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Monitoring Temporary Immunodepression by Flow Cytometric Measurement of Monocytic HLA-DR Expression: A Multicenter Standardized Study

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Background: **Single-center trials have shown that monocytic HLA-DR is a good marker for monitoring the severity of temporary immunodepression after trauma, major surgery, or sepsis. A new test for measuring monocytic HLA-DR is now available.**

Methods: **We evaluated a new test reagent set for monocytic HLA-DR expression (BD Quantibrite™ HLA-DR/Monocyte reagent; Becton Dickinson) in single-laboratory and interlaboratory experiments, assessing preanalytical handling, lyse-no-wash (LNW) vs lyse-wash (LW) values, reference values, and the effect of use of different flow cytometers and different instrument settings on test variance.**

Results: **For preanalytical handling, EDTA anticoagulation, storage on ice as soon as possible, and staining within 4 h after blood collection gave results comparable to values obtained for samples analyzed immediately after collection (mean increase of ~4% in monocytic HLA-DR). Comparison of LNW and LW revealed slightly higher results for LNW (~18% higher for LNW compared with LW; $r = 0.982$). Comparison of different flow cytometers and instrument settings gave CVs <4%, demonstrating the independence of the test from these variables and suggesting that this method qualifies as a standardized test. CV values from the interlaboratory comparison ranged from 15% (blood sample unprocessed before transport) to 25% (stained and fixed before transport).**

Conclusions: **For the BD Quantibrite HLA-DR/Monocyte test, preanalytical handling is standardized. Single-laboratory results demonstrated the independence of this test from flow cytometer and instrument settings. Interlaboratory results showed greater variance than single-laboratory values. This interlaboratory variance was partly attributable to the influence of transport and can be reduced by optimization of transport conditions.**

Temporary immunodepression is frequently associated with pathophysiologic events such as myocardial infarction, severe trauma, or major surgery *(1)*. Immunodepression in its severest form, immunoparalysis, can be life-threatening *(2)*, but there is no clinical sign to indicate its presence, and clinical signs of infectious complications

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may be absent because of the defective immune response. A laboratory marker is therefore needed that can recognize temporary immunodepression early enough to define patients at high risk for infectious complications.

HLA-DR belongs to the MHC class II molecules responsible for antigen presentation to T cells. Monocytes strongly express HLA-DR on their surfaces; it thus can easily be detected by flow cytometry. Monocytes with diminished or missing HLA-DR expression are strongly inhibited in their antigen-presenting function *(3)* and are inhibited in their ability to produce inflammatory mediators in response to a respective stimulus *(4)*. Compatible with this, diminished monocytic HLA-DR expression correlates with an increased risk for infectious and other complications in patients with severe injury *(5)*, patients with severe burns *(6)*, patients who have undergone cardiopulmonary bypass surgery *(7)*, patients with pancreatitis *(8)*, transplantation patients *(9–11)*, and patients who have undergone neurosurgical tumor extirpation *(12)*. Strongly diminished monocytic HLA-DR expression could therefore identify patients with temporary immunodepression who are at risk for infectious complications. Furthermore, decreased monocytic HLA-DR has been correlated with clinical outcome in septic patients *(2, 13)*.

The mechanisms responsible for down-regulation of monocytic HLA-DR are partly known. It seems clear that an imbalance between pro- and antiinflammatory responses in favor of antiinflammatory responses plays a role *(14, 15)*. Consequently, promising studies are underway to investigate the use of immunostimulatory agents, including interferon-γ, granulocyte-macrophage colony-stimulating factor, and glutamine-rich nutrition, in patients with severe and prolonged down-regulation of monocytic HLA-DR expression *(16–22)*. Controversial results regarding the diagnostic value of monocytic HLA-DR expression have also been reported, however *(23–25)*. One explanation for the controversial results may be methodologic differences. To date, no standardized method for measuring monocytic HLA-DR expression has been described in the literature. Laboratories differ in their procedures for preanalytical handling of samples, the antibodies used to detect HLA-DR expression, and monocyte gating and flow cytometers, and results are given as either "% positive monocytes" or "mean fluorescence intensity". Reported values have been laboratory specific, and interlaboratory comparisons have not been possible. These problems help to explain why no multicenter trials have investigated the use of monocytic HLA-DR for diagnosis of immunodepression or to monitor immunomodulating therapies. Here we report on the first test to determine monocytic HLA-DR expression that is independent of flow cytometer and instrument settings. This test was developed by Becton Dickinson in collaboration with the Institute of Medical Immunology/Charité Berlin. It is based on the generation of a calibration curve for phycoerythrin (PE)[14] fluorescence and an anti-HLA-DR antibody conjugated 1:1 with PE to allow the measurement of bound HLA-DR antibodies per cell independently from the flow cytometer and the instrument settings used.

Materials and Methods

HUMAN BLOOD SAMPLES

The patient blood samples used for this study were obtained from blood drawn for routine diagnostic procedures. No additional blood was taken from the patients; therefore, approval by the local ethics committee was not needed. Healthy volunteer donors who provided control samples gave informed consent before blood collection.

STANDARDIZED MEASUREMENT OF MONOCYTIC HLA-DR EXPRESSION

The test procedure is as follows: a mixture of beads to which defined amounts of PE molecules have been conjugated is measured at the same instrument settings as the cells incubated with a mixture consisting of anti-human HLA-DR-PE, anti-human CD14-PerCP-Cy5.5, and an inhibitor of HLA-DR turnover (BD Quantibrite™ HLA-DR/Monocyte reagent; Becton Dickinson). The PE beads facilitate conversion of the FL2 axis into PE molecules bound per cell. The known ratio of PE to anti-HLA-DR antibody (see below) is used to convert the PE molecules per cell into antibodies per cell (AB/c). The anti-HLA-DR antibody, clone L243, reacts with a nonpolymorphic HLA-DR epitope and is conjugated with PE molecules in a 1:1 ratio [for quantification of PE-conjugated antibodies, see Ref. *(26)*]. The anti-CD14 antibody, clone MoP9, is conjugated with PerCP-Cy5.5. CD14 is expressed by the majority of monocytes. In addition, the cyan dye recognizes CD64, as the ratio Cy5.5:PerCP is higher than in other PerCP–Cy5.5 conjugations. The anti-CD14 PerCP-Cy5.5 antibody therefore detects all monocytes (CD14 brightly positive and weakly positive) *(27, 28)*. The inhibitor of cytoskeletal transport partly prevents ex vivo up-regulation of monocytic HLA-DR. The method is fast; results are available within 1 h.

For the cellular assay, we incubated 50 μL of anticoagulated blood with 20 μL of the antibody mixture anti-HLA-DR-PE/anti-CD14-PerCP-Cy5.5 for 30 min at room temperature in the dark. After vortex-mixing, 500 μL of BD lysing solution was added to the blood for another 15 min, after which the cells were ready for measurement [lyse–no-wash method (LNW)]. Alternatively, after erythrocyte lysis by centrifugation at 200*g* for 5 min, cells were resuspended in 1000 μL of washing buffer (e.g., phosphate-buffered saline with azide plus 20 mL/L fetal calf

[14] Nonstandard abbreviations: PE, phycoerythrin; AB/c, antibody bound per cell; LNW, lyse–no-wash; LW, lyse–wash; FSC, forward scatter; SSC, side scatter; and CI, confidence interval.

serum) and centrifuged for another 5 min [lyse–wash method (LW)].

The advantages and disadvantages of LNW and LW were evaluated. After cell preparation, the flow cytometer was started and the acquisition program opened. Using the setup mode, we adjusted all settings for the cellular assay. The respective beads or cells may be used for this adjustment. The lyophilized PE beads (BD) were then reconstituted with 500 μL of buffer such as phosphate-buffered saline with azide plus 5 g/L bovine serum albumin and vortex-mixed. The tube was run thresholding on forward scatter (FSC) or side scatter (SSC), and 10 000 events were collected. The bead singlets were gated on the FSC-vs-SSC plot, and the singlet bead population was analyzed with the histogram plot of FL2 in linear values. Markers were adjusted around the 4 bead populations, and the histogram statistics with display of the geometric means were shown (Fig. 1A). Using the same instrument setting, we ran the cellular assay with respective thresholds on SSC and FL3, acquiring 500-1000 monocyte events, defined as cells with the respective SSC and PerCP-Cy5.5 staining characteristics (Fig. 1, B and C). The HLA-DR molecules per monocyte can be calculated either automatically (e.g., for Becton Dickinson flow cytometers, with BD CellQuest 3.1 and later versions) or manually by entering the geometric means of the 4 bead populations on a statistics spreadsheet. The lot-specific values for the PE molecules per bead population are then entered. The $\log_{10}$ values for the FL2 geometric means and for the PE molecules per bead population are calculated. A linear regression of $\log_{10}$ PE molecules per bead population against $\log_{10}$ FL2 fluorescence is plotted, using the equation:

$$y = mx + c$$

where y is the $\log_{10}$ FL2 fluorescence and x is $\log_{10}$ PE molecules/bead population. To determine bound anti-HLA-DR antibody (AB/c) per monocyte, the $\log_{10}$ FL2 geometric means are substituted with the $\log_{10}$ FL2 median value of the monocyte population, and the equation is solved for $x = \log_{10}$ AB/c. The antilog is determined to get AB/c. The detailed protocol can be read at http://www.bdeurope.com/temp/530721.pdf.

We assessed the influence of anticoagulant (EDTA, citrate, and heparin) and preanalytical storage temperature on HLA-DR stability (patient blood samples, n = 5) and of anticoagulant (EDTA, citrate, and heparin) on intraassay variance (each with 10 replicates of 1 patient sample), compared LNW vs LW cell preparation methods (patient blood samples, n = 100), and analyzed monocytic HLA-DR stability after LNW (healthy control blood samples, n = 4) and LW (patient blood samples, n = 5). We also performed single-laboratory and interlaboratory comparisons to test the influence on monocytic HLA-DR values of different flow cytometers, instrument settings, and sample transport and handling.

For single-laboratory comparisons, we prepared 20 replicates of a stained LNW sample from a healthy individual. The operator then made the following quantitative HLA-DR measurements: 5 measurements on flow cytometer A with instrument setting 1; 5 measurements on flow cytometer A with instrument setting 2; 5 measurements on flow cytometer B with instrument setting 1; and 5 measurements on flow cytometer B with instrument setting 2. Flow cytometers A and B were FACSCalibur instruments (Becton Dickinson). Instrument setting 1 was generated with commercially available Calibrite beads and FACSComp software (Becton Dickinson). Instrument setting 2 was adjusted manually with single-color stained cells. Both instrument settings were appropriate for simultaneous measurement of beads and cells stained with the respective fluorochromes. Intra- and interassay variances were determined.

For interlaboratory comparison of unstained samples

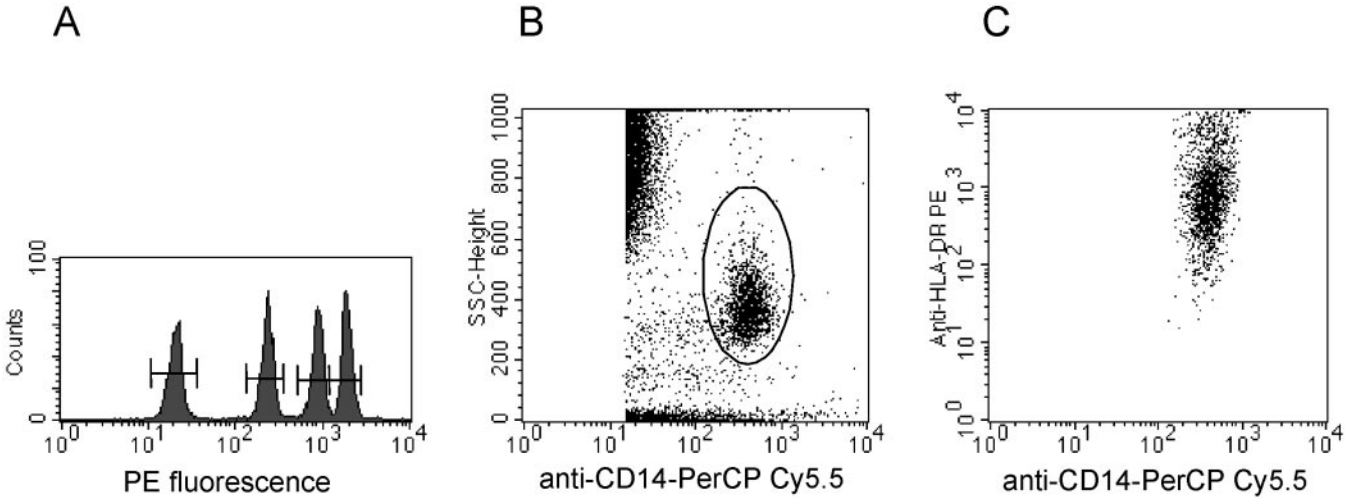

Fig. 1. Main steps in measurement of monocytic HLA-DR expression by the standardized test.

BD Quantibrite beads are gated on their SSC and FSC characteristics (data not shown), and the PE fluorescence is plotted (*A*). The patient's monocytes are gated by their CD14-binding and SSC properties (*B*), and HLA-DR expression is plotted against CD14 to calculate the median HLA-DR expression (*C*).

(samples 1 and 2) and stained samples (LNW; samples 3 and 4), samples from a patient in immunoparalysis (monocytic HLA-DR <5000 AB/c, samples 1 and 3) and a healthy control (samples 2 and 4) were packed (in cool packs and protected from light) at the Institute of Medical Immunology/Charité Berlin and sent to 9 different laboratories (including back to the Institute of Medical Immunology/Charité Berlin). Processing of the samples according to the LNW protocol was started at the same time (24 h after packaging). Seven laboratories used a FACSCalibur (Becton Dickinson), 2 used an Epics XL (Coulter), 5 calculated AB/c automatically, and 4 used the manual procedure described above.

Finally, reference values were established for the LNW method (healthy control blood samples, n = 100), and the LW method was compared with an old, nonstandardized test (patient blood samples, n = 80) to generate preliminary cutoff values for evaluating the severity of monocytic immunodepression with the standardized method.

statistical analysis

We calculated the median, mean, SD, CV, 2.5th and 97.5th percentiles, 95% confidence interval (CI), significant group differences, and correlation coefficient (Pearson) with SPSS software, Ver. 10. Group differences were tested for significance with the Friedman test, and if these results were positive, we used the Wilcoxon test for paired samples. Differences between groups were considered significant at $P < 0.05$.

Results

influence of different anticoagulants and preanalytic sample handling on ex vivo stability of monocytic hla-dr expression and on intraassay variance

We have found that monocytic HLA-DR expression commonly increases in vitro, probably because of endogenous interferon-γ production (our unpublished data), possibly influenced by the anticoagulant used as well as preanalytic sample handling. We therefore tested heparin-, citrate-, and EDTA-anticoagulated blood samples from 5 patients; samples were prepared with the LNW method and stored for 2, 4, or 24 h either at room temperature or on ice.

Samples analyzed immediately (time point zero) showed rather small differences with respect to HLA-DR expression independent of the anticoagulant and storage conditions [mean (95% CI), 6230 (5950–6520) AB/c; CV = 1.8%]. HLA-DR expression increased significantly after preanalytical storage of blood for 2 h at room temperature, particularly in heparin- and citrate-anticoagulated blood. The mean increase was 238% in heparin blood [mean (95% CI), 15 100 (11 400–18 800) AB/c; $P < 0.05$], 75% in citrate blood [10 700 (9320–12 100) AB/c; $P < 0.05$], and 21% in EDTA blood [7510 (7000–8020) AB/c; $P < 0.05$]. In contrast, the mean increase in HLA-DR values in EDTA blood stored on ice for 4 h was 4% [6500 (6090–6860 AB/c; $P = 0.225$], which was not significant (see Fig. 1A in the Data Supplement that accompanies the online version of this article at http://clinchem.org/content.vol51/isue12). To measure the influence of heparin, citrate, and EDTA anticoagulants on intraassay variance, we analyzed 10 replicates of the respective anticoagulated blood samples. Citrate- and EDTA-anticoagulated samples demonstrated much smaller intraassay variances than did heparin-anticoagulated samples (CV = 5.6%, 6.4%, and 14%, respectively; mean values, 7000, 5730, and 10 300 AB/c, respectively; see Fig. 1B in the online Data Supplement).

Because of the better monocytic HLA-DR stability and the lower intraassay variance, all later experiments and method optimizations were done with EDTA-anticoagulated blood stored on ice as soon as possible and analyzed within 4 h after collection.

comparison of lnw and lw methods

Comparison of the results obtained with the LNW and LW techniques in randomly collected patient blood samples sent to our diagnostic laboratory demonstrated excellent correlation between the 2 methods ($r = 0.982$; $P < 0.01$), with an only slightly increased mean value (18%) in LNW samples compared with LW samples [for LW samples, mean (95% CI), 14 600 (11 300–18 000) AB/c (set as 100%); for LNW samples, mean (95% CI), 15 700 (12 600–18 800) AB/c]. It is important to mention, however, that in single cases, differences between the LNW and LW results were as high as 120% (see Fig. 2 in the online Data Supplement).

Both LNW and LW samples [EDTA-anticoagulated blood samples from healthy individuals (LNW, n = 4) or patients (LW, n = 5)] were stable for at least 24 h when stored at 4 °C in the dark [for LNW, mean (95% CI) at 0 h, 28 400 (19 500–37 200) AB/c; at 24 h, 28 000 (19 600–36 400) AB/c ($P = 0.144$); for LW, at 0 h, 6230 (6200–6260) AB/c; at 24 h, 6290 (6170–6410) AB/c ($P = 0.225$); see Fig. 3 in the online Data Supplement]. In addition, we demonstrated that results for LW samples were independent of the anticoagulant used and the storage temperature after lysis (see Fig. 3B in the online Data Supplement).

single- and interlaboratory comparison of quantitative measurement of monocytic hla-dr

To test the influence of different flow cytometers and instrument settings on monocytic HLA-DR values, we prepared 20 replicates of a stained LNW sample from a healthy control and measured each replicate sample with 2 different flow cytometers at 2 different instrument settings. As seen in Table 1, interassay variance analysis revealed values <4% for all combinations tested. [For individual and SD values, see Table 1 in the online Data Supplement.]

For interlaboratory comparisons, we sent blood samples from a patient in immunoparalysis [monocytic HLA-DR <5000 AB/c the day before (samples 1 and 3)]

Table 1. Influence of different flow cytometers and instrument settings on monocytic HLA-DR values.[a]

	Flow cytometer/instrument settings			
	A/1	A/2	B/1	B/2
Mean monocytic HLA-DR, AB/c	41 300	40 700	40 100	38 000
Intraassay CV, %	1.3	2.7	1.7	2.0
Interassay CV, %				
A/1 vs A/2	1.1			
A/1 vs B/1	1.5			
A/1 vs B/2	3.2			
A/2 vs B/1	1.1			
A/2 vs B/2	3.6			
B/1 vs B/2	3.8			

[a] We prepared 20 replicates of an LNW stained sample from a healthy control. HLA-DR expression was quantified on 2 flow cytometers each time with 2 different instrument settings: A/1, flow cytometer A with instrument setting 1; A/2, flow cytometer A with instrument setting 2; B/1, flow cytometer B with instrument setting 1; B/2, flow cytometer B with instrument setting 2. At any time, 5 replicates were measured. Flow cytometers A and B both were FACSCalibur (Becton Dickinson). Instrument setting 1 was generated with commercially available Calibrite beads and FACSComp software (Becton Dickinson). Instrument setting 2 was adjusted manually with single-color stained cells. Both instrument settings were appropriate for simultaneous measurement of beads and cells. Intra- and interassay variances were determined.

and from a healthy volunteer (samples 2 and 4) to the 9 participating laboratories within 24 h. Samples 1 and 2 were unprocessed, whereas samples 3 and 4 were stained at the Institute of Medical Immunology/Charité Berlin before transport as described above. All laboratories started the processing at the same time (24 h after packaging). As seen in Table 2, the CVs ranged from 15% (sample 2) to 25% (sample 3).

Table 2. Interlaboratory comparison of monocytic HLA-DR expression.[a]

	Monocytic HLA-DR expression, AB/c			
	Sample 1	Sample 2	Sample 3	Sample 4
Institution				
1	10 400	35 900	5680	34 000
2	8000	35 200	5810	33 000
3	13 000	31 100	8050	23 300
Mean	10 600	34 800	7030	32 500
CV, %	20	15	25	18

[a] Samples 1 and 3 represent blood samples from a patient in immunoparalysis the day before (monocytic HLA-DR <5000 AB/c); samples 2 and 4 represent blood samples from a healthy control. Samples 3 and 4 were stained at the Institute of Medical Immunology/Charité Berlin according to the described protocol. All samples were then sent to the 9 participating institutions (packed in cool packs and protected from light), including back to the Institute of Medical Immunology/Charité Berlin, within 24 h. All institutions started the procedure at the same time. The results were sent to the Institute of Medical Immunology/Charité Berlin. Results from 3 representative institutions, mean values, and CVs are shown.

Reference values for standardized assessment of monocytic HLA-DR expression by LNW method

We generated reference values for the LNW method. (For characteristics of the sample donors, see Table 1 in the online Data Supplement.) Reference values were 13 200–42 500 AB/c for females and 15 300–40 100 AB/c for males (2.5th–97.5th percentiles, respectively) with median values of 26 200 AB/c for females and 25 300 AB/c for males (see Table 2 in the online Data Supplement).

Comparison of the LW method with a nonstandardized method

As mentioned earlier, different methods have been used to measure monocytic HLA-DR expression, making interlaboratory comparison impossible. Before establishment of standardized HLA-DR quantification at the Institute of Medical Immunology/Charité Berlin, results had been given in "% HLA-DR+ monocytes" with a reference value >60% considered indicative of immunocompetence, values of 60%–30% considered indicative of moderate to severe immunodepression, and values <30% considered strongly diminished expression or immunoparalysis [for details, see Ref. *(16)*]. We correlated this old method with the new standardized test (LW) in randomly collected blood samples from 80 patients sent to our diagnostic laboratory to generate preliminary cutoff values for the standardized method, with values of 10 000–15 000 AB/c representing moderate immunodepression and values <5000 AB/c representing immunoparalysis ($r = 0.594$; $P < 0.01$; see Fig. 4 in the online Data Supplement).

Discussion

A standardized method for measuring monocytic HLA-DR expression is urgently needed to enable multicenter testing of this marker for diagnosing temporary immunodepression and monitoring immunomodulating therapies. Previous attempts to standardize monocytic HLA-DR measurements addressed preanalytical handling but were unsuccessful because results were not independent from such variables as the flow cytometer used and instrument settings *(29, 30)*. The test described here is independent from these variables because the number of HLA-DR molecules per monocyte is quantified by use of beads conjugated with defined amounts of fluorochromes to calibrate the fluorescence scale of the flow cytometer in terms of numbers of fluorochrome molecules *(26, 31)*.

With respect to preanalytical conditions, we demonstrated that ex vivo up-regulation of monocytic HLA-DR expression can mask in vivo down-regulation, depending on the time between blood drawing and staining, storage temperature of the unstained blood, and the anticoagulant used. We found that use of EDTA blood, storage of unprocessed blood on ice as soon as possible before staining, and staining within 4 h after blood drawing were

the conditions that produced the lowest variance between ex vivo values.

The LNW and LW methods demonstrated excellent correlation; however, in single cases we observed marked differences between methods, possibly because of nonspecific antibody binding caused by inflammation-induced up-regulation of Fc-γ receptors. Although the LW method requires another working step, measurement is faster because of the increased cell concentration. These findings should be taken into account when deciding which method to use, but it is important to focus on a single method for multicenter trials. Because the LNW method has fewer working steps and is described in the instruction leaflet, we recommend that this method be used in multicenter studies.

The single-laboratory analysis of the influence of different flow cytometers and instrument settings on monocytic HLA-DR expression revealed CV values <4%. Thus, the test is independent from these external variables, a characteristic that is a prerequisite for successful standardization. In contrast, the interlaboratory comparison revealed CV values as high as 25%. For the unprocessed samples, CV values of 15% and 20% are promising considering the fact that ex vivo monocytic HLA-DR expression is rather unstable. The high CV values in the prestained fixed samples were rather surprising, because fixed samples should be stable for at least 24 h. Stability is an important issue when specimens must be transported, and specimen transport must be addressed in interlaboratory experiments. We therefore currently test different transport conditions to minimize their influence on the test results. In future interlaboratory comparisons, we plan to further improve transport conditions and to send replicate samples to each laboratory to analyze laboratory-specific intraassay variance.

With the LNW method we generated a reference value for the median number of HLA-DR–binding antibodies/monocyte of ~25 700 (2.5th–97.5th percentiles, 14 100–42 500). These values, however, were generated under optimal conditions, i.e., blood samples were stored on ice immediately and stained within 30 min after drawing; therefore, laboratories that cannot guarantee these conditions should eventually generate their own reference values.

We compared cutoff values generated with our old, nonstandardized method with the new method to generate preliminary cutoff values for monocytic HLA-DR expression for the LW method (>15 000 AB/c as indicative of immunocompetence, in accordance to the reference data presented above; 5000–15 000 AB/c as indicative of moderate to severe immunodepression; <5000 AB/c as indicative of immunoparalysis). Data from a single-center study using standardized measurement of monocytic HLA-DR expression in patients who have undergone cardiopulmonary bypass surgery support a cutoff of <5 000 AB/c to indicate patients with immunoparalysis and an enhanced risk for developing infectious complications *(7)* (Fig. 2). Another report of the same test, however, described the failure of monocytic HLA-DR to discriminate patients with postoperative complications *(25)*. The questions raised by such conflicting results may be answered by multicenter trials now underway in which the new, standardized test is being performed in parallel with laboratory-specific tests for monocytic HLA-DR expression.

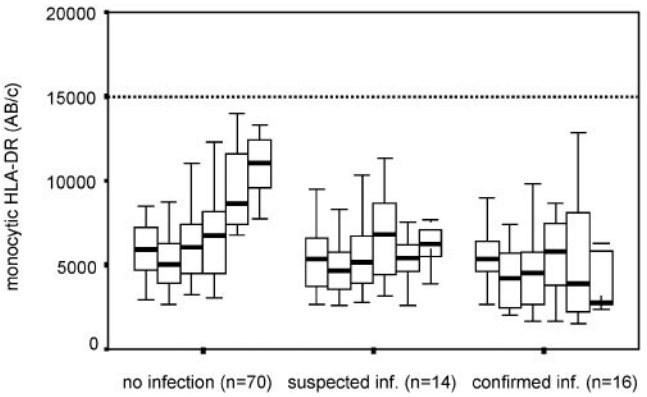

Fig. 2. Monocytic HLA-DR expression in patients >70 years of age and/or with an ejection fraction <25% who underwent selective cardiopulmonary bypass.

HLA-DR concentrations were analyzed for up to 6 days after surgery and correlated with infectious complications (*inf*). Data are presented as box plots with medians (*lines inside boxes*), 25th and 75th quartiles (*limits of boxes*), and 1.5 times the interquartile range from the lower/upper quartile (*whiskers*). The *dotted line* represents the lower preliminary cutoff value of 15 000 AB/c [adapted from Strohmeyer et al. *(7)*].

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