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Multiplexed infectious protein microarray immunoassay suitable for the study of the specificity of monoclonal immunoglobulins

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ABSTRACT

Enzyme-linked immunosorbent assays (ELISAs) used to detect antibodies specific for common infectious agents such as Epstein–Barr virus (EBV), cytomegalovirus (CMV), *Toxoplasma gondii (T. gondii),* and hepatitis C virus (HCV) are time-consuming and require large volumes of samples, which restrict their use. We propose a new assay based on a multiplexed infectious protein (MIP) microarray combining different epitopes representative of the four germs. Antigens and lysates were printed on nitrocellulose slides to constitute the microarray. First, the microarray was incubated with human serum samples. Then, the suitability of the microarray for analysis of the specificity of purified monoclonal immunoglobulin (mc Ig) was assessed using serum and mc Ig of HCV-positive patients. Bound human immunoglobulin G (IgG) was detected using fluorescently labeled secondary antibodies, and the signals were quantified. Results obtained in serum samples with the new MIP microarray immunoassay were compared with ELISAs; we observed concordances of 95% for EBV, 93% for CMV, 91% for *T. gondii,* and 100% for HCV. Regarding purified mc Ig of HCV-positive patients, 3 of 3 recognized antigens printed on the microarray. Hence, the novel EBV/CMV/*T. gondii*/HCV MIP microarray allows simultaneous diagnosis of polyclonal and monoclonal immune response to infectious diseases using very small volume samples.

Protein microarrays share methodological characteristics with other arrays such as robotic printing of probe targets in an indexed fashion, "hybridization" with samples of interest, and detection of interaction events by high-resolution scanning and image analysis [1]. This technology offers important tools for proteomic studies, and several applications based on antigen–antibody interactions have been described recently [2–5]. Protein microarrays are a particular format that allows the study of epitope–antibody interaction in a miniaturized and highly multiplexed fashion [6]. Microarrays allow high-throughput disease diagnosis [7,8]. In addition, the highly sensitive microarray technology facilitates detection of antibodies specific for antigens from viruses or bacteria, and they require only small volumes of samples. Protein microarrays are easy to handle and store, and they were shown to yield high-quality and reproducible results [9–12]. Thus, protein microarrays are suitable for rapid and cost-effective assessment of large series of small volume samples.

Over the past years, the development of protein microarrays has offered multiple applications for analysis of serum and other biological fluids in different contexts ranging from biochemistry, with analysis of protein–protein interaction, cancer profiling, and infectious diseases, with the detection of various microorganisms, to toxicology, with the detection of toxins and drugs of abuse [13–18]. Regarding infectious diseases, the serological status of patients for a list of infectious agents is usually determined using enzyme-linked immunosorbent assays (ELISAs).[1] Most of these tests are performed routinely in diagnostic laboratories using robotics. To obtain the serological status for a given germ, it is necessary to dispose of at

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[1] *Abbreviations used:* ELISA, enzyme-linked immunosorbent assay; MIP, multiplexed infectious protein; EBV, Epstein–Barr virus; CMV, cytomegalovirus; HCV, hepatitis C virus; mc Ig, monoclonal immunoglobulin; MGUS, monoclonal gammapathy of undetermined significance; MM, multiple myeloma; IgG, immunoglobulin G; VCA, viral capsid antigen; EBNA, EBV nuclear antigen; PBS, phosphate-buffered saline; BSA, bovine serum albumin; T-PBS, PBS and Tween 20; GST, glutathione *S*-transferase; FLU, fluorescence intensity; VZV, varicella–zoster virus.

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least 150 μl of serum per germ to be multiplied by the number of germs of interest. To minimize this constraint, we developed a new multiplexed infectious protein (MIP) microarray assay. The MIP microarray allows simultaneous determination of the serological status of patients against four common infectious agents: Epstein–Barr virus (EBV), cytomegalovirus (CMV), the parasite *Toxoplasma gondii (T. gondii),* and hepatitis C virus (HCV). The EBV/CMV/*T. gondii*/HCV MIP microarray would be useful in case of suspicion of infection before and during the follow-up of organ or bone marrow transplantation, in autoimmune diseases, in aplastic anemia, and to determine the serological status of patients with solid tumors or hematological malignancies. The main advantage is the multiplexed serological status determination combined with the drastic reduction of sample volume required, that is, only 1 to 3 μl of serum compared with at least 150 μl in classical ELISA techniques. The clinical performance of the MIP microarray assay was validated with human sera previously characterized with ELISAs for their reactivity against antigens and/or lysates from the four germs. Furthermore, the suitability of the MIP microarray for the study of the specificity of purified monoclonal immunoglobulin (mc Ig) from patients with monoclonal gammapathy of undetermined significance (MGUS) or multiple myeloma (MM) was also assessed.

Materials and methods

Serum samples

The study was performed on a panel of 70 human sera, provided by the Virology, Bacteriology, and Parasitology laboratories of the University Hospital of Nantes, and obtained from a heterogeneous group constituted of a majority of hospitalized patients and a few outpatients, including 34 females 36 males of ages ranging from 7 to 73 years ($M = 49.3 \pm 11.9$) and for whom one or several serological analyses had been prescribed. With consent, samples of venous blood were collected without anticoagulant. After coagulation, blood samples were centrifuged at 3500 rpm for 15 min at 4 °C, and sera were collected and stored at −20 °C until analysis.

Determination of serological status

Serological status for one or several pathogens, including EBV, CMV, *T. gondii,* and HCV, was determined according to the clinician's prescription using classical ELISAs, as described below.

EBV serological status was determined by quantitative sandwich chemiluminescent ELISA on a DiaSorin Liaison analyzer using the Liaison EBNA immunoglobulin G (IgG) kit (cat. no. 310520) and the Liaison VCA (viral capsid antigen) IgG kit (cat. no. 310510). These assays detect antibodies directed against EBV nuclear antigen (EBNA) and antibodies against the VCA as described in Table 1. The secondary antibody is a mouse monoclonal antibody conjugated with isoluminol, directed against human IgG.

CMV serological status was determined by quantitative sandwich chemiluminescent ELISA on a DiaSorin Liaison analyzer using the Liaison CMV IgG kit (cat. no. 310740). This assay detects antibodies directed against human CMV (Table 1). The secondary antibody is a mouse monoclonal antibody conjugated with isoluminol, directed against human IgG.

Determination of serological status against *T. gondii* was performed using quantitative ELISA on an AxSYM System from Abbott. This assay detects IgG directed against the whole tachyzoïte *T. gondii* (Table 1). The secondary antibody is an anti-human IgG conjugated with alkaline phosphatase; revelation is done by the addition of 4-methylumbelliferyl phosphate.

HCV serological status was determined by chemiluminescent ELISA on an Architect Abbott analyzer using the Abbott anti-HCV kit (cat. no. 6C37). This assay detects antibodies directed against structural (core protein) and nonstructural (NS-3 and NS-4) proteins of HCV, as described in Table 1.

Table 1
Antigens used in ELISA and antigens used to perform the MIP assay.

		ELISA	MIP microarray
EBV	EBNA	Synthetic peptide EBNA 1	Recombinant protein EBNA 1
	VCA	VCA p18 (BFRF3)	VCA p23 (BLRF2) VCA p23 (BLRF2) aa 1–162
CMV		Human CMV inactivated AD169 strain	Purified viral lysate from CMV AD169 strain aa 297–510 of pp65 (UL83) pp28 (UL99) pp52 (UL44) glycoprotein B pp38 (UL80a)
T. gondii		Whole tachyzoïtes	Purified tachyzoïtes lysate P24 protein (GRA1)
HCV	Core protein NS-3 NS-4	aa 1–150 aa 1192–1457 aa 1569–1931	aa 1–119 aa 1192–1459 aa 1691–1710 aa 1712–1733 aa 1921–1940 From genotypes1, 2, 3, and 5

Determination of IgG concentration

The IgG concentration of each serum sample was determined with an immuno-nephelemetric assay performed on a Beckman Immage Analyzer. Then, for each serum, IgG concentrations were adjusted from 12.5 to 800 μg/ml in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) and 0.1% Tween 20 (T-PBS) for further use on protein microarray (80 μl/incubation pad). Purification of mc IgG from MGUS and MM patients was performed as described previously [19,20]. IgG concentrations in eluates of purified mc Ig were determined using the same immuno-nephelemetric assay.

Design of the multiplexed protein microarray

Selected antigens and lysates

Antigens were supplied by Abcam (Cambridge, UK), Advanced Biotechnologies (Columbia, MD, USA), and Virogen (Watertown, MA, USA). Lysates were supplied by Advanced Biotechnologies.

For EBV, the three antigens used were VCA p23 (cat. no. ab43145, Abcam), p23 region 1-162aa (cat. no. 00211-V, Virogen), and EBNA recombinant protein EBNA-1 (cat. no. 10-523-001, Advanced Biotechnologies). For CMV, a mixture of five antigens was used: region 297-510 of CMV pp65 IE (cat. no. ab54103, Abcam), CMV pp28 (UL99) immunodominant region (cat. no. ab43038, Abcam), CMV pp52 (UL44) immunodominant region (cat. no. ab43044, Abcam), glycoprotein B immunodominant region (cat. no. ab43040, Abcam), and CMV pp38 (UL80a) immunodominant region (cat. no. ab73042, Abcam) as well as a purified viral lysate (cat. no. 10-144-000, Advanced Biotechnologies). For *T. gondii,* we used one antigen, p24 (GRA1) protein (cat. no. ab43137, Abcam), and a purified tachyzoïte lysate (cat. no. 10-279-001, Advanced Biotechnologies). For HCV, three antigens were used: core protein composed of 119 amino acids (aa 1–119) (cat. no. ab49015, Abcam), NS-3 protein recombinant fragment subtype 1c (aa 1192–1459) (cat. no. ab91395, Abcam), and NS-4 recombinant mosaic protein containing the HCV NS-4 immunodominant regions aa1691-1710, aa1712-1733, and aa1921-1940 from genotypes 1, 2, 3, and 5 (cat. no. ab49027, Abcam). It should be noted that some