derived from population samples containing individuals with ID. These findings together with earlier reports imply that there are grounds as well as means for improving the sensitivity of laboratory tests to diagnose ID. This is of clinical importance in the elderly, in whom ID often heralds severe underlying diseases, such as ulcers or malignancies. In practice, this should motivate the production and use of iron-replete reference values of sTfR and other markers of iron status.

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References

Analytical Requirements for Measuring Monocytic Human Lymphocyte Antigen DR by Flow Cytometry: Application to the Monitoring of Patients with Septic Shock, Guillaume Monneret,* Nadia Elmenkouri, Julien Bohe, Anne-Lise Debard, Marie-Claude Gutowski, Jacques Bienvenu, and Alain Lepape (Immunology Laboratory and Intensive Care Units, Lyon-Sud University Hospital, 69495 Pierre-Bénite, France; * address correspondence to this author at: Flow Cytometry Unit, Immunology Laboratory, Lyon-Sud University Hospital, 69495 Pierre-Bénite, France; fax 33-4-7886-3344, e-mail guillaume.monneret@chu-lyon.fr)

The concept of immunoparalysis has recently been proposed for explaining the failure of 20 years of clinical trials using antiinflammatory drugs in sepsis (1–3). Immunoparalysis is characterized mainly by the paralysis of monocytic functions. In particular, because of decreased expression of HLA-DR, antigen-presenting capacity cannot yet be considered a standardized tool, and many variables must be taken into account for ensuring the technical quality of results (9). This is especially required.
in clinical research when clinicians and immunologists are assessing the potential value of a new marker, as is presently the case for monitoring septic patients, and it is particularly true for the determination of HLA-DR, which is considered a rapidly up- or down-regulated marker (5, 10). The present study was designed to determine whether different protocol procedures could lead to discrepant results for HLA-DR measurements. After establishing a reliable protocol, the second objective was to demonstrate immunoparalysis in monitoring patients with septic shock.

Samples of peripheral blood were collected in EDTA anticoagulant tubes. Staining was performed on whole blood using PC5-labeled CD45, fluorescein isothiocyanate (FITC)-labeled CD14 (Immunotech), and phycoerythrin-labeled HLA-DR (clone L243; Becton Dickinson). Samples were lysed manually by use of FACS lysing solution (Becton Dickinson) or the automated Q-Prep system (Beckman-Coulter). Cells were analyzed on a Coulter EPICS XL flow cytometer (System II software; Beckman-Coulter). After debris was excluded by means of a leukogate (11), monocytes were gated out from other cells on the basis of labeling with FITC-CD14 according to the latest consensus conference (12). All results are expressed either as percentages of HLA-DR-positive monocytes in the total monocyte population (a threshold was defined with the isotype control) or as the mean of fluorescence intensities (MFI) related to the entire monocyte population. Results are presented as the mean ± SE. The Wilcoxon matched-pairs test and the Mann–Whitney test were used for the statistical study.

The patient group consisted of patients with septic shock. We used the diagnostic criteria for septic shock of the American College of Chest Physicians/Society of Critical Care Medicine consensus conference (13). Because we focused our interest on the secondary stage (immunoparalysis) of septic shock, we excluded patients who did not survive more than 48 h after being admitted to intensive care units. Severity was assessed by the Simplified Acute Physiologic Score II (SAPS II) (14). Mortality was defined as death occurring within 28 days after diagnosis. To provide a panel of reference values, we also included 58 apparently healthy individuals from our laboratory.

Regarding the expression of results as a percentage of HLA-DR-positive monocytes or as MFI, we rapidly noticed that these two values, although correlated, could provide different information. It remains to be demonstrated which of these two results will better correlate with clinical resolution or improved survival. Furthermore, MFI results provided the opportunity to convert data as numbers of HLA-DR sites per monocyte by use of calibrated beads, which constitutes a first approach to standardizing results. Because using MFI implies working with antibodies at saturating concentrations, we had to ensure this point. We tested serial dilutions of the antibody directed against HLA-DR. As hypothesized, dilutions led to artificially lower results. We statistically confirmed these data in eight healthy donors by comparing the two highest concentrations of antibodies (1/1 vs 1/2). Regarding the percentages of HLA-DR-positive monocytes, we found no difference. In contrast, significantly different MFI results (17 ± 4 vs 23 ± 1 MFI; P < 0.01) supported the fact that antibodies must be used at saturating concentrations (20 μL/100 μL of whole blood) when expressing results as MFI.

Because HLA-DR is a marker that is rapidly regulated, the storage temperature before staining may be an important issue. We compared results from fresh whole blood and those obtained after storage for 24 h at 4°C. In healthy donors, storage significantly increased MFI results (Table 1). In septic patients, both the percentage values and MFI were increased after storage (Table 1). We also stored blood from five healthy donors at 37°C for 1 h and found a dramatic increase in the HLA-DR values measured for each sample, which supports a rapid non-specific in vitro modulation of this expression (Table 1). Finally, we investigated the stability of HLA-DR expression at room temperature in five healthy patients (Table 1). Our results indicated that samples should be stained within 1 h after sampling. We also demonstrated that temperature variations during staining led to discrepant results (data not shown). Thus, we chose to work as recommended by the manufacturer (i.e., at room temperature).

To ensure that the lysis step did not induce an analytical bias, because different lysis systems are used in multicenter studies, we compared an automated lysis system to a manual reagent. We found discrepant results (data not shown). Therefore, we chose to work as recommended by the manufacturer (i.e., at room temperature).

Table 1. Effect of storage temperature before staining. a

<table>
<thead>
<tr>
<th>Expression, b %</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy donors (n = 10)</td>
<td></td>
</tr>
<tr>
<td>Fresh blood</td>
<td>90 ± 6 (77–97)</td>
</tr>
<tr>
<td>After 24 h at 4 °C</td>
<td>89 ± 7 (76–98)</td>
</tr>
<tr>
<td>Septic patients (n = 10)</td>
<td></td>
</tr>
<tr>
<td>Fresh blood</td>
<td>37 ± 19 (5–63)</td>
</tr>
<tr>
<td>After 24 h at 4 °C</td>
<td>48 ± 19 (30–84)c</td>
</tr>
<tr>
<td>Healthy donors (n = 5)</td>
<td></td>
</tr>
<tr>
<td>Fresh blood</td>
<td>82 ± 8 (74–90)</td>
</tr>
<tr>
<td>After 1 h at 37 °C</td>
<td>95 ± 2 (93–96)d</td>
</tr>
<tr>
<td>Healthy donors (n = 5)</td>
<td></td>
</tr>
<tr>
<td>Fresh blood</td>
<td>85 ± 6 (77–91)</td>
</tr>
<tr>
<td>After storage at room temperature</td>
<td></td>
</tr>
<tr>
<td>1 h</td>
<td>87 ± 6 (79–94)</td>
</tr>
<tr>
<td>2 h</td>
<td>93 ± 4 (89–96)d</td>
</tr>
<tr>
<td>3 h</td>
<td>93 ± 2 (91–93)d</td>
</tr>
<tr>
<td>4 h</td>
<td>92 ± 2 (91–93)d</td>
</tr>
</tbody>
</table>

a Blood was either immediately stained at room temperature for 30 min or after storage for 24 h at 4 °C, after storage for 1 h at 37 °C, or after storage for 1–4 h at room temperature.

b Percentage of monocytes expressing HLA-DR.

c,d Versus fresh blood (Wilcoxon paired test): c P < 0.01; d P < 0.05.
protocol usable for a multicenter study, we used the manual lysis procedure, as automated lysis systems are not available worldwide. In many hospitals, flow cytometers are usually not available 24 h a day for rapid measurement. An alternative is to immediately stain and fix cells after sampling and analyze them afterward. We verified this procedure by analyzing 25 samples just after staining and after 24 h at 4 °C and found no discrepant results.

On the basis of our results, we defined the most reliable protocol for measuring monocytic HLA-DR as follows. Fresh EDTA whole blood is immediately stained at room temperature for 30 min. Samples are lysed with the FACS lysing solution (Becton Dickinson) and immediately analyzed. After debris is excluded by means of a leukogate, monocytes are gated out from other cells on the basis of labeling with FITC-CD14. Results are expressed as the percentage of monocytes expressing HLA-DR (a threshold is defined with the isotype control) and as MFI.

We also performed two sets of analyses to study intraassay precision: one sample stained once and analyzed 10 times or one sample stained 10 times and analyzed 10 times. The intraassay CVs ranged from 3.9% to 5.0% (percentage of HLA-DR-positive monocytes) and from 2.0% to 2.3% (MFI).

The present study confirmed that HLA-DR is a marker whose determination is extremely dependent on analytical procedures. Consequently, a common protocol should be defined to make data from different centers as comparable as possible. The elapsed time between blood collection and staining constitutes a critical step: we observed a rapid nonspecific increase in measured HLA-DR values after sampling. Our precision results also illustrate that despite critical steps in the measurement, accurate determination of HLA-DR on monocytes allows day-to-day comparisons. When MFI and calibrated beards for fluorescence quantification are used, results become comparable between different laboratories.

For our clinical findings, we studied 17 patients with septic shock (mean age, 58 years; 13 males and 4 females) with a mean SAPS II admission score of 48 (range, 32–89) and a global mortality of 47%. We also included 58 apparently healthy donors (age range, 25–65 years). At 48 h after admission, the expression of HLA-DR on monocytes was severely and significantly reduced in septic patients (25% ± 4%; MFI value, 6 ± 1) compared with healthy donors (89% ± 1%; MFI value, 35 ± 3) without overlap. When survivors and nonsurvivors were compared, there was no difference in HLA-DR monocytic expression at 48 h. However, HLA-DR concentrations 5 days after admission were significantly higher values in survivors, suggesting an ongoing recovery of their immunologic status (Fig. 1).

In clinical reports, poor prognosis is associated with lower expression of HLA-DR on monocytes in the first days after admission, and it has been suggested that poor outcome might be attributable to this monocyte deactivation (6–8). The present study confirms and extends these data. We report that 5 days after admission, the persistence of a very low concentration of monocytic HLA-DR (<40%) is associated with a fatal outcome. Clinical and experimental results suggest that patients presenting with a low monocytic HLA-DR concentration should receive therapy, such as interferon-γ, to stimulate immune function. However, despite a promising trial using this approach in 10 septic patients with <30% HLA-DR-positive monocytes (6), there is no definitive evidence that boosting the immune system can reduce mortality from sepsis in immunocompromised patients. A placebo-controlled multicenter trial based on monocytic HLA-DR concentrations is necessary. As we have shown in the present study, a critical issue in such a trial will be the capacity for each center to perform standardized measurements of HLA-DR.

We thank Justin Kingsley for greatly assisting in the preparation of this manuscript.

References


Fig. 1. Changes in HLA-DR expression on monocytes in patients with septic shock after admittance to the intensive care unit. Results are expressed as the mean ± SE (error bars) percentage of monocytes expressing HLA-DR (a threshold was defined with the isotype control) in patients who survived (n = 9) or died (n = 8). *P < 0.01 vs nonsurvivors (Mann–Whitney test). The mean (± SE) value for 58 healthy donors was 89% ± 1%.
Identification of Major CYP2C9 and CYP2C19 Polymorphisms by Fluorescence Resonance Energy Transfer Analysis, Jürgen Borlak and Thomas Thum (Fraunhofer Institute of Toxicology and Aerosol Research, Center of Drug Research and Medical Biotechnology, 30625 Hannover, Germany; * address correspondence to this author at: Fraunhofer Institute of Toxicology and Aerosol Research, Center of Drug Research and Medical Biotechnology, D-30625 Hannover, Germany; fax 49-511-5350-573, e-mail Borlak@ita.fhg.de)

CYP2C9 and CYP2C19 monoxygenases (EC 1.14.14.1) are responsible for the metabolism of a variety of drugs and other xenobiotics, including proton pump inhibitors, certain tricyclic antidepressants, barbiturates, beta-blockers, nonsteroidal anti-inflammatory drugs, warfarin, and others (1). More than 12 variants of CYP2C9 and CYP2C19 are known, some of which can be linked to altered drug metabolism and to potential severe side effects (2,3). CYP2C9*2 (430C→T), CYP2C9*3 (1075A→C), CYP2C19*2 (681G→A), CYP2C19*3 (636G→A), and CYP2C19*4 (1A→G) account for >90% of Caucasian poor-metabolizer alleles (nucleotide changes in parentheses after the allele) (4,5). The nucleotide changes in the CYP2C9*2 and CYP2C19*3 alleles lead to changes in the amino acid sequence (R144C for CYP2C9*2 and I359L for CYP2C9*3) and thus to decreased enzyme activity. In the case of CYP2C19*2, *3, and *4, the nucleotide changes lead to a splicing defect, stop codon, and GTG initiation codon, respectively, and therefore to a protein with no activity.

We developed a new assay based on fluorescence resonance energy transfer (FRET). We labeled oligonucleotides with donor and acceptor fluorophores for mutation detection and applied this assay to the LightCycler (Roche Diagnostics). Single base alterations can be identified on the basis of different melting temperatures (Tm), and we used this method to screen genotypes of healthy unrelated individuals from Southern Germany. We report a robust and swift genotyping assay to permit analysis of major CYP2C9 and CYP2C19 alleles within 60 min of blood collection for each allele. This assay can be used in routine clinical practice to provide guidance for dose adjustments of drugs metabolized by CYP2C9/19.

We examined 189 healthy males and females from a Human Pharmacology Unit for participation in various clinical research trials. After giving written informed consent, these individuals were genotyped for CYP2C9*2, CYP2C9*3, CYP2C19*2, CYP2C19*3, and CYP2C19*4. DNA from whole blood was isolated using the NucleoSpin Blood DNA Extraction Kit (Macherey-Nagel) according to the manufacturer’s instructions.

CYP2C9 alleles were genotyped as follows. The fluorogenic adjacent hybridization probes were obtained from TIB-MOLBIOL. The sequences for the various PCR oligonucleotides are shown in Table 1. Hybridization probes were designed in such a way that their Tm, were marginally higher than the Tm, of the primers. The sensor probes of the CYP2C9*2 and CYP2C9*3 alleles were labeled with fluorescein at the 3′ end, and the anchor probes were coupled with LightCycler Red 640 (CYP2C9*2) or LightCycler Red 705 (CYP2C9*3) at the 5′ end (see Table 1). Each of the corresponding probes recognized adjacent sequences, with the shorter probe lying over the mutation site, and probes were separated by one base. Fluorescein was used as the donor fluorophore and blocked extension from the probe during PCR. LightCycler Red 640 and LightCycler Red 705 were used as acceptors in the FRET process, with the 3′ ends of the anchor probes phosphorylated to block extension. The greater stability of the longer anchor probe meant that loss of fluorescence occurred as the shorter probe (sensor) melted off the template. The probes were designed such that the two different mutation sites could be detected simultaneously (duplex PCR).

PCR was performed with 100 nM CYP2C9 primers (see Table 1) in a standard PCR reaction containing 400 nM each of the anchor and sensor hybridization probes, 100 ng of DNA, 4.0 mM MgCl2, and 2 μL of LightCycler DNA master hybridization mixture (LightCycler-DNA Master Hybridization Probes; Roche Diagnostics Inc.) in a total of 20 μL. The reaction was started with a denaturation step at 95 °C for 30 s, and amplification was performed for 50 cycles of denaturation (95 °C for 0 s; ramp rate, 20 °C/s), annealing (55 °C for 7 s; ramp rate, 20 °C/s), and extension (72 °C for 12 s; ramp rate, 20 °C/s).

PCR products were identified by monitoring DNA melting curves in the glass capillary. DNA was denatured at 95 °C for 30 s, and maximum fluorescence was acquired by holding the reaction at 52 °C for 30 s. Data for the melting curves were generated by heating slowly to 80 °C with a ramp rate of 0.1 °C/s, and were collected continuously during that time. When the shorter probe melted off the template, FRET no longer took place, and fluorescence was converted to melting peaks by software that plotted the negative derivative of fluorescence with respect to temperature (−dF/dT vs T). The sequence-specific hybridization probes melted off the target sequences at characteristic temperatures: 68 °C (variant allele) and 73 °C (wild-type allele) in the case of CYP2C9*2 (channel F2 in the LightCycler), and 60 °C (wild-type allele) and 66 °C (variant allele) for CYP2C9*3 (channel F3 in the LightCycler). The mutations produced a minimum Tm shift of 5 °C, allowing easy detection of a wild-type from a variant allele. A typical example is given in Fig. 1. Reverse complementary oligonucleotides of the anchor and sensor probes were used as positive controls (see Table 1). Certain amplification products were also se-