that variations in cellular cytokine mRNA levels would indicate similar changes in protein production. This is not always true. For example, post-transcriptional regulatory mechanisms can operate to modulate cytokine production by cells. The use of this technique is restricted to research purposes. It is argued that it is valid to determine both cytokine mRNA and intracellular cytokines in peripheral blood mononuclear cells in diseases restricted to the central nervous system. Support for this argument derives from the fact that encephalitogenic clones are first activated outside the central nervous system, in lymphoid organs, and subsequently migrate into the central nervous system, where they act.

In a longitudinal study on multiple sclerosis, which involved blood drawing every 2 weeks for one year, we demonstrated that: a) mRNA coding for TNF-α, TNF-receptor(R)1, and TNF-R2 from peripheral mononuclear blood cells peaked 4-6 weeks before acute disease, and that b) absolute values for these parameters fluctuated within the ranges obtained from healthy controls (14). To summarise, beyond their scientific value, and from a practical point of view, these data do not favour the use of these determinations in clinical practice.

Flowcytometry is a well-established technique that can also be used to determine, within a cell population, the proportion of cells that contain cytokines. Double or multiple labeling methods combine cytokine-specific antibodies with antibodies that detect surface markers. These latter antibodies provide information on the cell types that synthesise cytokines. Therefore, the combination of an intracellular staining technique with cytofluorimetry renders possible the leap from the detection of circulating cytokines to the direct evaluation of the cells that produce them, and that contain them prior to their release. The technique of intracellular cytokine staining comprises a) a brief in vitro stimulation of T cells, b) their permeabilisation with appropriate reagents (to allow cytokine-specific antibodies to enter the cells), c) the reaction with antibodies to membrane antigens, and d) the count with a cytofluorimeter. The main drawback is the method’s sensitivity, as in cytofluorimetry it is often necessary to stimulate cells to detect cytokines. Since this technique requires specific training and experience, its use is restricted to research purposes.

Autoimmune diseases are typically associated with a cytokine imbalance. This imbalance is sustained by pro-inflammatory cytokines, such as TNF-α, IFN-γ, and IL-12, which, in turn, promote T-cell differentiation towards the Th1 phenotype. In cytofluorimetry, with a cytokine intracellular staining technique, we typed Th1 and Th2 cells with, respectively, IFN-γ and IL-4 as defining cytokines (18). The study was carried out on patients with multiple sclerosis with an active or stable disease. Th2 cell percentages were higher in stable than in active multiple sclerosis. This fact indicates that these cells could be involved in downmodulating immune response. Th1 cell percentages were lower in active than in
stable multiple sclerosis. Thus, it is unlikely that these cells play a role in exacerbations of the disease. Alternatively, pathogenetic Th1 cells could possibly migrate into the central nervous system when the disease is acute, and, as a result, could be reduced in the peripheral blood, where we find them in low percentages.

Cytokines play a fundamental role in the pathogenesis of multiple sclerosis, as well as in other inflammatory diseases of the central nervous system. Our studies bear witness to our efforts, over the past years, to identify which laboratory techniques produce reliable data about cytokines in neurological diseases. To penetrate the intricacies of the cytokine network is particularly important for the development of counteracting therapies. Whatever the methods used for their detection (those described here and those not mentioned here), the state of the art tells us that cytokine determinations, are not part of common laboratory practice, at least in neuroimmunology. This is due not only to the considerable problems, inherent in their nature, mentioned earlier, but also to shortcomings in both pre-analytical and analytical procedures: for example, the lack of standardisation in immunometry in the cytokine field.

In conclusion, knowledge grows by a never-ending process of approximation to truth. At least from the laboratory point of view, the cytokine network deserves further investigation in order to build on current knowledge.

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