T-CELL RESPONSE PROFILING TO BIOLOGICAL THREAT AGENTS INCLUDING THE SARS CORONAVIRUS

C. GIOIA, D. HOREJSH[1], C. AGRATI, F. MARTINI, M.R. CAPOBIANCHI[1], G. IPPOLITO[2] and F. POCCIA

Unit of Cellular Immunology, [1]Laboratory of Virology, [2]Department of Epidemiology, National Institute for Infectious Diseases (I.N.M.I.) "Lazzaro Spallanzani" I.R.C.C.S., Rome, Italy

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The emergence of pathogens such as SARS and the increased threat of bioterrorism has stimulated the development of novel diagnostic assays for differential diagnosis. Rather than focusing on the detection of an individual pathogen component, we have developed a T cell profiling system to monitor responses to the pathogens in an array format. Using a matrix of antigens specific for different pathogens, a specific T cell profile was generated for each individual by monitoring the intracellular production of interferon-gamma by flow cytometry. This assay allows for the testing of multiple proteins or peptides at a single time and provides a quantitative and phenotypic assessment of CD4(+) and CD8(+) responding cells. We present profiling examples for several positive individuals, including those vaccinated with the smallpox and anthrax vaccines. We also show antigen optimization for the SARS-hCoV, as studies revealed that these proteins contain peptides which cross-react with more common coronaviruses, a cause of the common cold. The T cell array is an early and sensitive multiplex measure of active infection, exposure to a pathogen, or effective, recent vaccination.

With the continual emergence of new pathogens and the increased threat of bioterrorism attacks, the differential diagnosis or identification of etiological agents in infection is the first important step for controlling the diffusion of a disease. The SARS coronavirus (SARS-hCoV) tested the ability of the scientific community to develop methods to isolate, identify, and characterize an emerging virus. The anthrax scare after September 11th highlighted the need to develop assays that also detect the intentional release of pathogens intended to "terrorize" the public. The key to an effective public health response is the early diagnosis of infection and the identification of the biological agents to curb outbreaks and secondary spread of these diseases.

For the differential diagnosis of infection, there are several methods currently in use. Pathogen, isolation, PCR detection of nucleic acids, and antibody detection by ELISA, immunofluorescence, or neutralization have been used to confirm infection (1-3). Practical laboratory preparedness problems limit the use of isolation of infectious agents in many institutions, especially for high-risk pathogens. Therefore, more rapid a PCR-based and antibody detection methods have been developed as commercial products for diagnosis. During the acute phase of infection, PCR-based nucleic acid detection method may be the most effective, but the results are not always reliable due to individual variability, sampling time, and sample type (4). Antibody detection by ELISA is a more precise method and offers the advantage of detection in exposed, uninfected individuals, but an effective

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Mailing address: Dr. Douglas Horejsh
National Institute for Infectious Diseases "L. Spallanzani", I.R.C.C.S., Via Portuense, 292
00149 Rome, ITALY
Tel +39 0655170907 - FAX +39 0655170918
e-mail: horejsh@inmi.it

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antibody response can take weeks to develop (the SARS-hCoV exclusion criteria used by the CDC suggests that this response can take up to 28 days to develop).

The optimal assay should incorporate the detection at an earlier time point, with the sensitivity of an immune-based assay to detect exposed individuals. In the absence of a detectable serology, antigen-specific T cell responses could be detected in exposed, but uninfected persons, as shown for HIV and HCV contacts (5-6). Also, the initial development of delayed hypersensitivity, an index of cell-mediated immunity, occurs as early as two days after a smallpox vaccination (7). Thus, monitoring a T cell response profile to a diverse panel of antigens may allow an earlier identification of the infecting agent. In addition, this assay also may be used to testassa the robustness of specific immunity after vaccination (8). We evaluated the feasibility of an easy, rapid, and sensitive assay to monitor T cell responses to a composite-diverse panel of pathogens.

MATERIALS AND METHODS

Antigens

Antigen preparations are fully described in Table I, including the commercial or academic source and the quantity used in these analyses.

Antibodies

Unconjugated mouse-anti-human CD28 (IgG1, clone CD28.2); unconjugated mouse-anti-human CD49d (IgG1, clone 9F10); fluorescein (FITC)-conjugated mouse-anti-human IFN (IgG1, clone B27); PE-cyanine-5 (Cy-5)-conjugated mouse-anti-human CD3 (IgG1, clone RPA-T3); AlloPhycoCyanin (APhC)-conjugated mouse-anti-human CD8 (IgG1, clone RPA-T8) monoclonal antibodies, and FITC-conjugated IgG1 isotype-matched control (IgG1 clone MOPC-21) were obtained from Becton Dickinson Immunocytometry Systems (San Jose, CA).

Cell stimulation

Peripheral blood mononuclear cells (PBMC) were obtained using standard Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation. Stimulation was performed as already described with minor modifications (8). $1x10^6$ freshly or $1x10^6$ lived thawed PBMC in 1 ml of complete RPMI 1640, 10% v/v heat-inactivated FCS, 2 mM L-Glutamine, 10 U/ml penicillin/streptomycin, were incubated with 1 μg each of anti-CD28 and CD49d monoclonal antibodies and the antigenic preparations listed in Table I. To control the spontaneous cytokine production, cells incubated with only anti-CD28 and -CD49d were included in each experiment. The IFN-γ release induced by PMA (50 ng/ml) plus ionomycin (10 mg/ml) was used as a positive control. The cultures were incubated at 37 °C in a 5% CO_2 incubator for 1 h, followed by an additional 5 hours of incubation with 10 μg/ml of the secretion inhibitor Brefeldin-A (Sigma, St. Louis, MO).

Immunofluorescent staining

Antigen-stimulated and control cultures were washed in cold Dulbecco's phosphate-buffered saline (dPBS) containing 1% bovine serum albumin and 0.1% sodium azide. Cells were washed twice in PBS, 1% BSA, and 0.1% sodium azide, and then stained with monoclonal antibodies specific for the membrane antigens described above for 15 min at 4°C. Samples were fixed in 1% paraformaldehyde for 10 min at 4°C, incubated with anti-interferon (IFN)-γ monoclonal antibody diluted in PBS 1X, BSA 1% and saponin 0.5%. The cells were finally washed twice in PBS 1X, BSA 1%, 0.1% saponin and resuspended in FACS FLOW prior to acquisition using a FACScalibur cytometer (Becton Dickinson). Controls for non-specific staining were monitored using an isotype-matched monoclonal antibody and non-specific staining was always subtracted from specific results.

Flow cytometric analysis

Six-parameter flow cytometric analysis was performed on a FACScalibur flow cytometer (Becton Dickinson Immunocytometry Systems), using FITC, PECy-5 and APhC as the fluorescent parameters. At least 100,000 live events were acquired, gated on small viable lymphocytes. Data files were analyzed using CellQuest software (Becton Dickinson). Data were compiled in a Microsoft Excel spreadsheet (Microsoft Corporation, Seattle, WA) for array analyses.

Statistical analysis

Grouped T cell response data are presented as means ± standard deviations (SD) of the mean.

RESULTS

T cell response profiling of individuals using the antigen array

A T cell response profile was developed for several individuals (Fig. 1a). There was a wide individual variability, but sample duplicates confirmed specificity. A marked, specific response to CMV antigens was seen in each of the healthy

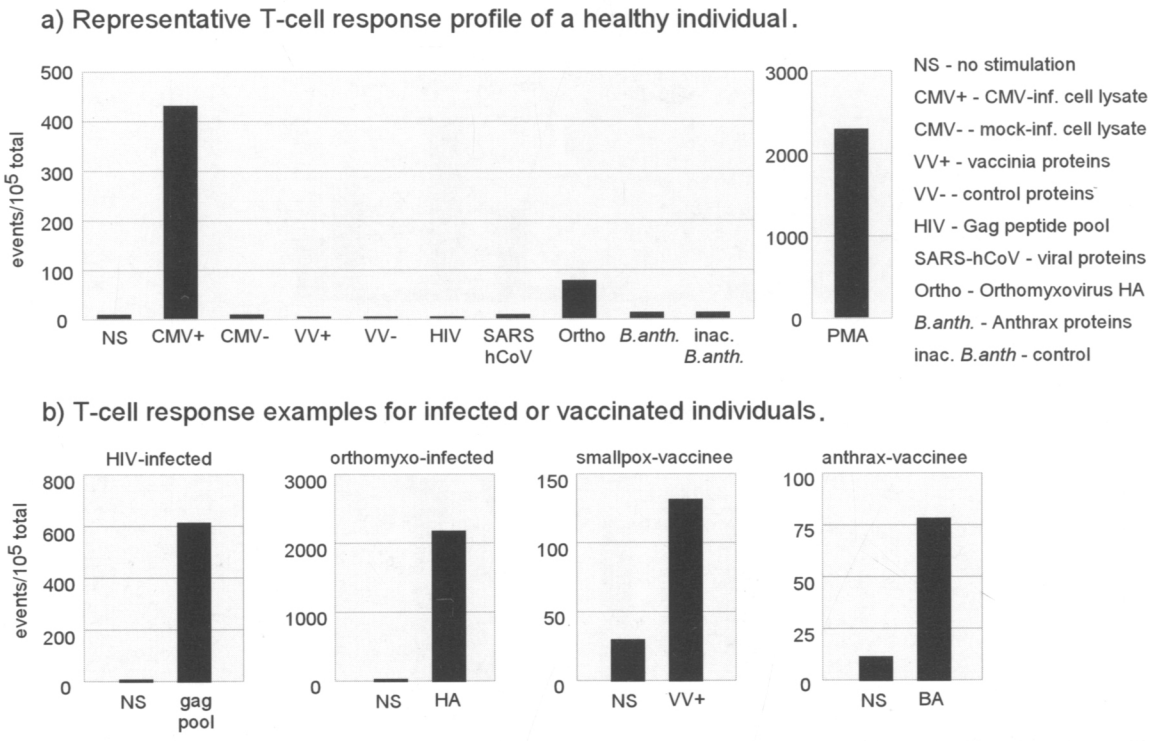

Fig. 1. *T cell response profiling measures recent infection or vaccination response. a) The T cell response profile of this representative, healthy individual indicates exposure to orthomyxovirus and CMV. Non-stimulated and PMA-stimulated controls are shown at the typically expected levels. b) The T cell responses of infected or vaccinated individuals are positive for the expected, corresponding pathogen.*

donor panels. In several individuals, a response was also detected to influenza virus, albeit at low levels in subjects neither recently vaccinated, nor recently infected. Neither of these results were unexpected, as the prevalence of sero-positivity for CMV in Italy is quite high and the response levels were expected to vary, depending on the individual. Also, influenza virus is recurrent on a yearly basis, so virtually all individuals have been exposed to this virus and respond to various strains due to antigenic sin. Pathogen-infected or recently vaccinated individuals were used as controls to confirm the reactivity of the antigen mixes for the response panel. As shown in Fig. 1b, a robust response was observed in representative examples of infected or vaccinated individuals for their respective pathogens.

Development of the SARS-CoV antigen for the antigen mix

A small, but reproducible response was seen to the recombinant SARS CoV protein pool in a number of healthy donors (Fig. 2a). The SARS-CoV epitopes in our preparation are not unique to group IV coronavirus, but are instead conserved among the other classes of coronaviruses that cause the common cold or gastroenteritis (Fig. 2b). It is, therefore, not unexpected that the recombinant proteins for SARS-CoV E and N2 contain cross-reactive epitopes, as these proteins stimulated a response above background in donors not exposed to the SARS CoV (9). Peptide design and optimization will further tune the SARS-CoV component of the assay as more reactive peptides are defined as being specific for SARS-CoV without cross-reaction to the more common and less dangerous coronaviruses.

DISCUSSION

An important aspect in diagnostic assay development is the availability of a rapid and easily automated system. that works on virtually all persons who carry the disease.Cultivation of high-risk pathogens is impractical for biosafety and/or technical reasons in the routine laboratory. Also, early detection using PCR or pathogen protein ELISA can be difficult due to individual host and pathogen acute phase variability. Host antibody detection

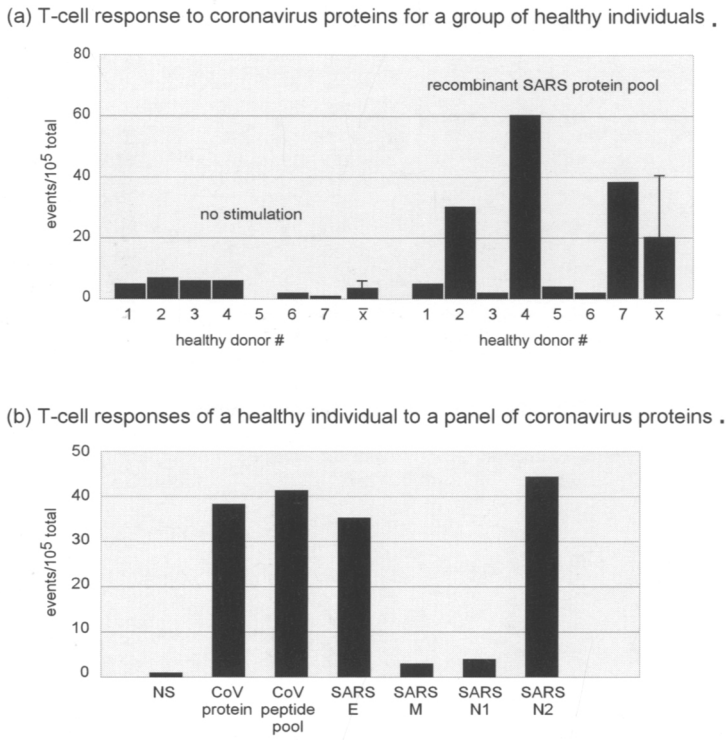

Fig. 2. *Cross-reaction between phylogenetically-defined classes is identified through the use of different coronavirus antigens. a) The T cell response profile of seven healthy controls to coronavirus protein reveals the presence of cross-reactive epitopes. Column "x" is the mean of the group, with the indicated standard deviation. b) The T cell responses of a healthy individual to a panel of coronavirus proteins indicated that coronavirus proteins E and N2 contain epitopes that are conserved among coronaviruses. Coronavirus proteins that tend to be more class-specific were seen to be negative in the healthy donor group.*

gives the added advantage of detection in exposed, uninfected individuals, but the antibody response can take weeks to develop. As cell-mediated immunity is stimulated almost immediately after infection or vaccination, we chose to focus our detection methods on the immune cells that directly respond to the pathogen antigens. Intracellular T cell cytokine staining by flow cytometry presents several advantages compared with other techniques such as tetramer staining and ELISpot. In fact, flow cytometry allows for testing multiple antigens simultaneously in array format and provides at the same time a quantitative and phenotypic assessment of CD8(+) and CD4(+) responding T cells (10-11). We have now been able to show that it is possible to develop an array of these antigens to screen for exposure, infection, or effective vaccination by a given pathogen.

Moreover, optimization of the antigen preparations with peptide pools designed to be pathogen-specific, highly conserved, and independent of HLA haplotypes, may allow for the development of a second generation of more sensitive flow cytometric T cell assays, extending the possibility also to perform retrospective studies using cryopreserved samples (12). Accurate monitoring of these cells is crucial in differential diagnosis or in determining the effects of HIV therapy and vaccine efficacy. Using an intracellular cytokine staining based assay, we are able to directly quantify functional antigen-specific CD8+ T cells. This assay is highly reproducible, and can be performed using both fresh and cryopreserved peripheral blood cells. Importantly, this assay can be used to examine multiple peptide epitopes simultaneously, and can be designed to be independent of patient HLA haplotype. We find that when using mixes of multiple peptides, the CD8+ T cell response to the mixture is equivalent to the sum of the responses to the individual peptides contained therein. Although some patients sharing HLA alleles occasionally recognize common peptides, rarely are responses to those peptides dominant within the same group of patients. These results confirm our previous findings that the responses to single HIV-peptides are rarely representative of the entire HIV response.

The technique could be easily automated through the

Table I. *Antigenic preparations used in the T-cell response profile.*

Microbe	Antigens	Source	Amount/ml
CMV Ag (AD 169)	Infected Cell lysate	BioWhittaker (Walkersville, USA)	20µl (2µg)
CMV negative control	Control Ag	BioWhittaker (Walkersville, USA)	20µl (2µg)
Vaccinia Ag	Infected Cell extract	Main Biotechnology Service, Inc (Portland, USA)	10µl (dil 1:100)
Vaccinia negative control	Control Ag	Main Biotechnology Service, Inc (Portland, USA)	10µl (dil 1:100)
HIV-1	GAG Peptide Pool	Sigma-Genosys (Cambridge, UK)	1µl/pept (1µg/pept)
Bacillus anthracis	Toxin	Kindly provided by Dr. A. Fasanella (Bari, Italy)	20µl (5µg)
Coronavirus, SARS assoc., M Protein	Recombinant Protein	Biodesign International (Saco, USA)	10µl (2µg)
Coronavirus, SARS assoc., E Protein	Recombinant Protein	Biodesign International (Saco, USA)	10µl (2µg)
Coronavirus, SARS assoc., N Protein (aa.1-49)	Recombinant Protein	Biodesign International (Saco, USA)	10µl (2µg)
Coronavirus, SARS assoc., N Protein (aa.192-220)	Recombinant Protein	Biodesign International (Saco, USA)	10µl (2µg)
Coronavirus	Peptide Pool	Adaltis (Montreal, Canada)	10µl (2µg)
Orthomyxovirus	Viral lysate	L. Spallanzani - Virology Lab	10µl (dil 1:100)

use of analytical instruments already available in most clinical laboratories (13) that use flow cytometry. The recent availability of mobile flow cytometer units may even allow use of this assay for field diagnostic and epidemiologic investigation (14). More T cell response panels are being completed on healthy, vaccinated, or infected subjects to continue our evaluation and development of this assay.

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