Detection of Human Monocyte-Reactive Alloantibodies by Flow Cytometry After Selective Downmodulation of the Fc Receptor I


Monocyte-reactive human alloantibodies may be of importance in situations such as transfusion reactions and bone marrow and kidney transplantation. So far, only complement-binding monocyte-reactive antibodies can be detected with a cytotoxicity assay. No antoglobulin assays are yet available that also detect noncomplement-fixing monocyte-reactive antibodies. The binding of monomeric IgG with high affinity to the Fc receptor I (FcRI) on monocytes has severely hampered the development of such an assay until now. We report on the selective removal of the FcRI from monocytes to test human sera in a flow cytometry assay for the presence of monocyte-reactive IgG alloantibodies. Selective downmodulation of FcRI was accomplished by incubating the cells with murine monoclonal antibodies against FcRI followed by a second incubation with goat-antimouse IgG polyclonal antibodies. With such modified cells, human complement-binding and noncomplement-binding IgG and IgM alloantibodies against polymorphic determinants of the HLA class I and II glycoproteins, the human monocyte antigen system and polymorphic antigenic determinants of the LFA complex, can be detected in a sensitive and reproducible manner.

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MATERIALS AND METHODS

Sera. Sera were obtained from healthy women immunized by pregnancy, from patients immunized by blood transfusion, or from patients with autoimmune neutropenia. These sera are routinely used as typing reagents for HLA- and granulocyte-specific antigens. The specificities of the granulocyte-reactive antibodies were NA1, NA2, NB1, ND1, 5a, 5b, and 9a. The antibodies in all typing sera were alloantibodies, except anti-ND1, which is an autoantibody.8 The anti-HLA sera used had the following specificities: anti–HLA-A1, -HLA-A2, -HLA-A3, -HLA-B7, -HLA-B35, -HLA-B41, -HLA-Cw1, -HLA-Cw4, -HLA-DR1, -HLA-DR2, -HLA-DRw11, and -HLA-DRw52. Monocyte-reactive anti-HMA sera were also applied.14 Two other well-defined sera used in this study were the serum Mart, which recognizes the Mart antigen, and serum Ond, which recognizes the same antigen as serum E27 described by Pischel et al.1 These two alloantigens are localized on the CD11a and CD11b chain of the LFA complex, respectively (van der Schoot et al, submitted). Sera from pregnant women and selected patients (patients who rejected an HLA-matched kidney graft or developed a febrile transfusion reaction) were also studied; negative control sera were taken from healthy male volunteers with blood group AB. Anti-HMA sera were provided by Dr M. Jager and serum Mart was kindly provided by M. Clay and Dr J. McCullough.

Antibodies. The MoAbs applied in our study are listed in Table 1. The MoAb 10.11 was a generous gift of Dr N. Hogg (Imperial Cancer Research Fund, London, UK); MoAb 32.21 was obtained from Medarex (W. Lebanon, NH). Appropriate dilutions of MoAb in phosphate-buffered saline (PBS)/0.1% (wt/vol) bovine serum albumin (BSA) were used in the indirect immunofluorescence test together with an optimal dilution of fluorescein isothiocyanate (FITC)-labeled goat-antimouse antiserum (GM17-01F; CLB, Amsterdam, The Netherlands). Diethyl aminoethyl (DEAE)-purified polyclonal goat-antimouse IgG (GM17-01P; CLB) was used for cross-linking the MoAb.

Binding of human IgG and IgM alloantibodies was shown using an optimal dilution of FITC-labeled F(ab')2 fragments of polyclonal rabbit-antihuman IgG (K26H14F; CLB) and FITC-labeled polyclonal rabbit-antihuman IgM (K26H15 21F2; CLB), respectively.

Absorption with platelets. To remove anti-HLA class I antibodies, sera were absorbed with pooled platelets by standard procedures.2 In summary, random leukocyte-poor donor platelets prepared by differential centrifugation of acid disodium citrate-anticoagulated blood were washed and suspended in PBS/0.01%
NaNO₂ and stored for at least 1 month at 4°C for optimal HLA expression. Serum samples were absorbed twice at 37°C for 1 hour with an equal volume of packed platelets.

**Blood cells.** Monocytes and lymphocytes were isolated from the pooled buffy coats of 85 U of citrated blood from healthy volunteer blood donors or from theuffy coats of single donations of 1 U of blood by means of density centrifugation followed by elutriation. For screening purposes and standardization of the test, pooled monocytes and pooled lymphocytes were preferred. For more detailed analysis, cells from a single donor were used. Monocytes and lymphocytes were stored in plastic ampoules in liquid nitrogen. Freezing and thawing were performed as described by De Boer et al. The time that elapsed from the bleeding of the blood donors to the freezing of the cells was less than 15 hours. After thawing, viability of the cells was over 95%, as shown by trypan blue exclusion.

Lymphocytes of single donors were typed for HLA class I phenotype according to National Institutes of Health (NIH) standard protocol. Typing for HLA class II was performed by the Department of Transplantation Immunology and Tissue Typing of the CLB (head, Dr L.P. Leeuwen). Typing was performed by the Department of Transplantation Immunology and Tissue Typing of the CLB (head, Dr L.P. Leeuwen).

**TESTS**

**Monocyte immunofluorescence test (MIFT).** The monocytes were suspended in RPMI-1640 (GIBCO, Paisley, Scotland) with 10% (vol/vol) fetal calf serum (FCS; GIBCO) (RPMI/FCS) at a concentration of 2.5 × 10⁶/mL. A volume of 0.05 mL (ascites diluted 1:500 in RPMI/FCS) of the MoAb 10.1 was added to 0.05 mL of cell suspension (final dilution of the MoAb 1:1,000 in RPMI/FCS). After incubation for 30 minutes at RT, followed by three washings with PBS/BSA, Fluorescence of the samples was measured as described for the MIFT using forward and sideward scatter gates for selecting the lymphocytes.

**Lymphocyte cytotoxicity test/monocyte cytotoxicity test (LCT/ MCT).** The LCT was performed according to the standard protocol of the NIH. In summary, a suspension with 5 × 10⁶/mL of PFA-fixed lymphocytes was prepared in PBS/BSA. This cell suspension (0.05 mL) was placed in the well of a round-bottom microtiter plate and mixed with 0.01 mL of a serum sample and incubated for 30 minutes at RT, followed by three washings with PBS/BSA. Fluorescence of the samples was measured as described for the MIFT using forward and sideward scatter gates for selecting the lymphocytes.

![Fig 1. Schematic presentation of the downmodulation of the monocyte FcRl by cross-linking with MoAb. Details are given in the text.](www.bloodjournal.org)
RESULTS

Downmodulation of the FcRI. The results in Table 2 show that the membrane expression of the FcRI after cross-linking is strongly reduced and cannot thereafter be detected by means of immunofluorescence with specific MoAbs. The binding of control MoAb had also diminished. This result is probably also due to the downmodulation of the FcRI, because the Fc part of murine MoAb also binds to human FcRI. IgG2b MoAb have been shown to have the same affinity for the FcRI as IgG1 MoAb and were not studied separately. The binding of anti-FcRIII MoAb to the modulated monocytes is reduced, but, after modulation, is higher than that caused by control MoAb of the same isotype. The membrane expression of FcRI after sequential incubation of monocytes prefixed with PFA with MoAb 10.1 and goat-antimouse antibodies remains unaltered, as shown by the binding of MoAb 32.2 relative to the binding of the control MoAb (Table 2). The binding of MoAb 10.1 is reduced, which may be explained by blocking of the epitopes by MoAb 10.1 and unlabeled goat-antimouse antibodies, used for the cross-linking. Moreover, binding of monomeric human IgG is not diminished (data not shown).

Comparison of the MIFT for IgG alloantibodies with and without modulation. Figure 2A shows the effect of modulation on the aspecific binding of monomeric IgG to the monocytes. To study the effect of downmodulation of the FcRI on the sensitivity of the MIFT, a serum containing IgG alloantibodies recognizing a high-frequency HLA class II alloantigen expressed by monocytes (HLA-DRw52) was used with pooled monocytes and single donor monocytes (HLA-DRw52-positive). The single donor monocytes retained their high fluorescence intensity after modulation of the FcRI (Fig 2B). Using modulated pool monocytes, two populations of monocytes were seen, one with a high mean fluorescence and one with a low mean fluorescence. The

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<th>Table 2. Reactivity of the MoAbs</th>
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<td>Mean Fluorescence Intensity</td>
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<td>MoAb Specificity</td>
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<tr>
<td>10.1 FcRI</td>
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<td>32.2 FcRI</td>
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<td>IV.3 FcRI</td>
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<td>CLB-gran/l FcRIII</td>
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<td>Control IgG2a</td>
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<tr>
<td>W6/32 HLA I</td>
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<td>CIA2 HLA II</td>
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<td>TB133 αβ</td>
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<td>Bear-1 αα</td>
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<td>LeuM5 αγ</td>
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<td>CLB-CD18 β</td>
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Reactivity of MoAb with monocytes before and after modulation of the FcRI and with prior PFA fixation and sequential incubation with MoAb 10.1 and goat antimouse IgG. Modulation was induced by incubating monocytes with MoAb 10.1 and cross-linking the MoAb with goat antimouse IgG (see Materials and Methods for details). Binding of MoAb was shown with FITC-labeled antimouse antibodies. The mean fluorescence intensity (±SD) is given of three experiments.

population with a high mean fluorescence presumably represents the HLA-DRw52-positive monocytes in the pool. The mean fluorescence of the other population was similar to that caused by the negative control serum. These results are shown in Fig 2C. The single donor monocytes were also incubated with twofold serial dilutions of this serum or a negative control serum. Results are shown in Table 3. Due to the strongly decreased aspecific binding of
IgG to the monocytes after the modulation, a significant difference in fluorescence intensity was found. Thus, the sensitivity of the MIFT for detecting IgG alloantibodies was markedly increased by modulation.

**Sensitivity of the MIFT versus MCT, LIFT, and LCT.** To ascertain that HLA class I and II alloantigens were still expressed after modulation, experiments were performed with MoAb against nonpolymorphic determinants of HLA class I and class II molecules. After modulation, the binding of anti-HLA class I MoAb decreased about 30%, while the binding of MoAb against HLA class II molecules remained unaltered (Table 2).

The sensitivity of the MIFT for detecting anti-HLA IgG alloantibodies was compared with that of the MCT, LIFT, LCT, and TCF. Modulated monocytes isolated from single-donor buffycoats were incubated with typing sera in a series of twofold dilutions in negative control serum. The typing sera contained either anti-HLA class I or class II antibodies. Before the investigation, sera with anti-HLA class II antibodies were absorbed with pooled platelets to remove contaminating HLA class I antibodies. Results of these experiments are shown in Table 4. The titers of all the typing sera except one (anti-DQw1) were higher in the MIFT than in the MCT, LCT, and/or TCF and those of the anti–HLA-A2 and anti–HLA-Cw1 sera were also higher than those in the LIFT.

We observed that the specificity of the typing sera as defined in LCT or TCF was not identical to that found in the MIFT (Table 4). This finding could be due either to a higher sensitivity of the MIFT or to the presence of noncomplement-fixing alloantibodies with a different specificity.

**Complement-binding versus noncomplement-binding alloantibodies.** As mentioned before, some lymphocyte-reactive IgG alloantibodies are not capable of activating complement. Therefore, such antibodies will not be detected in techniques based on complement activation. This has been shown in the LIFT in comparison with the LCT. We investigated whether such antibodies reacted with monocytes, ie, whether they could be detected with the MIFT but were also noncomplement-fixing when bound to monocytes. Therefore, we selected sera from six patients with febrile transfusion reactions, which were positive in the LIFT but negative in the LCT. These sera were tested in the MIFT and the MCT. In all cases, IgG alloantibodies were detectable in the MIFT, while the MCT was negative.

**MIFT for detecting monocyte-reactive IgM alloantibodies.** Tests were performed with sera of pregnant women and negative control sera using modulated and nonmodulated monocytes. The mean background immunofluorescence in four assays using an anti-IgM conjugate was 43 ± 3.2. This value is much lower than that found with an IgG conjugate. No effect of the modulation was seen. Positive reactions were found with 2 of 10 sera, whereas no monocyte-reactive IgG antibodies were detectable. No further tests were performed to identify the nature of these antibodies.

**Monocyte-specific blood group antigens.** Jager et al showed the existence of a biallelic monocyte-specific antigenic system that was named HMA. HMA typing sera were tested in MIFT after absorption with pooled platelets on pooled monocytes. A bimodal curve was obtained with both the anti-HMA1 and the anti-HMA2 sera. There was no reactivity with the lymphocytes from the same donors. The calculated phenotypic frequencies of HMA1 and HMA2 (0.55 and 0.60, respectively) are close to those found by Jager et al, which suggests that the positive reactions obtained with these typing sera were caused by anti-HMA alloantibodies. No testing with monocytes of single donors was possible, because insufficient typing sera were available.

**Sharing of neutrophil antigens.** The expression of neutrophil-specific polymorphic antigens defined by human anti-sera on monocytes has not been investigated in detail. With the cells of six typed donors, we have studied in the MIFT whether these systems are present on monocytes. The NA/NA$_2$ system antigens were not expressed on monocytes. Neither the NB nor the ND, antigens were found to be present on monocytes from donors whose neutrophil granulocytes were positive for these antigens. Anti-Sa/Sb sera did not react with monocytes from positive donors. Positive reactions were found with some donors not related to their Sa/Sb type, suggesting the presence of contaminating antibodies. These sera remained positive in immunofluorescence with monocytes and granulocytes after platelet absorption. No further tests were performed to identify the nature of these antibodies.

**ABo blood groups.** With four sera containing anti-A.
and four sera containing anti-B alloantibodies of the IgG as well as the IgM class, as determined by routine serology, no positive results were obtained with an anti-IgG nor with an anti-IgM conjugate in the MIFT with monocytes from donors with blood group A and B, respectively. We conclude from these results that the A and B antigens are not significantly expressed on monocytes.

Polymorphism of the LFA antigens. Recently, a new polymorphic determinant has been described on the LFA α1-chain. To investigate whether the MIFT is suitable for the detection of IgG alloantibodies reactive with polymorphic determinants on the LFA complex, we used MoAb to measure the expression of the LFA molecules before and after modulation. All the CD11/18 MoAb remained reactive with the modulated monocytes (Table 3). This result implies that the MIFT may be suitable for detection of LFA-reactive alloantibodies.

The sera Ond and Mart, which contain such alloantibodies, were positive in the MIFT with the cells of all donors tested (n = 64) as well as with all the cells of the pooled monocytes. The mean fluorescence intensity ranged from 290 to 460 and from 642 to 1,276 obtained with Ond serum and Mart serum, respectively.

Occurrence of monocyte-reactive antibodies in patient sera. IgG alloantibodies against not yet defined polymorphic antigens were detected in sera from four patients with a kidney graft transplantation with unexplained periods of rejection. Kidney donors were HLA-identical with their recipients and no donor-reactive anti-HLA antibodies could be detected in techniques based on complement activation. The lymphocyte crossmatches were also negative. However, one serum was known to contain antibodies reactive with endothelial cells.

The sera were tested in the MIFT before and after absorption with pooled platelets. Three of the sera were positive before absorption and two of these sera remained positive after absorption. These latter two were also tested for reactivity with endothelial cells from a single donor (human umbilical vein endothelial cells [HUVEC]) and gave positive reactions using an anti-IgG conjugate (C. Hoek, personal communication, May 29, 1989).

DISCUSSION

Two different Fc receptors for IgG are expressed on the membranes of peripheral blood monocytes, the FcRI (CD64 antigen) and the FcRII (CD32 antigen) (reviewed by Anderson and Looney). These receptors play a role in the recognition and binding of IgG-sensitized target cells and immune complexes, resulting in the activation of extracellular cytotoxic lysis and phagocytosis. The FcRI is a 70-kD glycoprotein with a high affinity for monomeric IgG (Ka = 5 × 10^9 M^-1) with approximately 45,000 molecules per monocyte. The FcRII is a 40-kD glycoprotein with no detectable affinity for monomeric IgG, but immune complexes will readily bind to it. The high affinity of the FcRI causes "aspecific" binding of IgG; this has previously made monocyte immunofluorescence impracticable as a serologic test.

Our results indicate that 'downmodulation' of the FcRI with anti-FcRI MoAb and goat anti-MoAb is possible. The FcRI is no longer detectable with MoAb against the FcRI, either with MoAb 10.1 used for the modulation or with MoAb 32.2 directed against a different epitope on the FcRI. After modulation, the aspecific binding of IgG was reduced to such an extent that demonstration of specific binding of IgG alloantibodies became possible. We showed the necessity of an active modulation of the FcRI by showing that sequential incubation with MoAb 10.1 and goat anti-MoAb of prefixed monocytes did not affect the expression of the FcRI as measured with MoAb 32.2. Moreover, background binding of monomeric IgG was also not diminished using prefixed monocytes.

To study the selectivity of modulation, the expression of other membrane antigens was measured with a panel of MoAbs. Some decrease of the FcRII expression was found. Yet, complete comodulation of the FcRII could be induced only by using an anti-FcRII MoAb together with an anti-FcRI MoAb before the cross-linking. However, this did not result in a further reduction of the fluorescence intensity obtained with negative control sera and, therefore, does not contribute to the sensitivity of the MIFT (data not shown). The binding of the IgG2a anti-FcRII MoAb CLB-gran/1 also diminished. However, a low but specific expression of FcRII could then be detected, as has also been reported by others.

The expression of HLA and LFA (CD11a, b, c, and CD18) antigens remained high, although the fluorescence intensity caused by the W6/32 MoAb directed against HLA class I glycoproteins decreased about 30%. In part, this result could be due to a decreased aspecific binding of this IgG2a MoAb. However, the extent of the decrease suggests that some comodulation of HLA class I molecules may have occurred as well.

Although a decrease of HLA class I antigens was found, our results show that after FcRI modulation specific binding of anti-HLA class I IgG alloantibodies could easily be shown. Also, human alloantibodies against HLA class II antigens gave strongly positive results as compared with negative control sera. HLA class I and class II antibodies could be detected even in a more sensitive manner compared with other techniques, such as MCT and TCF. Thus, modulated monocytes can be used for HLA typing of class I and class II antigens. However, because in established typing sera also noncomplement-fixing HLA antibodies are detected in the MIFT, which are not detected in the cytotoxic tests used to select such sera, they should be checked for the presence of such "contaminating" antibodies. The detection of noncomplement-fixing class I antibodies is also possible by LIFT, but not class II antibodies. Because such antibodies may be involved in transfusion reaction or graft rejection, an assay to show them has potential clinical value.

Apart from HLA antibodies, also non-HLA monocyte-reactive antibodies exist. These are directed against monocyte-specific antigens or antigens shared by monocytes and other cells. The only monocyte-specific antigens reported so far are the HMA antigens. The MCT was used to define these antigens. Unfortunately, the HMA-antigen...
system could not be investigated in detail because of a shortage of test sera. However, our results show that anti-HMA sera contain monocyte-reactive non-HLA class I antigens with a population reactivity that is concordant with the HMA-phenotype frequencies. Thus, the MIFT is a suitable method to detect antibodies against HMA antigens as well. Antisera against the 9a antigen, said to be similar to the HMA-1 antigen, did not react positively in the MIFT. However, these antisera have been shown to be nonreactive in granulocyte immunofluorescence as well.

The LFA glycoprotein complex (CD11/18 complex) is expressed on monocytes and consist of gp180/95, gp155/95, and gp150/95 molecules, also denoted as αβ, αβ, and αβ, respectively. A polymorphic determinant has been described on the α chain as detected by the serum E27 obtained from a multiple transfused patient with SLE. A serum from a multiple transfused patient (Ond) identified in our own laboratory appeared to have the same specificity as E27. Furthermore, we found that serum Mart, which reacts with an alloantigen of neutrophils, monocytes, and a subset of lymphocytes, is directed against a polymorphic determinant of the α chain (Van der Schoot et al, submitted). In the present study, we show that these sera are not only applicable for granulocyte serology, but also in monocyte immunofluorescence.

No expression of the neutrophil-specific NA antigens was found on monocytes. This result is compatible with the finding that monocyte/macrophage FcRIII is the transmembrane form and is different from the PIG-linked neutrophil FcRIII, which express the NA1/NA2 epitopes.

Other neutrophil-specific antigens could not be detected in the MIFT either. The 5a/5b-typing sera reacted positively with the monocytes of some of the donors different of their granulocyte phenotype. This result was probably due to contaminating alloantibodies of different specificity. After absorption with platelets, concordant reactions were found with the granulocytes of all donors. This result indicates that the 5a/5b antigens are not expressed by platelets as has been suggested, which implies that 5a/5b antigens are neutrophil-specific.

ABO antigens are expressed not only by red cells, but by other blood cells as well. On platelets, the ABO antigens are weakly expressed. No ABO antigens have been shown on neutrophils. With the MIFT, we studied the status of the ABO antigens on monocytes. We could not show any binding of IgG anti-A or anti-B nor of IgM anti-A or anti-B alloantibodies to monocytes. Thus, like neutrophils, monocytes do not carry ABO blood group antigens, as has also been reported before by Dunstan et al.

Using an anti-IgM conjugate we were also able to detect monocyte-reactive IgM alloantibodies. The specificity and clinical relevance of these antibodies is not clear and has not been investigated so far. Modulation of the FcRII does not increase the sensitivity of the MIFT to detect these antibodies. This finding is not surprising because the FcRII does not bind IgM and no IgM receptor has been reported to be expressed by monocytes. It is possible, though, to use modulated cells for the demonstration of monocyte-reactive IgM alloantibodies as well.

With the MIFT we could detect monocyte-reactive antibodies in sera from patients with a kidney graft rejection. Especially in the case of transplant rejection of an HLA-matched donor organ, minor transplantation antigens can be of importance. The role of monocyte-specific antigens is not established so far, mostly because of the intricate procedures to show them. The underlying mechanism by which monocyte-reactive alloantibodies could be able to induce a graft rejection is not clearly understood either. An attractive theory is that these antigens are shared with endothelial cells. The reaction with HUVEC of two sera supports this "endothelial cell/monocyte antigen" theory. However, studies with cells of the donors should be performed to clarify this issue.

In conclusion, we describe a new and simple immunofluorescence assay for the detection of membrane-bound monocyte-reactive human antibodies. The assay is sensitive and reproducible. With this assay, monocyte-reactive alloantibodies that are of clinical importance can be detected and characterized in more detail. Moreover, monocyte-specific antigen systems can be (re)defined using this method.

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