Investigation of Cell-Mediated Immunity

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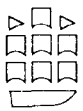

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Foreword

This ninth and last volume of the series *Practical Methods in Clinical Immunology* is the most ambitious so far in dealing with cell-mediated immunity in its totality as it applies to the diagnosis, management and understanding of human disease. Dr Yoshida has brought a team of 30 collaborating immunologists to the task of writing a practical manual about the full spectrum of cell-mediated immunity in the light of the most modern concepts of molecular immunology.

The authors are from diverse biomedical laboratories in the United States with abundant practical experience of their particular topics. They have produced an up-to-date work of reference for clinicians and for laboratory workers. For the latter, the book provides clear, practical guidance to undertake cell immunity investigation given a properly equipped immunology laboratory.

Melbourne, 1985 R.C.N.

Preface

This volume deals with a variety of techniques and approaches currently available for evaluation of the cell-mediated immunity in humans. With the rapid increase in our knowledge concerning the mechanisms of cell-mediated immunity in general (outlined in Chapter 1), attempts to apply such basic information for clinical diagnosis in human diseases have also been accelerated tremendously for the last decade or so. Some procedures and methods including the delayed type skin reaction and several assays to detect specific lymphokines have been established completely as a valid way of evaluating cell-mediated immune responsiveness in patients with various diseases. However, the practical value of some in vitro assays detecting lymphokines and/or cytokines has not been so clear in terms of their predictability of cell-mediated immune reactivity in vivo, specifically in humans, although their potential is considerable.

Thus, this volume contains both types of chapters: those describing the completely established and practical methods in routine use, and others dealing with the promising methods still being clinically evaluated. Authors of each chapter discuss the significance of each method so that readers are told how to perform the assays described and to interpret the results appropriately. The volume is notionally divided into four main sections: Chapters 2 and 3 deal with methods detecting effector lymphocytes (namely T cells) in blood and tissues; Chapters 4 to 9 discuss the reactions directly mediated by mononuclear cells; Chapters 10 to 16 describe the lymphokine-mediated reactions; the final Chapter is concerned with delayed type skin reactions.

As a whole, this book attempts to provide the clinician with practical methods to evaluate cellular immunity in humans, to interpret the results and at the same time, supplies background information about the state of the art in this field of science.

Tokyo, 1985 T.Y.

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Contents

1
Cell-Mediated Immunity: An Overview

T. Yoshida

Immunological reactions are conveniently divided into two mechanistically different types: Humoral (or antibody-mediated), and cellular (or cell-mediated). The former type is mediated by specific antibodies and the latter, which is the theme of this volume, by immune lymphocytes or more specifically T cells without apparent humoral antibodies. This type of reaction is mediated through specific interaction of their cell surface receptors with antigens. The structure of T cell receptor is known to be similar to that of immunoglobulins, although it seems not to be chemically the same as any of the known types of immunoglobulins. The gene structure controlling the T cell receptor was revealed recently, and its exact chemical nature may soon be discovered.

CELL-MEDIATED CYTOTOXICITY AND LYMPHOKINE-MEDIATED REACTIONS

T-cell-mediated immune reactions fall into two broad categories: those that involve direct participation of immune T cells and those mediated through non-antibody soluble factors (lymphokines) generated by immune lymphocytes after their specific interaction with antigens. The former reactions are essentially limited to cell-mediated cytotoxicity, whereas the latter comprise the broader group of cell-mediated immune reactions.

Cell-mediated cytotoxic reaction results from interaction between cytotoxic T cells and a variety of target cells. Thus, the cell surface antigen stimulates the specific receptor on the immune cytotoxic T cells which can eventually kill the target cells through a complex chain of events such as adherence, lethal hit and lysis of the target cell. Cytotoxic T lymphocytes belong to a specific subpopulation of T cells, i.e. Ly 1^- 2^+ 3^+ cells in mice. In contrast to those mentioned below for lymphokine-producing cells, cytotoxic T lymphocytes are not known for their ability to generate any soluble mediator after their interaction with specific target cells. No common soluble mediator for target cell killing is detectable in this type of reaction. Instead, direct cell-cell interation is essential for the effect although there still remains the possibility that intercellular cytotoxic substance may mediate the killing.

In addition to cytotoxic T cells, other types of cells (non-T lymphocytes and macrophages) may function as effector cells for cell-mediated cytotoxicity in a broader sense. Antibody-dependent cell-mediated cytotoxicity (ADCC) may not be

included in the cell-mediated immune reactions in a narrow sense, because humoral factors are also involved. Nevertheless, assay systems for ADCC and for activated macrophages will be considered because of their application in clinical immunology. The significance of NK cells in this context is complicated; NK cells are recognized as a non-T and non-B cell population, representing a new subpopulation namely Large Granular Lymphocytes (LGL), and although NK activity does not require the presence of antibody, LGL could show ADCC activity.

In contrast to cell-mediated cytotoxicity, another major class of cell-mediated reactions is effected by soluble factors collectively called 'lymphokines' rather than by direct cellular contact. In other words, immune T lymphocytes stimulated with specific antigen generate and release the biologically active soluble substances which can affect a variety of functions of target cells. These lymphocytes are known to belong to a subpopulation of T lymphocytes differing from cytotoxic lymphocytes; in mice for example, their cell surface markers are Ly 1^+ 2^- 3^-, instead of Ly 1^- 2^+ 3^+.

Table 1.1 Target cells of lymphokines

Inflammatory (efferent) lymphokines
Macrophages (monocytes)
Neutrophils
Eosinophils
Basophils
Lymphocytes, T and B
Fibroblasts
Endothelial cells
Platelets
Immunoregulatory (afferent) lymphokines
Lymphocytes, T and B
Macrophages (monocytes)

As summarized in Table 1.1, lymphokines can exert their effects on various target cell types at several functional stages. Overall, the outcome of these effects is far-reaching, from the induction of chronic inflammation to the protection of the host against malignancy and the regulation of almost all aspects of immune responses. Most of the lymphokines are defined in terms of specific and complicated in vitro assays. Clinically one can employ any of these assays as described in subsequent chapters of this book to assess a specific immunological capability of the host. As discussed later, however, the extrapolation of such in vitro results to any in vivo situation is not simple, but requires cautious interpretation of the data.

Kinds of lymphokines

The lymphokines have been defined in terms of in vitro assays. Although more than 100 activities have been described, some of these may represent the same molecule showing different biological activities according to the in vitro assay employed (reviewed Yoshida, 1979). Even with recent technologies including hybridoma techniques, monoclonal antibodies and gene clonings, the identity of some

lymphokines still remains an enigma. For example, the question continues to be asked whether or not macrophage-activating factor(s) represent the same molecule as gamma-interferon, though the latter is most likely one of many heterogenous molecules with macrophage-activating activity.

Until we can classify lymphokines according to their molecular structures and identities, it is convenient to classify them on their functional characteristics. As already alluded to in Table 1.1 and shown more specifically in Table 1.2, lymphokines can be categorized into two major groups: inflammatory lymphokines which affect many functions of various cell types involved in inflammation and immunoregulatory lymphokines which can modulate immune responses through their effects mainly on lymphocytes and macrophages. Particularly in case of inflammatory lymphokines, most of them have names as a simple combination of both target cells (Table 1.1) and their functions affected (Table 1.2), such as macrophage migration inhibition factor (MIF), eosinophil chemotactic factor (ECF), fibroblast activation factor (FAF), macrophage growth factor (MGF), and so on. Inflammatory lymphokines may also be classified as efferent lymphokines and the immunoregulatory group of molecules as afferent lymphokines. Afferent lymphokines may recruit and stimulate lymphocytes to produce more efferent and afferent lymphokines, or may suppress lymphocytes to turn a reaction off.

Table 1.2 Cellular functions affected by lymphokines

Cellular functions	*Examples of lymphokines*
Motility	Migration inhibition factors
	Chemotactic factors
Proliferation	Mitogenic (or growth) factors
	Cytostatic and cytotoxic factors
Differentiation	Differentiation factors
Phagocytosis	Activation factors
Secretion	Stimulation factors
Immune functions	Helper factors
	Suppressor factors
	Growth and differentiation factors

Biochemical nature of lymphokines

Despite progress with the use of cell-hybridization techniques, monoclonal antibodies and gene clonings, only a few lymphokines have been chemically purified or well-defined according to their biochemical characteristics. In general, most lymphokines are proteins or glycoproteins and rather stable (a few days at 4°C, more than a year at −70°C, intact after heating at 56°C for 30 min). Molecular weights of most of them are around 25 000 to 80 000, rarely more than 100 000 or less than 10 000. Recent studies indicate the presence of small molecular species for MIF and/or macrophage chemotactic factor (MCF) of humans, and also suggest the existence of polymeric forms.

Lymphokine-producing cells, monokines and cytokines

As discussed above, cell-mediated immune reactions are manifestations of T lymphocyte function, and it is assumed that T cells are the source of all different kinds of lymphokines. However, we reported for the first time that B cells also could produce lymphokines under certain circumstances (Yoshida et al, 1973). Evidence for the association of cell-mediated immunity and T cell function is overwhelming. Part of the explanation for this apparent paradox may be in the difference in the requirements for lymphokine production by T cells and B cells. T cells can be activated by either specific antigen or mitogens, whereas B cells require polyclonal stimuli or mitogens for generation of lymphokines. Thus, T cells are still considered to be crucial for specific reaction with antigen for the production of lymphokines. An endogenous mitogenic factor (a lymphokine) from such T cells may trigger B cells to start producing extra lymphokines for acceleration and potentiation of cell-mediated immune reactions.

Besides lymphocytes, it has been shown that a variety of cell types can produce substances which have similar biological functions as lymphokines; some are reported to be biochemically and antigenically the same as corresponding lymphokines. When produced by monocytes or macrophages, they are called 'monokines'. Generalizing further, 'cytokines' represent products of other cell types such as fibroblasts and non-lymphoid tissue culture cell lines (reviewed by Ewan & Yoshida, 1979). Cytokine networks may be operating at the cellular level for the potentiation and modulation of the results of immune reactions, just as hormones do for various physiological functions in the body.

In vivo significance of lymphokines

Lymphokines are generally defined in terms of complex, semiquantitative in vitro biological assays, but it is not hard to imagine that they may be actively involved in the in vivo cell-mediated immune reactions, although data to support this contention have been surprisingly scanty.

In delayed type hypersensitivity skin reactions, several lymphokines have been extracted, including skin reactive factor (SRF) and macrophage chemotactic factor (MCF). Similarly, lymphokines such as MIF, MCF and interferon (IFN) have been recovered either from peritoneal cavity in the macrophage disappearance reaction (MDR) or from peripheral blood in the monocyte disappearance phenomenon (MDP). Both MDR and MDP are known to represent in vivo manifestations of cell-mediated immunity (Yoshida & Cohen, 1974). Recently, the lesion of hypersensitivity granulomas, yet another manifestation of cell-mediated immunity, was shown to contain many lymphokine-active materials including MIF, MCF and macrophage fusion factor (MFF), as well as an abundant amount of IL1 (monokine). Conversely it was shown that lymphokines produced in vitro could induce such in vivo reactions as DTH skin reaction, MDR, MDP and granuloma formation when injected appropriately into normal animals. Furthermore, if inhibitors of in vitro lymphokine activities are injected into immune animals, the expression of cell-mediated immune reactions is suppressed significantly. This clearly indicates that lymphokines mediate a variety of in vivo manifestations of cell-mediated immunity.

Interestingly, it has been shown that the local manifestation of cell-mediated immune reactions such as DTH skin reaction is suppressed when a large quantity of

lymphokines are circulating in the peripheral blood. It was suggested that a feedback regulatory mechanism is operating in vivo to suppress the manifestation of the cell-mediated immunity (Yoshida & Cohen, 1982).

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2

Analysis of Human T Cells in Blood with Monoclonal Antibodies and Flow Cytometry

R. A. Moscicki, A. L. Luttinger and R. B. Colvin

T lymphocytes have discrete, apparently preprogrammed, functions in immune regulation. The consequences of T cell interaction with antigen include lymphokine production, cytotoxicity, and activation of other cell types such as B cells and macrophages. Functional specificity is not revealed by any unique morphological features. Specialization into 'T cell subsets' or functional phenotypes is manifested most clearly by their differential response to antigen in conjunction with Class I or Class II histocompatibility antigens, and by their expression of distinct differentiation antigens on the cell surface.

Techniques for the identification of individual T cells have advanced considerably since 1980, by the development of monoclonal antibodies to several of the functionally relevant differentiation antigens and the application of immunoperoxidase and immunofluorescence techniques. Flow cytometry has emerged from the basic immunology laboratory to become a practical clinical tool for the analysis of surface markers on cells in suspension. This review will concentrate on the methodology and interpretation of flow cytometric analysis of human T cell subsets in the blood. Step-by-step protocols are given in the Appendix. With appropriate antibodies, these techniques are applicable to other cells, other suspensions, and other species.

E ROSETTES

The first specific marker to be widely used for T cell identification was the E rosette receptor (Jondl, 1972). Human T cells incubated at 37°C with sheep erythrocytes (SRBC) form delicate rosettes, easily counted with a conventional light microscope, a simplicity that remains its greatest asset. The binding is mediated by a 50 kilodalton glycoprotein on the T cell surface which can be identified by monoclonal antibodies (OKT11, Leu-5, LFA-2).

The SRBC method is cumbersome, labour intensive and subject to many variables. The fraction of mononuclear cells in normal peripheral blood that form SRBC rosettes ranges from 72–93%, depending on the technique (Ross & Winchester, 1980). The binding requires metabolic activity, but is fragile and can be disrupted by prolonged incubation at 37°C or mechanical agitation. The sialic acid content of the red cell interferes with the attachment, and pretreatment with neuraminidase (Bianco et al 1971) or 2-aminoethylisothiouronium bromide (Pelle-

grino et al 1975) enhances rosette formation. Unfortunately, such treatment also increases the tendency of the red cells to bind to the monocytes. Use of SRBC stored less than 14 days is important (Bentwich et al 1973), as well as the addition of SRBC-absorbed AB or fetal calf serum to prevent disintegration, and sequential incubation at 37°C and 4°C. The assay requires prior separation of mononuclear cells, usually done with Ficoll-Hypaque. This necessarily leads to a variable contamination with monocytes, which dilute the E rosette values, expressed as a percentage of the recovered mononuclear cells with attached SRBC. Specific diseases may give anomalous results. A dysfunction of the SRBC receptor has been described in Hodgkins disease (Strober & Bobrove, 1975), and anti-lymphocyte antibodies in SLE or JRA can inhibit rosette formation (Winchester et al, 1974). Ficoll-Hypaque separation often results in low yields and high contamination when patients are on steroids, as is the case for renal transplant recipients. Ox erythrocytes, which do not bind to human T cells, have been used to measure IgC and IgM Fc receptors on T cells, which were originally thought to distinguish suppressor and helper T cell subsets (Moretts et al, 1977). A portion of the IgG binding by the mononuclear cells has been shown to be due to contaminating monocytes or NK cells, however, and the utility of this approach is limited (Reinherz et al, 1980).

While the rosette technique retains historic interest, from a practical standpoint, T cells can be more readily and accurately measured with monoclonal antibodies to the 50 kilodalton receptor molecule (Verbi et al, 1982), a point recently acknowledged by the FDA in their approval of OKT11 as a diagnostic reagent for the detection of T cells.

ANTIBODIES TO T CELL SURFACE MOLECULES

Polyclonal antibodies

Heteroantisera reactive to human T cells (antithymocyte globulin, ATG) have been raised in a variety of species and are currently used as immunosuppressive agents to treat renal allograft rejection, aplastic anemia, and other conditions (Russell et al, 1984). Their use as diagnostic reagents has been limited partly because of the difficult absorption steps required to obtain even small amounts of antibody specific for subsets of T cells. One such antibody, anti-TH2, reacts selectively with the suppressor subset of T cells (Reinherz & Schlossman, 1979), and helped encourage attempts to develop monoclonal antibodies to the same subset ($T8^+$ cells).

Complement-fixing IgG auto-antibodies have been detected in certain patients with systemic lupus erythematosus and juvenile rheumatoid arthritis (JRA) that recognize a surface antigen on 30–40% of normal blood T cells (Morimoto et al, 1981). JRA^+ cells include those able to proliferate in response to soluble antigen and able to induce suppressor function in other T cells, corresponding to a subpopulation of $T4^+$ cells defined with monoclonal antibodies. This auto-antibody is of interest primarily for the insight it provides into a pathogenetic mechanism of abnormal immune regulation.