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Research paper

The duration of in vitro stimulation with recall antigens determines the subset distribution of interferon-γ-producing lymphoid cells: A kinetic analysis using the Interferon-γ Secretion Assay™

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Abstract

Analyses of cellular immune responses during natural infections and following vaccination with established or candidate vaccines are becoming increasingly important and so are the research tools used to achieve this goal. During a recent evaluation of the analytical performance characteristics of one of these techniques, the interferon-γ secretion assay, we noticed that following overnight incubation of PBMC with recall antigens (varicella-zoster antigen, *Candida albicans* antigen or hepatitis B surface antigen) NK cells are frequently the most predominant interferon-γ-producing cell population. In this study, we monitored the subset distribution of interferon-γ-producing cells following more extended in vitro culture periods and found that, irrespective of the antigen applied, the contribution of NK cells decreased whereas the importance of T cells and NKT cells rose. Analysis of the subset distribution showed that HBsAg stimulated CD4 cells predominantly whereas *Candida* antigen and varicella-zoster antigen were better inducers of CD8 responses. No correlation was found between the kinetics of total number of interferon-γ-producing cells and the changes of concentrations of interferon-γ in the culture supernatants. Interferon-γ levels in culture supernatants correlated strongly with the kinetics of T_H lymphocytes ($CD3^+$, $CD4^+$), CTL ($CD3^+$, $CD8^+$), and NKT cells ($CD3^+$, $CD56^+$). These observations lead us to conclude that methods that enumerate cytokine-secreting cells without determining their phenotype should be interpreted with great care and that an 'elispot' should not be directly considered as the footprint of a T lymphocyte.

Keywords: Flow cytometry; Cytokine secretion; Kinetics; Vaccines; IFNγ; VZ Ag; HBsAg; T cell subsets

Abbreviations: BC, buffy coat; CTL, cytotoxic T lymphocyte; Elisa, enzyme-linked immunosorbent assay; Elispot, enzyme-linked immunospot; FACS, fluorescence activated cell sorter; HBsAg, hepatitis B surface antigen; IC, intracellular cytokine staining; IFNγ, interferon-γ; ISA, IFNγ secretion assay; LPA, lymphoproliferation assay; 2-ME, 2-mercapto-ethanol; moAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells; PI, propidium iodide; Th, T helper; VZ Ag, varicella-zoster virus antigen.

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1. Introduction

Most, if not all, existing vaccines that are efficient protect via 'protective antibodies.' It is becoming increasingly clear that for certain pathogens, protective antibodies cannot be induced by a vaccine or, when induced, will not suffice to convey protection against infection or disease. This holds in particular for human immunodeficiency virus (HIV) (Koup et al., 1994), hepatitis C virus (HCV) (Wodarz, 2003), *Chlamydia trachomatis* (Perry et al., 1997), *Plasmodium falciparum* (Nardin and Nussenzweig, 1993), and *Mycobacterium tuberculosis* (Stenger and Modlin, 1999). Although the immune correlates for protection against these pathogens remain undefined, it is generally accepted that successful vaccines against these agents will need to induce and maintain protective T cell responses in addition to 'protective antibodies.' Therefore, the development and clinical evaluation of vaccine candidates will largely depend on reliable tools to monitor the kinetics and magnitude of cellular immune responses.

Several techniques have been developed to detect and quantify pathogen-specific T cell responses. Taking advantage of the antigen-induced cytokine release by activated $CD4^+$ helper T (T_H) cells and $CD8^+$ cytotoxic T lymphocytes (CTL), the presence and activity of these cells can be monitored in different ways. The simplest but least informative assay consists of the quantification of cytokines (interferon-γ, Il-5, and others) in the supernatants of antigen-activated lymphocyte cultures using enzyme-linked immunosorbent assays (ELISAs). This method is unable to define the frequency and the phenotype of cytokine-producing cells. In the past decade, several methods have been developed to detect cytokine production at the single cell level and these are now widely used to study cellular immune responses induced by acute or chronic microbial infections (Byl et al., 1999; Badovinac and Harty, 2000; Asemissen et al., 2001; Karlsson et al., 2003; Listvanova et al., 2003) and prophylactic (Fernandez et al., 1994; Byl et al., 1999) or therapeutic vaccines (Scheibenbogen et al., 2000b, 2002; Banchereau et al., 2001; Walker and Disis, 2003). The most commonly used technique is the enzyme-linked immunospot or elispot assay which enables the direct visualization of single reactive cells by labelling of captured cytokine on a membrane surface of the culture well. Another technique, known as the cytokine secretion assay (commercialized by Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) immobilizes cytokines on the surface of the secreting cell using bi-specific antibodies that attach to the cell surface with one binding side and capture the cytokine with the other. Subsequently, the immobilized cytokine is labelled for flow cytometric analysis with a fluorochrome-tagged, cytokine-specific antibody (Assenmacher et al., 1998; Brosterhus et al., 1999; Oelke et al., 2000). Intracellular cytokine staining is an alternative method that enables flow cytometric assessment of reactive cells (Andersen et al., 2000; Koehne et al., 2002). Flow cytometric methods allow visualization of single cells, the intensity of their cytokine production and their frequency within the examined cell suspension, and have the additional advantage of enabling phenotypic characterization of responding cells. These methods, however, are not yet adapted for routine analysis of numerous samples.

Recently, we noticed that in many subjects and, irrespective of the antigen applied in the overnight culture, the majority of interferon-γ-producing cells were NK cells (Desombere et al., 2004). To examine whether extended exposure to antigen changes the subset distribution of interferon-γ-producing cells, we performed the experiments described herein. We have monitored the responses of PBMC from different individuals against a variety of antigenic stimuli that relate to their 'natural' infections experienced and vaccines received. We have not only examined the responses following a short-term in vitro re-stimulation as is usually done, but we have also monitored the interferon-γ production of different lymphoid populations after extended incubation periods.

2. Material and methods

2.1. Isolation and freezing of human peripheral blood mononuclear cells (PBMC)

Buffy coats (BC) were obtained from the Blood Transfusion Center, Red Cross Flanders, Ghent, Belgium. PBMC were prepared by standard Ficoll-Isopaque (Lymphoprep™, Nycomed Pharma AS, Oslo,