



MOLECULAR IMMUNOTOXICOLOGY TESTING *IN VITRO*

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Abstract—Analysis of the modulation of expression of key immunoregulatory cytokines has significant potential for the development of *in vitro* immunotoxicological screening assays. Many techniques are available to analyse cytokine mRNA expression in cultures derived from specific immune cell populations; in our hands reverse transcription–polymerase chain reaction (RT–PCR) analysis was extremely sensitive but had low reproducibility, whereas dot-blot analysis gave acceptable levels of sensitivity and specificity and was highly reproducible. Certain cytokines such as IL-1 and IL-2 are pivotal to the normal development of an immune response. We have shown using murine *in vitro* systems that macrophage IL-1 mRNA can be induced by levels of Biostim (an immunostimulatory drug) as low as 10 pg/ml. We have also shown that concentrations of cyclosporin A (an immunosuppressive drug) down to 1 ng/ml can inhibit the expression of lymphocyte-derived IL-2 mRNA. Other immunomodulatory compounds such as azathioprine (1 µg/ml) and tributyltin oxide (1 nM) caused a partial inhibition of IL-2 and/or IL-2 receptor mRNA within rodent mixed lymphocyte cultures. Based on these results we are proposing a series of simple tests to predict immunomodulatory potential of new compounds. These techniques are equally applicable to cell cultures of human origin and current research in our laboratory is focused on the use of human peripheral blood mononuclear cells to explore the allergenic potential of drugs and chemicals by analysing characteristic modulation of patterns of normal cytokine expression.

Introduction

The subject of immunotoxicology covers both the effects of agents stimulating the immune system and those that depress the immune response (Miller *et al.*, 1991). In some cases the manifestations of this dysfunction may be primary, such as in allergic reactions or in autoimmune disease. In other cases, the consequences are likely to be secondary in that perturbation or impairment of immune function may lead to inflammatory reactions or increased susceptibility to infection or perhaps to cancer. Immunosuppression is the most extensively studied area and there is ample experimental evidence that the mammalian immune system can be damaged and that immune reactivity can be altered following exposure to a diverse range of drugs and chemicals (Luster *et al.*, 1988; Miller, 1985).

These findings are in the main based on rodent studies, many of which are used in conjunction with a subchronic exposure regimen, and there is a lack of agreement at present as to whether such immunotoxicological testing can be performed within the context of required routine toxicity testing or whether stand-alone tests are required. Animal laboratory studies have a number of disadvantages, and the immune system, which consists of a disseminated moving system with specialized lymphoid structures,

cannot be easily dissected and subjected to classic toxicological analysis.

There is limited human information concerning chemically induced suppression of immune function and such information is available only from studies in which individuals have been exposed to large doses over a relatively short period of time. Evidence in humans relating to the effects of low-level exposure over longer periods of time is by its very nature hard to come by and might be apparent only in longitudinal epidemiological studies, which are difficult to design and carry out given the diversity of human behaviour, the role of genetic factors, and the influence of age and nutritional status. For example, young children who are malnourished and have recently had measles infection will have a double reason to be immunosuppressed and thus are more susceptible to further insults to their immune system by chemical agents. Findings from the many clinical studies of patients treated with immunosuppressive agents and those with human immunodeficiency virus infections, however, justify concerns about the potential of chemicals or drugs to impair immune function. Recent clinical data on biotechnology-derived products primarily designed to act on the immune system (monoclonal antibodies and recombinant cytokines) also demonstrate the need to assess the potential of such products to induce unwanted (adverse) immune reactions and to determine the extent of their immunosuppressive effect.

In contrast to immunosuppression, there is ample evidence in humans of chemically mediated hypersensitivity reactions with the respiratory tract and

Abbreviations: IFN = interferon; PBMC = peripheral blood mononuclear cells; RT–PCR = reverse transcription–polymerase chain reaction; Th = T helper; TNF = tumour necrosis factor.

the skin as the two primary routes of exposure. On the other hand, apart from animal models for the predictive testing of potential contact sensitizers, there is at present no validated animal model that provides information on immunological events and functional changes in other types of hypersensitivity reactions. The preclinical assessment of compounds with respect to allergic and/or autoimmune responses is therefore limited to assessing their ability to induce a specific immune response and mechanistic studies have been limited to the diagnosis of allergy in individuals who are already sensitized.

The various categories of immunotoxicity (suppression, allergic, autoimmune, non-specific immune response) display different characteristics with regard to dose-response relationships, mode of antigen presentation, and dependence on genetic variation in determining susceptibility. However, there is a wealth of evidence that the immune system is tightly regulated by cytokines and related intercellular polypeptide messengers. These molecular targets offer important new avenues to explore in developing *in vitro* screening assays for potential immunotoxic effects. Development of new biotechnological tools such as cDNA probes, oligonucleotides and reverse transcription-polymerase chain reactions (RT-PCR) methodology also represents an innovation and should contribute greatly to developing *in vitro* model systems. The application of molecular biological techniques to the analysis of immune cell populations will be of value not only as potential immunotoxicological screens (molecular immunotoxicology) but also to give insights into the biochemical mechanisms that underlie immunotoxic effects.

Molecular immunotoxicology

The immune response to antigen is regulated by a variety of products both from cells of the immune system and from cells of the microenvironment of the immune system. These soluble mediators (cytokines) are responsible for the amplification, maintenance and limitation of the immune response. A considerable proportion of the effort in molecular biology over the last two decades has been directed towards a better understanding of the regulation of the immune system. This has resulted in a large number of cytokines, interferons and growth factors, most often derived from mice or humans, being cloned at both cDNA and genomic level, thus enabling molecular analysis of gene expression at the RNA level. All cytokines are important although some have a more pivotal role in the induction of the immune response [e.g. interleukin 2 (IL-2) in the clonal expansion of T cells], whereas others appear to have a more specialized role (e.g. IL-5 in recruitment of eosinophils). T lymphocytes are now known to play a key role in directing effector mechanisms through differentiating into two activated subsets of T-helper cells, Th1 and Th2 which, although they express the

same surface markers, secrete different cytokines and have different functional activities. *In vivo* and *in vitro* allergens and helminthic antigens selectively induce the development of Th2 cells, whereas bacterial and viral antigens induce Th1 cells (Romagnani, 1990). The factors that influence the differentiation of T-helper cells into Th1 or Th2 cells are not so well established, but cytokines present during the induction phase of the immune response (e.g. IL-1) and the nature of the antigen presenting cells (dendritic cells or macrophages) appear to be particularly important. Th2 cells produce IL-4, which is responsible for inducing B lymphocytes to secrete IgE and therefore is critical for the development of immediate allergic reaction; together with other B cell-tropic cytokines (i.e. IL-6), IL-4 is also involved in humoral autoimmunity since these mediators regulate the activation and proliferation of autoreactive B cells. On the other hand interferon- γ (IFN γ) is a key mediator of cell-mediated reactions including type IV hypersensitivity reactions through the activation of macrophages. These two subsets are cross-regulated; Th1 cells negatively influence Th2 through the production of IFN γ , whereas IL-10 secreted by Th2 cells blocks Th1 functions. Immunosuppression could therefore also occur as a consequence of an agent inducing the prolonged expression of IL-10 or by interference with T-cell receptor mediator signalling.

As stated earlier, analysis of the modulation of gene expression is a very powerful technique, and three different types of macromolecules can be analysed to determine expression or altered expression within a cell. Analysis of DNA and the intricate control system within DNA relates to what the cell is programmed to perform; studies on polypeptides or proteins released into the culture media using either ELISA or biological assays allow the monitoring of changes in the expression of proteins, particularly after they have undergone post-translational processing, and can be an important analytical tool. However, we feel that the most direct form of analysis is the expression of mRNA species within the cell. Since most mRNA species are relatively short-lived, the analysis of mRNA expression can pinpoint precisely what a cell is doing at any point in time and how it is responding to external stimuli. Thus the opportunity now exists to study the effect of compounds on the immune system *in vitro*, under conditions that mimic *in vivo* immune responses, by determining the expression and activities of relevant immunoregulatory cytokines and their receptors at the level of mRNA.

Defined cell populations can be pre-incubated with a test compound before immune stimulation and expression analysis. In our laboratory the *in vitro* systems we have chosen to study most closely are primary cultures of murine peritoneal macrophages and splenocytes, although we have established *in vitro* exposure systems using cells from other tissues

relevant to the immune system, as well as using peripheral blood mononuclear cells from human donors. As stated earlier, IL-1 and IL-2 can be considered as central to the development of the immune response, whereas other cytokines appear to have more specialized roles. In developing *in vitro* screening systems we have therefore, in the first instance, sought to analyse expression of those cytokines which are pivotal to the immune response, although in certain cases analysis of other more specific cytokines may be indicated (for details see Meredith, 1992).

In terms of overall strategy, this molecular immunotoxicological analysis is relatively straightforward. RNA is extracted from cells after exposure to test compound and/or *in vitro* stimulation and the extracted RNA is purified and immobilized on a membrane support. Bound mRNA species are detected using radiolabelled probes derived from the cDNA. However, great care must be taken to shield the mRNA species from endogenous or exogenous ribonuclease activity to which they are extremely susceptible. In our experience, the most effective technique for minimizing ribonuclease activity is to vigorously disrupt the cells in the presence of guanidinium salts. Other techniques have proved

inadequate in our hands, particularly with macrophage cultures that appear to have a high endogenous ribonuclease activity. After extraction and ethanolic precipitation the RNA is recovered by repeated centrifugation at 13,000 *g* and can then be stored and subsequently prepared for dot-blotting or Northern-blot analysis. In our laboratory, Northern blots are used to check on technique and specificity of hybridization; once satisfied, we use dot-blotting for rapid multiple sample analysis. Dot-blotting involves direct transfer of the RNA dissolved in an appropriate formaldehyde buffer to a membrane support, and detection of different mRNA species using cDNA probes; these probes are radiolabelled or coupled to enzyme-substrate detection system. In our laboratory we normally use ^{32}P -labelled cDNA probes; the advantage of the less sensitive non-isotopic methods stems from safety considerations, which are important in some laboratories without containment facilities. After stringent washing to reduce background and eliminate non-specific binding, the membrane is subjected to autoradiography or other detection systems by which the presence of specific mRNAs can be confirmed and semi-quantitative analysis can be performed by densitometry. The basic technique is summarized in Fig. 1.

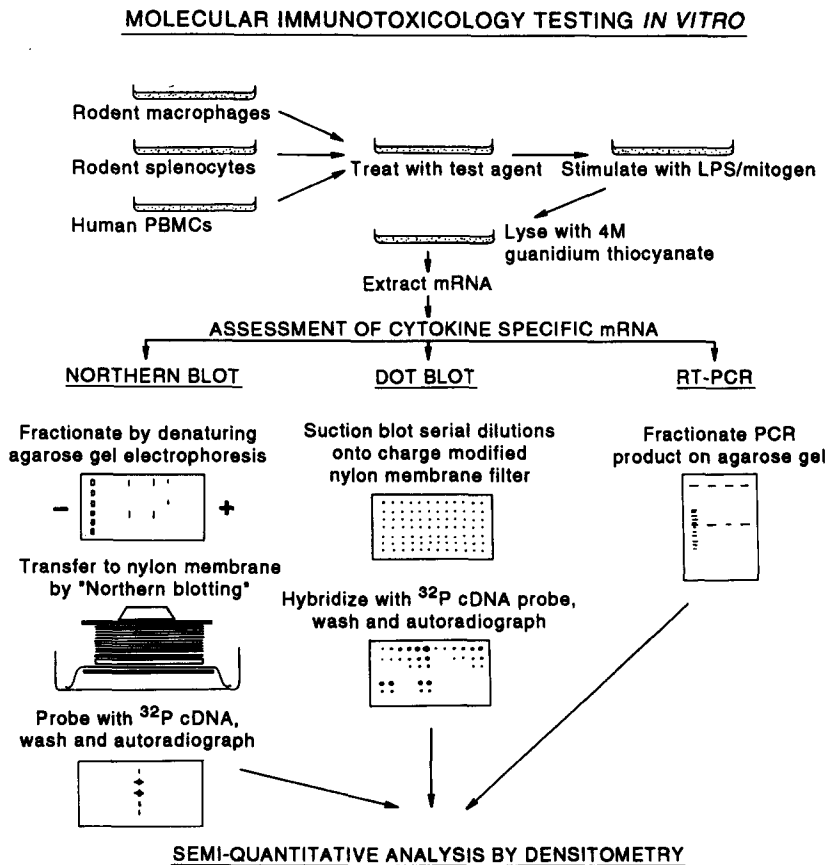


Fig. 1. Molecular immunotoxicology *in vitro*.

Application of *in vitro* molecular immunotoxicology techniques

In order to characterize any test system, it is necessary to select model compounds to define the response. We used two pharmaceutical agents with intended immunomodulatory effects and collaborated with the Mario Negri Institute under the auspices of the EC Biotechnology Action Programme in order to ensure correlation between *in vitro* and *in vivo* results. The two agents were Biostim, a bacterial glycoprotein extract with immunopotentiating properties, and the immunosuppressant, cyclosporin A. We chose to examine the effects of the drugs on peritoneal macrophages in culture and on the ability of cultured splenocytes to participate in a mixed lymphocyte reaction. This would allow comparison with the functional *in vitro* and *ex vivo* assays performed at the Mario Negri Institute. Murine C3H strain peritoneal macrophages were isolated and cultured with Biostim (1 pg/ml–100 µg/ml) or cyclosporin A (1–1000 ng/ml) for periods of 3 or 24 hr. Reaction to allogenic determinants (C57/B1 stimulator cells *v.* C3H responders) was assessed by incubating splenic lymphocytes with test compounds for periods of 24 or 48 hr.

Initial results showed that isolated peritoneal macrophages displayed transient expression of cytokine mRNAs as a result of adherence to plastic culture dishes; overnight incubation (18 hr) allowed them to become essentially quiescent with respect to cytokine and enhanced the sensitivity of the assay. Biostim had no effect on the turnover of actin (a housekeeping gene) but dramatically elevated IL-1 α , IL-1 β , tumour necrosis factor- α (TNF- α) and IL-6 mRNA transcripts at levels as low as 1–10 pg/ml. The expression was transient, peaking after 2–3 hr of exposure and declining rapidly after 8 hr. Only transcripts for IL-1 β mRNA were detectable after 24 hr (Meredith *et al.*, 1990). Cyclosporin A had no effect on IL-1 α , IL-1 β or actin expression in mixed lymphocyte reactions, but dramatically reduced the expression of IL-2 mRNA at both 24 and 48 hr, presenting levels as low as 10% of the control at concentrations of 100 and 1000 ng/ml at 48 hr.

We have applied this type of analysis to the compounds azathioprine and tributyltin oxide and we can demonstrate partial inhibition of the expression of mRNAs for IL-2 and IL-2 receptor in mixed lymphocyte cultures *en route* to the development of cytotoxic T cells. The use of a highly specific probe for cytotoxic T cells, based on the DNA sequence for a serine esterase unique to this cell population (Lobe *et al.*, 1986), allows confirmation of the ultimate development or failure of development of functional cytotoxic lymphocytes. The inclusion of determination of expression of a high turnover housekeeping gene such as actin or a metabolic gene allows discrimination between those compounds which exert a selective immunotoxicity and compounds such as

azathioprine which act by way of a non-specific cytotoxic mechanism.

On the basis of our experience in the field of molecular immunotoxicity, we propose the following simple experiments for the investigation of the immunomodulatory potential of a drug or chemical.

- (1) Ability to induce expression of monokine mRNAs (e.g. IL-1 α/β , IL-6, TNF- α) in cultured macrophages.
- (2) Ability to modulate expression of these monokine mRNAs in lipopolysaccharide-stimulated cultured macrophages.
- (3) Ability to induce expression of lymphokine mRNAs (e.g. IL-2, IL-4, IL-2rec, IL-6) in cultured splenocytes.
- (4) Ability to modulate expression of those lymphokine mRNAs in cultured splenocytes stimulated with alloantigen or mitogen. Analysis of mRNAs specific to cytotoxic T cells (protease or lipases).
- (5) Analysis of total cytokine mRNA levels in selected cells or tissues (e.g. spleen, thymus, lymph nodes, alveolar/peritoneal macrophages) following *in vivo* exposure.
- (6) Analysis of *ex vivo* cells as in (2) and (4).

This scheme is liable to modification as the amount of data available expands and indicates priorities to be evaluated; there may also be specialized steps, such as biotransformation systems, that merit inclusion in a preliminary screen. At present, however, this scheme represents a framework within which pertinent information can be obtained and which can point the way to other more specific experiments.

Future applications

Clearly, if immunotoxicology is to play a significant role in the safety evaluation of drugs and chemicals, it is important that it can offer tests that are sensitive, specific and reproducible. Several laboratories, including our own, are currently studying ways in which human or murine cDNA probes can be modified to detect all cytokine populations from the rat, in order to design a test battery of molecular biological reagents for cytokine analysis. We believe that the extent of animal studies can be significantly decreased if *in vitro* immunotoxicological assessment using both rat and human cells serves as a pre-screen.

As far as drug-induced immunosuppression is concerned, possible mechanisms to be considered include the interference of T cell receptor-mediated transduction signalling, modulation of cytokine transcription, and mRNA degradation and the induction of apoptosis. For classical drugs the major adverse effects on the immune system to be considered are autoimmunity and Type I hypersensitivity. Experimental

autoimmune manifestations induced by mercurials, gold salts and D-penicillamine in rodents have been extensively characterized, especially in terms of genetic factors and T-cell involvement. Interestingly, the autoimmune abnormalities observed in these models are quite similar to those caused by the same agents in humans (Goldman *et al.*, 1991). These animal models could therefore form the basis for developing predictive *in vitro* assays using lymphoid cells from animals that are genetically susceptible to or resistant to drug-induced autoimmunity *in vivo*.

There are no validated animal models for preclinical assessment of the potential of a drug to give rise to adverse reactions, and studies have therefore been limited to diagnosis of allergy in individuals who are already sensitized. Some *in vitro* tests such as measurement of proliferative responses of human lymphocytes in response to drugs are used, but they are rather insensitive and their reliability is doubtful. There is evidence that determination of cytokines (i.e. IL-2, IFN- γ , IL-10, IL-4, IL-5 and IL-6) is much more reliable when analysing drug-specific human T-cell responses in allergic drug reactions. Test systems are being developed—at least in one laboratory—to use a primary autologous mixed lymphocyte reaction in which responders peripheral blood mononuclear cells (PBMC) from normal individuals are cultured with autologous drug-treated stimulator PBMC. This test has potential for screening any compound for its capacity to generate T-cell determinants—although for some drugs a metabolic generating system will need to be incorporated.

The recent introduction of biotechnology-derived products as therapeutic agents raises new and difficult issues in this area and stresses the importance of developing new methods to predict immunotoxic reactions. In the case of OKT3, for example, the antibody triggers a massive polyclonal activation of CD3⁺ cells and monocytes, resulting in the systemic release of cytokines such as TNF, which has been shown to be responsible for severe first-dose reactions. This activating effect can be reproduced *in vitro* in isolated PBMC.

Conclusion

It is important to develop and standardize *in vitro* tests for inclusion in the evaluation of drugs and chemicals. One possible design could be to incubate PBMC from normal donors with the new drug or chemical at different doses and at different times points, and then to measure cytokine levels in culture supernatants and at the mRNA level. Such investigations, as well as other studies using recent advances in molecular immunology, should lead to the establishment of *in vitro* assay systems that are compatible with prenormative requirements. Indeed, we believe that molecular immunotoxicology has an immediate contribution to make and that its contribution will become increasingly more important as *in vitro* studies gain acceptance in testing protocols.

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