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SCIENCE DIRECT

Clinical Immunology 117 (2005) 161 – 167

Clinical IMMUNOLOGY

www.elsevier.com/locate/yclim

Quantitative and qualitative assessment of circulating NY-ESO-1 specific CD4$^+$ T cells in cancer-free individuals

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Received 23 May 2005; accepted with revision 14 July 2005
Available online 15 August 2005

Abstract

The germ cell antigen NY-ESO-1 is characterized by its frequent expression in patients bearing cancers of various histological types, that positively correlates with stage of disease, together with its frequent spontaneous immunogenicity in patients with advanced cancer. Because of these features, NY-ESO-1 is presently viewed as a prototype antigen for the development of cancer vaccines aimed at preventing disease progression. To gain a global view of the CD4$^+$ T cell repertoire available for NY-ESO-1 in individuals of different genetic background, in this study, we have addressed the presence, frequency, and fine specificity of CD4$^+$ T cells reactive against NY-ESO-1-derived sequences among circulating lymphocytes from healthy donors. NY-ESO-1 specific CD4$^+$ T cells were present among circulating lymphocytes at a frequency between 0.5 and 5 precursors per million CD4$^+$ T cells. In the majority of the cases, the reactivity of NY-ESO-1 specific CD4$^+$ T cells was directed towards immunodominant regions located in the carboxyl-terminal half of the protein. Interestingly, immunodominant regions were confined to parts of the NY-ESO-1 protein containing hotspot sequences with predicted high binding for multiple frequently expressed MHC class II molecules. In contrast, no reactivity was found against the amino-terminal part of the protein, which was concomitant with the paucity, in this region, of sequences with predicted high binding to MHC class II molecules.

Keywords: Human; Tumor immunity; T cell epitopes; CD4$^+$ T cells

Introduction

The NY-ESO-1 antigen [1] is a member of the cancer/testis antigen family and one of the most spontaneously immunogenic tumor antigens described so far. In the adult testis, NY-ESO-1 is strongly expressed in spermatogonia and in primary spermatocytes, but not in post-meiotic cells or in testicular somatic cells, nor in other somatic tissues [2]. Frequent expression of NY-ESO-1 has been reported in tumor types among the most frequent in humans, including melanomas, sarcomas, prostate, and ovarian cancer and numerous others [1,3–5]. Interestingly, a growing body of evidence indicates that the expression of NY-ESO-1 increases with disease progression [3,6,7]. A significant fraction of patients bearing NY-ESO-1 expressing tumors develop specific and integrated immune responses to the antigen during the course of their disease [8,9]. These responses are more frequently found in the presence of relatively large tumor masses, often in advanced stages of the disease. In addition, the magnitude of these immune responses has been shown to correlate with the evolution of disease. For example, increase in the titer of naturally occurring NY-ESO-1 specific antibodies has been observed concomitantly with disease progression and decrease in association with regression [10]. It is conceivable that the induction of earlier NY-ESO-1 specific immune responses, than it naturally occurs, could impact on the evolution of the disease and possibly control disease progression.

Parallel single variable clinical trials assessing NY-ESO-1-based immunogens are currently under way at hospitals and medical centers around the world under the sponsor-

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doi:10.1016/j.clim.2005.07.004

ship of the Cancer Vaccine Collaborative (CVC, http://www.cancerresearch.org). The aim of these early phase trials is primarily to assess the immunogenic potential of different antigenic variants and formulations. The immunogens tested include NY-ESO-1-derived peptides, recombinant NY-ESO-1 encoding viruses, recombinant proteins, and others. The molecular monitoring of these trials, particularly when full-length antigens are used, is challenging and requires the use of appropriate methodologies that allow, as much as possible, the quantitative and qualitative evaluation of overall vaccine-induced responses in individuals of various genetic backgrounds. A prerequisite for the analysis of NY-ESO-1 specific T cell responses in patients with vaccine-induced immunity is the assessment of the complete NY-ESO-1 specific T cell repertoire available in humans. To gain insight into this repertoire, in this study, we have assessed the presence, frequency, and fine specificity of NY-ESO-1 specific $CD4^+$ T cells among circulating lymphocytes of individuals without cancer.

Materials and methods

Assessment of NY-ESO-1 specific $CD4^+$ T cells

Peripheral blood from healthy donors was obtained from the New York City Blood Bank. $CD4^+$ T cells were highly enriched (>90%) from PBMC by magnetic cell sorting using a miniMACS device (Miltenyi Biotec, Auburn, CA, USA) and stimulated with irradiated autologous antigen presenting cells from the $CD4^-$ fraction in the presence of a pool of partially overlapping peptides spanning the entire NY-ESO-1 protein sequence (2 μM each, NeoMPS Inc., San Diego, CA, USA). Culture medium for lymphocytes was IMDM (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 8% heat-inactivated pooled human serum (CTL medium), recombinant human (rh) IL-2 (10 IU/ml) and rhIL-7 (10 ng/ml). At day 8 after stimulation, cultures were tested for intracellular cytokine secretion using the same peptide pool. To this purpose T cells were stimulated in the absence or presence of the peptide pool during 4 h as described previously [11]. Brefeldin A (10 μg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added 1 h after the beginning of the incubation. After incubation, cells were stained with anti-CD4 mAb (BD Biosciences, San Jose, CA, USA) for 20 min at 4°C and fixed using formaldehyde, permeabilized with saponin (0.1% in PBS and 5% FCS; Sigma-Aldrich), stained with anti-IFN-γ mAb (BD Pharmingen, San Jose, CA, USA), and analyzed by flow cytometry. Data analysis was performed using Cell Quest software. The frequency (F) of NY-ESO-1 specific precursors was calculated as follows: $F = X/2^n$, where X is the number of cells producing IFN-γ in presence of the NY-ESO-1 peptide pool − the number of cells producing IFN-γ in absence of the NY-ESO-1 peptide pool and n is the number of days of culture. Based on previous experiments, the calculation was done taking into account an estimated division rate of one per day after TCR mediated stimulation of $CD4^+$ T cells in these culture conditions. $CD4^+$ T cells secreting IFN-γ in response to stimulation with the peptide pool were isolated by cytokine guided magnetic cell sorting using the cytokine secretion detection kit (Miltenyi Biotec) and stimulated in the presence of irradiated autologous feeder cells from the IFN-γ negative fraction and rhIL-2 (100 IU/ml). After in vitro expansion, aliquots of the derived cell lines were assessed for reactivity and fine specificity by stimulation with the NY-ESO-1 peptide pool or with single peptides. The concentration of IFN-γ in the supernatant of these cultures was assessed after 48 h by ELISA as described previously [11]. For the limiting dilution assay (LDA), highly enriched $CD4^+$ T cells were stimulated with the NY-ESO-1 peptide pool, at 10^5 lymphocytes/well in 96-well round bottom plates in medium containing rhIL-2 (10 IU/ml) and rhIL-7 (10 ng/ml) together with 10^5 irradiated autologous feeder cells from the $CD4^-$T cell fraction/well. A total of 19.2×10^6 $CD4^+$ T cells (i.e., 192 single cultures) were assessed for each donor. After 1 week, cultures were stimulated again with the NY-ESO-1 peptide pool and assessed 1 week later. To this purpose, cultures were first washed extensively. Each single culture was then divided in two and cultured in the presence or in the absence of the NY-ESO-1 peptide pool. IFN-γ was assessed in the supernatant of these cultures 24 h after stimulation. Cultures were scored as positive when the concentration of IFN-γ produced in the presence of the NY-ESO-1 peptide pool was 3 times higher than that obtained in the absence of the peptide pool. The frequency of NY-ESO-1 specific precursors was calculated using the Poisson distribution analysis [12] according to the following formula: Precursor frequency = −ln [(number of wells tested − number of positive wells) / number of wells tested] / number of $CD4^+$ T cells/well. According to Poisson statistics, in the cases in which <20% of the tested wells are positive, there is a 95% confidence that specific cells in a positive culture derive from a single precursor. In the cases for which >20% of cultures were positive, we performed a repeat experiment by assessing cells seeded at 2×10^4 $CD4^+$ T cells/well. In these experiments, the percentage of positive wells was always lower than 20%.

Results

Assessment of the frequency of NY-ESO-1 specific $CD4^+$ T cells among circulating lymphocytes of healthy donors and their isolation

To obtain a first evaluation of NY-ESO-1 specific $CD4^+$ T cells in cancer-free individuals, $CD4^+$ T cells from 6 healthy donors were highly enriched from circulating lymphocytes using magnetic cell sorting and stimulated