HLA-B27 phenotyping with flow cytometry: further improvement by multiple monoclonal antibodies

Johannes J.M.L. Hoffmann* and Willy C.M. Janssen

To establish the optimal flow cytometric method for HLA-B27 phenotyping, we compared several strategies, using three monoclonal anti-B27 antibodies (from the HLA-ABC-m3, GS145.2, and FD705 clones). We used a triple-color direct immunofluorescence assay, including a T-lymphocyte-specific antibody as an internal control and an anti-HLA-Bw4 antibody. Blood samples from > 400 subjects were tested. From ROC curve analysis none of the three antibodies appeared to be suitable for use as a single typing reagent. The efficiency of the test was affected by cross-reactions with other HLA antigens, notably the HLA-B7 antigen. Preincubation with anti-B7 serum efficiently inhibited this cross-reaction and raised the test efficiency considerably. We concluded that none of the anti-B27 antibodies investigated is suitable for use as a single typing reagent. Additional typing of Bw4 is not valuable, whereas inhibition of cross-reactions due to the B7 antigen will considerably improve the performance of the test. We recommend that two different monoclonal anti-B27 antibodies be used for accurate and reliable HLA-B27 phenotyping with flow cytometry.

The HLA system comprises the major histocompatibility complex in humans. This system is highly polymorphic; several hundreds of antigens have been recognized to date. Several HLA antigens are associated with various diseases, and probably the strongest association known is that between the HLA-B27 antigen and rheumatic diseases such as ankylosing spondylitis (also known as Bechterew disease), Reiter syndrome, and acute anterior uveitis [1]. Whereas the frequency of HLA-B27 in the Caucasian population is ~9%, at least 90% of patients with ankylosing spondylitis are HLA-B27 positive. Indeed, a diagnosis of ankylosing spondylitis becomes very unlikely for a patient whose HLA-B27 phenotype is negative [2]. Since the prevalence of ankylosing spondylitis is <0.1%, the presence of the HLA-B27 antigen is no diagnostic proof of the disease. Therefore, the main clinical use of the HLA-B27 antigen is to exclude ankylosing spondylitis.

The standard method of HLA typing is the classical microlymphocytotoxicity test, developed by Terasaki [3]. This test is not well-suited for application in clinical laboratories performing only HLA-B27 typing because it is a time-consuming assay requiring a great deal of experience. Now that several monoclonal antibodies to HLA-B27 have become available and flow cytometers are increasingly in operation in clinical laboratories, various flow cytometric applications for HLA-B27 typing with monoclonal antibodies have been described [4–12]. Most of the available antibodies are not specific for the HLA-B27 antigen and also react with other HLA-B antigens, notably HLA-B7, B22, and B40. On the other hand, some antibodies do not recognize all subgroups of the B27 antigen and may not be sensitive enough for diagnostic purposes.

The lack of specificity has stimulated investigators to find ways for improving the discrimination between HLA-B27 and cross-reacting antigens [4, 6, 7, 10, 11]. Some authors hold the assumption that cross-reactions may be identified on the basis of fluorescence intensity, which is generally lower than that of a truly positive reaction [4, 5, 7]. We have previously shown that the major cross-reaction due to the B7 antigen can effectively be suppressed by an anti-B7 antiserum [6]. Recently, we have extended these observations with the use of a monoclonal anti-B7 serum in a method applicable to whole blood. In theory, cross-reaction due to the HLA-B7 antigen might be recognized by simultaneous HLA-Bw4 typing: The B27 antigen belongs to the Bw4 superfamily of antigens, whereas the B7 antigen is part of the Bw6 supertype, and therefore an apparent positive reaction of any anti-B27 antiserum can be reliably classified as false positive when the cells are Bw4 negative. Although this
method of additional HLA-Bw4 typing is currently in use, it has not been adequately validated in the literature.

None of the flow cytometric methods currently seems to be sufficiently specific and sensitive for use as the sole method of HLA-B27 typing. Therefore, and because it is not yet clear how the different methods compare, we have performed a study in which several approaches for HLA-B27 typing by flow cytometry were investigated, using different monoclonal antibodies. Our results show that none of the available antibodies is appropriate for use as a solitary typing reagent, but that a combination of two different monoclonal antibodies can achieve complete specificity and sensitivity in flow cytometric HLA-B27 typing.

**Materials and Methods**

**REAGENTS**

The monoclonal antisera used were: anti-HLA-B27 (clone HLA-ABC-m3) [13] obtained from Behringwerke in purified form as well as labeled with fluorescein isothiocyanate (FITC); anti-HLA-B27 (clone GS145.2) [14], FITC labeled and mixed with phycoerythrin (PE)-labeled CD3 (a pan-T-lymphocyte marker) obtained as the HLA-B27 screening kit from Becton Dickinson; and anti-HLA-B27 (clone FD705) [15], FITC labeled (produced by One Lambda) purchased from EuroBiochem S.C.R.L. 1

In addition, we used nonconjugated anti-HLA-B7 and PE-labeled anti-HLA-Bw4 (both monoclonal; Behringwerke) and peridinin–chlorophyll protein (PerCP)-labeled CD3 (Leu-4) from Becton Dickinson. Polyclonal anti-HLA-B7 serum (human) was obtained from Biotest. Secondary antisera labeled with FITC or PE were from various suppliers, as were irrelevant monoclonal antisera, also FITC- or PE-conjugated, which served as the negative controls for the anti-HLA sera. FACS lysing solution was purchased from Becton Dickinson and Lymphoprep (density 1.077 g/L) from Nycomed Pharma.

**METHODS**

For all flow cytometric methods blood was anticoagulated with K$_2$-EDTA (final concentration 1 g/L); the samples were kept at ambient temperature and the analyses performed within 24 h from blood collection. The microlymphocytotoxicity typing was carried out on blood samples anticoagulated with heparin (15 kU/L). Blood was obtained from healthy volunteers and from material left over from the daily workload of the laboratory, in agreement with the regulations of the hospital’s ethical committee.

**Screening with HLA-ABC-m3 antibody.** To 50 µL of whole blood we added 10 µL of a mixture of anti-B27-FITC (HLA-ABC-m3), anti-Bw4-PE, and CD3-PerCP. All antisera were used in dilutions that had previously been shown to give optimal results. After incubating for 30 min in melting ice in the dark, 2.0 mL of lysing solution was added; the lysis step took 10 min at ambient temperature. The cells were washed once with PBS (67 mmol/L phosphate, 0.15 mol/L NaCl, pH 7.40) and suspended in 0.3 mL of PBS. Within 1 h, the samples were analyzed as described below.

**Screening with the B27 screening kit.** To 50 µL of whole blood we added 30 µL of a ready-for-use mixture of anti-B27-FITC (GS145.2) and CD3-PE, following the directions for use provided by the manufacturer of the kit [11]. The incubation lasted for 15 min at ambient temperature, and after lysis and washing, the cells were counted.

**Screening with FD705 antibody.** To 50 µL of whole blood we added 5 µL of anti-B27-FITC (FD705) and CD3-PerCP and processed as for screening with HLA-ABC-m3 antibody.

**Confirmation after preincubation with anti-B7.** Ten microliters of nonconjugated monoclonal anti-B7 were added to 50 µL of whole blood and incubated for 30 min in melting ice in the dark. Without washing, either of the anti-B7 antisera was added and the procedure was carried out as stated above.

**FLOW CYTOMETRIC ANALYSIS**

The cells were analyzed in a five-parameter FACScan flow cytometer (Becton Dickinson). The flow cytometer was calibrated and controlled daily according to standard procedures; special care was taken to compensate for spectral overlap between the three fluorescence signals. The two light-scatter parameters (forward and side scatter) were used to identify the lymphocyte population, and the fluorescence measurements were limited to the cells within this electronic gate. At least 5000 lymphocytes were counted, with the FACScan Research software (version 2.6, Becton Dickinson). The fluorescence measurement in the first (FITC) and second (PE) fluorescence channels was limited to T lymphocytes, which were recognized by a positive reaction in the third fluorescence channel with CD3-PerCP.

With appropriate control antibodies, a fluorescence intensity was defined so that at least 99.5% of the lymphocytes analyzed were below this threshold; all lymphocytes with a higher fluorescence intensity than this threshold were regarded as positive and were expressed in percent of lymphocytes analyzed. The strength of the fluorescence signal of the positive lymphocytes was expressed as mean fluorescence intensity (MFI), on a scale of 1024 channels encompassing four log decades. In addition, the median fluorescence intensity (MdFI) was similarly calculated.

For screening with the B27 screening kit, we used the dedicated HLA-B27 software package (Becton Dickinson),

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1 Nonstandard abbreviations: FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin–chlorophyll protein; MFI, mean fluorescence index; and MdFI, median fluorescence index.
following the directions for use as provided by the manufacturer. In this case, ~15,000 blood cells were counted and the lymphocytes were gated on the basis of their forward scatter and CD3 intensity [11]. The software performed the interpretation of the B27-associated fluorescence and reported a sample either as negative or as possibly positive (called “retest”). Because of the software specifications, the fluorescence strength was expressed as MdFI on a scale of 256 channels, encompassing two log decades.

SAMPLES
Blood samples from 410 subjects were investigated. Most samples were randomly selected from the population served by our hospital; 16 samples were taken from patients known to be HLA-B27 positive. Each donor was included only once in the study. The blood collection procedure was in accordance with the guidelines of the local ethical committee.

DATA ANALYSIS
Flow cytometric reference method. This was performed exactly as described previously [6]. Briefly, lymphocytes were isolated from blood by density gradient centrifugation over Lymphoprep. Twenty microliters of the lymphocyte suspension, adjusted to contain $5 \times 10^9$ cells/L, was preincubated with 40–50 µL of polyclonal human anti-B7 serum for 30 min in melting ice in the dark. Then, without washing, 10 µL of nonconjugated anti-B27 (HLA-ABC-m3) was added and incubated for a further 10 min in melting ice. After washing twice with PBS, the cell pellet was incubated with a mixture of anti-mouse IgG-PE and anti-human IgG-FITC for demonstrating the HLA-B27 and B7 antigens, respectively [6].

Cytotoxicity reference method. In all cases where there was a discrepancy between any of the screening methods and the flow cytometric reference method or any of the screening methods between them, all HLA class I antigens were typed by the standard microlymphocytotoxicity method [3] as a reference (L.P. de Waal, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam).

Standard statistical methods were used for evaluating and comparing the data. The diagnostic performance of the approaches investigated was assessed by using ROC plots, with GraphROC software [16].

Results
The general technique used for analyzing lymphocytes by flow cytometry was followed here. In brief, the stained lymphocytes in lysed whole blood were clustered into an electronic gate on the basis of their forward and sideward light-scatter properties. All fluorescence analyses were confined to the cells within this electronic gate. In the second step, T lymphocytes were recognized in the third fluorescence channel (FL3: PerCP fluorescence) by virtue of their reaction with CD3 antibodies. Finally, the T lymphocytes were analyzed for their signals in the first (FL1: FITC) and, if applicable, the second (FL2: PE) fluorescence channels and the fluorescence intensities plotted as histograms (Fig. 1). The MFI and MdFI were calculated.

To establish which of the fluorescence parameters obtained yielded the best discrimination between HLA-B27-negative and B27-positive samples, we constructed ROC plots by using the percentage of positive lymphocytes, the MFI, and the MdFI. As an example, in screening with the HLA-ABC-m3 monoclonal, the area under the
ROC curves (±SE) was 0.9970 ± 0.0023 (percentage), 0.9957 ± 0.0027 (MFI), and 0.9959 ± 0.0026 (MdFI) (Fig. 2); these differences were not statistically significant. Also, with the other antibodies, the highest area under the ROC curve was always obtained by using the percentage of lymphocytes, but the differences were never statistically significant (results not shown). Therefore, only the percentage of positive lymphocytes is used throughout this report.

**TESTING WITH THE HLA-ABC-M3 MONOCLONAL**

Blood from 406 subjects was screened with the HLA-ABC-m3 monoclonal, and from the ROC plot the cutoff point was calculated, which gave the highest test efficiency (the proportion of correctly classified samples at a given cutoff limit), 0.990. At a cutoff value of 97% positive lymphocytes, the sensitivity was 0.960 and the specificity 0.994. When the results of Bw4 typing were used in addition, the test characteristics did not change (Table 1).

Next, screening was repeated after preincubation with the anti-B7 monoclonal (Fig. 1). The MFI of B27-negative samples significantly decreased from 322.2 ± 62.2 (mean ± SD) to 240.8 ± 48.9 ($P < 0.0001$ by paired $t$-test). The fluorescence of B27-positive samples slightly decreased from 494.2 ± 39.2 to 472.3 ± 40.3 ($P = 0.01$). The test efficiency increased slightly after preincubation with anti-B7, and again these data remained unaltered when Bw4 typing was used in conjunction (Table 1).

**TESTING WITH THE B27 SCREENING KIT (GS145.2 MONOCLONAL)**

A total of 201 samples was tested with the GS145.2 monoclonal. When we used the dedicated software supplied with this kit, we found the maximal sensitivity at a

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Cutoff value (anti-B27)</th>
<th>n</th>
<th>Sensitivity*</th>
<th>Specificity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-ABC-m3</td>
<td>97%</td>
<td>406</td>
<td>0.960 (48/50)</td>
<td>0.994 (354/356)</td>
</tr>
<tr>
<td>HLA-ABC-m3 + Bw4</td>
<td>97%</td>
<td>406</td>
<td>0.960 (48/50)</td>
<td>0.994 (354/356)</td>
</tr>
<tr>
<td>B7 + HLA-ABC-m3</td>
<td>90%</td>
<td>244</td>
<td>1.000 (53/53)</td>
<td>0.994 (190/191)</td>
</tr>
<tr>
<td>B7 + HLA-ABC-m3 + Bw4</td>
<td>90%</td>
<td>244</td>
<td>1.000 (53/53)</td>
<td>0.994 (190/191)</td>
</tr>
<tr>
<td>GS145.2</td>
<td>“Retest”</td>
<td>201</td>
<td>1.000 (35/35)</td>
<td>0.904 (150/166)</td>
</tr>
<tr>
<td>GS145.2 + Bw4</td>
<td>“Retest”</td>
<td>201</td>
<td>1.000 (35/35)</td>
<td>0.934 (155/166)</td>
</tr>
<tr>
<td>B7 + GS145.2</td>
<td>“Retest”</td>
<td>191</td>
<td>1.000 (31/31)</td>
<td>0.950 (152/160)</td>
</tr>
<tr>
<td>B7 + GS145.2 + Bw4</td>
<td>“Retest”</td>
<td>191</td>
<td>1.000 (31/31)</td>
<td>0.964 (154/160)</td>
</tr>
<tr>
<td>GS145.2</td>
<td>151b</td>
<td>201</td>
<td>0.971 (34/35)</td>
<td>0.988 (164/166)</td>
</tr>
<tr>
<td>GS145.2 + Bw4</td>
<td>151b</td>
<td>201</td>
<td>0.971 (34/35)</td>
<td>0.988 (164/166)</td>
</tr>
<tr>
<td>B7 + GS145.2</td>
<td>151b</td>
<td>191</td>
<td>1.000 (31/31)</td>
<td>0.944 (151/160)</td>
</tr>
<tr>
<td>B7 + GS145.2 + Bw4</td>
<td>151b</td>
<td>191</td>
<td>1.000 (31/31)</td>
<td>0.944 (151/160)</td>
</tr>
<tr>
<td>FD705</td>
<td>91%</td>
<td>209</td>
<td>0.950 (38/40)</td>
<td>0.988 (167/169)</td>
</tr>
<tr>
<td>FD705 + Bw4</td>
<td>91%</td>
<td>209</td>
<td>0.950 (38/40)</td>
<td>0.988 (167/169)</td>
</tr>
</tbody>
</table>

* Number of observations in parentheses.

b MdFI.
speciﬁcity of 0.904. Addition of Bw4 typing somewhat improved the test efﬁciency by reducing the number of false-positive results (Table 1). When the samples were preincubated with anti-B7, the speciﬁcity improved and it could be slightly increased by adding the results of Bw4 typing (Table 1).

When we used the MdFI instead of the interpretation provided by the software, the speciﬁcity of the GS145.2 monoclonal was considerably higher (0.988 at a cutoff value 151), at the expense of a small decrease in sensitivity. Addition of Bw4 typing did not essentially change these test characteristics (Table 1). Preincubation with anti-B7 decreased the MdFI of B27-negative samples with the highest ﬂuorescence intensity signiﬁcantly from 130.7 ± 31.1 to 126.0 ± 30.4 (P = 0.005), whereas the decrease in MdFI B27-positive samples was much smaller, from 168.1 ± 8.3 to 167.0 ± 7.8 (P = 0.04). Preincubation with anti-B7 increased the sensitivity to maximal and gave a slight decrease in speciﬁcity (Table 1).

TESTING WITH THE FD705 MONOCLONAL
In 209 samples tested with this antibody, the optimal discrimination was obtained at a cutoff value of 91% positive lymphocytes (test efﬁciency 0.981). Addition of Bw4 typing did not inﬂuence these data (Table 1). Preincubation with anti-B7 could be performed in only 18 cases. In six B27-negative samples exhibiting the highest ﬂuorescence intensity, the MFI decreased from 303.7 ± 104.0 to 202.3 ± 65.5 (P = 0.036). In 12 B27-positive samples MFI dropped from 558.9 ± 57.0 to 531.3 ± 57.2 (P = 0.01). These 18 cases were considered too few for calculation of sensitivity and speciﬁcity.

TESTING WITH COMBINATIONS OF TWO ANTIBODIES
Since none of the three antibodies studied appeared completely sensitive and speciﬁc, we investigated whether any combination of two antibodies could improve the test characteristics to maximal. Table 2 shows that this was indeed possible, but at the expense of a few percent discordant results. For example, the best combination (HLA-ABC-m3 after preincubation with anti-B7 and FD705) classiﬁed 201 of 205 (98%) samples correctly, whereas four samples (2%) gave discrepant results (these four were all B27 negative; one sample appeared positive with HLA-ABC-m3, the other three were FD705 positive).

CROSS-REACTIONS OF THE MONOCLONAL ANTIBODIES
From the comparison of the ﬂow cytometric reactions with the HLA class I phenotypes, discrete patterns emerged of non-B27 antigens, with which the different monoclonal antibodies were reacting; only phenotypes that were observed at least twice are noted. For the HLA-ABC-m3 antibody, cross-reactions were observed with HLA-B7, B37, B44, and possibly B49. The GS145.2 antibody cross-reacted with HLA-B7, B37, B44, and B55. Finally, the FD705 antibody reacted with HLA-B7, B37, B44, and B57. We made no attempts to block the non-B7 cross-reactions with speciﬁc antibodies. Cross-reactions with HLA-A antigens were not conspicuous.

Discussion
Monoclonal antibodies and ﬂow cytometry for HLA-B27 phenotyping are gaining increasing acceptance [4–12]. Initially, indirect methods with isolated lymphocytes were used [4–6], but nowadays the availability of FITC-labeled monoclonal anti-B27 antisera makes direct methods in whole blood the method of choice [10–12, 17, 18]. Yet, none of the published methods can be considered deﬁnitive, and comparative data on this application are scarce [10, 18]. Therefore, the present study was designed with the aim of ﬁnding the best approach for ﬂow cytometric HLA-B27 typing.

First we investigated which parameter was best suited for discriminating B27-positive from B27-negative samples. This item has not been addressed in the literature; most authors worked with either MFI [5, 7, 8, 10, 18] or MdFI [12, 17], while others used the percentage of positive cells [4, 18]. From the ROC curve analysis of our data it appeared that the percentage of positive lymphocytes yielded the highest areas under the ROC curve, irrespec-
tive of the antibody used. Another advantage of this parameter is that it is much less dependent on ﬂow cytometer settings, calibration, and stability of the conjugated monoclonal antibody than the MFI of MdFI are. Even with stringent calibration and quality-control proce-
dures, small variations in the settings are inevitable and will result in changes in MFI or MdFI, which may affect the correct classiﬁcation of samples. This has been recognized by Hulstaert et al., who compensated their decision level for these variations, but ostensibly at the expense of diagnostic speciﬁcity [11].

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Cutoff value (anti-B27)</th>
<th>n (total)</th>
<th>n (discordant)</th>
<th>Sensitivity*</th>
<th>Specificity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>B7 + HLAABC-m3/GS145.2</td>
<td>90%/<em>Retest</em></td>
<td>198</td>
<td>15</td>
<td>1.000 (32/32)</td>
<td>1.000 (151/151)</td>
</tr>
<tr>
<td>B7 + HLAABC-m3/GS145.2</td>
<td>90%/151b</td>
<td>198</td>
<td>4</td>
<td>1.000 (32/32)</td>
<td>0.994 (161/162)</td>
</tr>
<tr>
<td>B7 + HLAABC-m3/FD705</td>
<td>98%/80%</td>
<td>205</td>
<td>4</td>
<td>1.000 (39/39)</td>
<td>1.000 (162/162)</td>
</tr>
<tr>
<td>GS145.2/FD705</td>
<td><em>Retest</em>/80%</td>
<td>57</td>
<td>11</td>
<td>1.000 (22/22)</td>
<td>1.000 (24/24)</td>
</tr>
<tr>
<td>GS145.2/FD705</td>
<td>151b/80%</td>
<td>57</td>
<td>6</td>
<td>1.000 (21/21)</td>
<td>1.000 (30/30)</td>
</tr>
</tbody>
</table>

* Number of observations in parentheses.

b MdFI.
To our knowledge, only two groups have studied the comparison of different monoclonal antibodies using flow cytometry. Orr et al. concluded that the FD705 monoclonal appeared to be monospecific, whereas their other antisera (of which the clone identification was not provided, but probably was HLA-ABC-m3) cross-reacted with the B7 antigen [10]. These authors did not use any measures to minimize this well-known cross-reaction and unfortunately failed to provide data on sensitivity and specificity of both antisera.

Also, Ward and Nikaein compared two monoclonals, one of which was most probably the FD705, but the other could not be identified with reasonable certainty from their paper [18]. The FD705 monoclonal gave only 0.5% indeterminate results, as opposed to 12% indeterminate results for their other monoclonal. Again, no data on sensitivity and specificity were given in this study.

Test characteristics were given or could easily be inferred from the data presented in three other articles. All three concerned descriptions of a single antibody, which was evaluated against the reference method. A study involving the HLA-ABC-m3 antibody gave a sensitivity of 1.00 and specificity 0.85 [12]. Two studies on the GS145.2 monoclonal indicated a sensitivity of 1.00 and 0.976 with specificity of 0.974 and 0.959, respectively [11, 17]. It should be realized that in none of these studies was anti-B7 used for preventing cross-reactions with the B7 antigen. The data compare very well with our findings, but we were able to considerably improve the test efficiency by inhibiting the major cross-reaction by using anti-B7 antiserum (Table 1). Our data indicate that typing with HLA-ABC-m3 or FD705 is more or less equivalent, but we were able to considerably improve the test efficiency by inhibiting the major cross-reaction by using anti-B7 antiserum (Table 1). Our data indicate that typing with HLA-ABC-m3 or FD705 is more or less equivalent, but we were able to considerably improve the test efficiency by inhibiting the major cross-reaction by using anti-B7 antiserum (Table 1). Our data indicate that typing with HLA-ABC-m3 or FD705 is more or less equivalent, but we were able to considerably improve the test efficiency by inhibiting the major cross-reaction by using anti-B7 antiserum (Table 1).

Notwithstanding the very high sensitivity and specificity achievable, none of the three antibodies is entirely specific nor can be made specific by blocking the B7 cross-reaction. For the HLA-ABC-m3 antibody, the most important cross-reacting antigen is B7, but also B37 and B44 seem to react with the antibody. The GS145.2 monoclonal significantly reacts with B7, and to a lesser extent with B37 and B39 [11, 17, present study]. Finally, the FD705 antibody, which is generally regarded as highly specific for B27 antigen [15], also appears to react with B44 and B57 antigens. On the other hand, not all B27 variants are recognized to the same degree by all antibodies. For instance, HLA-ABC-m3 fails to react with the B2703 variant, but as this variant is not encountered in a Caucasian population, false-negative typing is quite unlikely [19]. The FD705 antibody reacts equally with all seven B27 variants and thus no false-negative reactions are to be expected [15].

Because no single monoclonal antibody is sufficiently sensitive and specific for use as a single typing reagent, we recommend performing flow cytometric B27 typing with two different antibodies (Table 2). When both antibodies yield the same result, the B27 phenotype can reliably be determined. In the rare case of discordant results (<2%), another method is necessary for definitive typing of the HLA-B27 antigen. Although the microlymphototoxicity test is considered the reference method, false-negative results of this technique have been reported [20]. As long as the simple and practical assay of soluble B27 antigens in serum is not thoroughly validated [21], typing at the DNA level [22] provides a useful alternative for the few samples that cannot accurately be typed with flow cytometry.

We thank B. Aalderink (Behring Diagnostica, The Netherlands) for supplying reagents, P. van der Meyden (Becton-Dickinson Benelux) for reagents and loan of the HLA-B27 screening software, Prof. Ax (Behringwerke AG, Marburg, Germany) for valuable discussions, A. Hoffmann for statistical advice, and all volunteers for giving blood.

References