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Characterizing the individual B cells that participate in the production of anti-HLA Abs requires isolation and culture of these cells and a suitable assay for detection of Abs produced in these B cell cultures. We previously showed that B cell precursors, programmed for anti-HLA Ab secretion, are present at measurable frequencies in peripheral blood of women immunized by pregnancy. In this study, we show that tetrameric HLA-A2, although designed for characterization of CTLs, provides a suitable affinity ligand for isolation of alloantigenic B cells, which subsequently can be induced to produce HLA-A2 Ab in a CD40-driven culture system. The validity of this concept was established by assaying human hybridomas, producing anti-HLA Abs, for specific tetrameric HLA-A2 binding. The availability of anti-HLA Ab-producing B cell cultures that are established without immortalization will be of value when T-B cell interaction is studied at an alloantigen-specific level. The Journal of Immunology, 2003, 171: 6599–6603.

The characterization of B cells that participate in a specific Ab response is hampered by their low frequency even during active immunization. In attempts to circumvent the constraints imposed by low frequencies, investigators have turned to immortalization of B cells by EBV transformation or hybridoma technology and selection of EBV lines or hybridomas based on the specificity of the secreted Ab. Beside their low efficiency, these approaches do not provide a genuine representation of the entire B cell response as immortalization is confined to certain subpopulations of B cells (1, 2). Ag-guided selection of B cells provides the yields that are necessary to establish specific Ab-producing B cell lines without the need for immortalization (3).

MHC class I tetramers have emerged as powerful means for characterization of Ag-specific T lymphocytes. CTLs, responsible for a variety of immune responses have been identified, quantified, and isolated using tetramers that included peptides derived from specific pathogens, even when the frequency in the peripheral circulation is low (reviewed in Ref. 4). Historically, the interaction of MHC class I with serum alloantibodies, induced by pregnancy or transfusion, was the basis for definition of the MHC system in humans (5); however, the lack of a suitable synthetic ligand was an obstacle for the characterization of B cells secreting these alloantibodies. We reasoned that tetrameric MHC class I, irrespective of the peptide bound in its groove, could be exploited to isolate allo-MHC-reactive B cells from women immunized by pregnancy. The availability of alloantigen-reactive B cell lines, established without immortalization, enables the study of interaction of these B cells with T cell populations with similar alloreactivity.

Short-term culture of affinity-isolated, Ag-specific B cells is facilitated in CD40/CD40 ligand-driven systems (6–8). The EL4.B5 system (6), which is based on CD40-CD40 ligand interaction, has enabled the characterization of affinity-isolated, single B cells producing Ab against U1A protein (9) and tetanus toxoid (10). In a previous study, we used the EL4.B5 system to culture B cells of anti-HLA Ab seropositive, multiparous females in limiting dilution format. This enabled us to determine B cell precursor frequencies (BCPF) toward HLA class I Ags (11). In the present study, we used tetrameric HLA-A2 as an affinity ligand to identify and isolate alloantigen-specific B cells and confirmed their HLA-A2 specificity by induction of specific Ab secretion in culture. To verify the validity of the concept of interaction of tetrameric MHC class I molecules with specific B cells, we showed that human monoclonal HLA-A2 Ab-producing hybridomas are specifically stained with tetrameric HLA-A2.

Materials and Methods

Tetrameric HLA-A2

Biotinylated monomeric recombinant HLA-A2 (based on the HLA-A*0201 allelic with a biotinylation site engineered in the α3 domain) containing an HPV16 E7-encoded synthetic peptide, TLGVCPV (aa 86–93) was kindly made available by Dr. F. Romagne (Immunotech, Marseille, France) and was incubated with streptavidin-PE (Sigma-Aldrich, St. Louis, MO) to form tetrameric HLA-A2/HPV. Tetrameric HLA-A2/M1 complex (containing the influenza A matrix 1 protein-encoded peptide, GILGFVFTL), tetrameric HLA-A1/HPV complex (containing the male HY-encoded peptide, IVDCIITEMY) and monomeric HLA-A2/HA1 complex (containing the minor histocompatibility Ag, HA1-encoded peptide, VLHDDILLEA) were produced locally according to Ref. 12.

Hybridomas

Hybridomas were derived from anti-HLA Ab-seropositive women by EBV transformation, followed by electrofusion of Ab-secreting EBV lines and subcloning. Human HLA mAb specificities had been determined by complement-dependent cytotoxicity (CDC) against large panels of serologically HLA-typed cells. mAbs produced by these hybridomas were used as crude supernatants.

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B cell isolation

Donor blood was obtained with informed consent under guidelines issued by the Medical Ethics Committee of the Leiden University Medical Center (Leiden, The Netherlands). Mononuclear cell fractions were isolated by ficoll-adiutorzio centrifugation. B cells were immunomagnetically isolated by positive selection (Dynal, Oslo, Norway) and the CD19 positively selected cells were released from the beads using Detach-a-Bead (Dynal). Alternatively, B cells were isolated with a Dynal kit based on depletion of non-B cells (negative selection). Yields of positive and negative selections were 98.5 ± 0.5% (n = 6) and 93.8 ± 1.3% (n = 2) pure B cells, respectively, as assessed by staining with a FITC-labeled anti CD19 mAb (BD Biosciences, Mountain View, CA) and flow cytometric analysis of lymphocyte gated cells on a FACSVantage SE (BD Biosciences) equipped with CellQuest software.

Flow cytometric staining with tetrarmers

CD19+ B cells, enriched by positive selection, were prepared for flow cytometry by incubation with MHC class I tetrarmers or streptavidin-PE at room temperature. Hybridomas were incubated with FITC-labeled F(ab′)2 of rabbit anti-human IgM Abs or anti-human IgG Abs (DAKO, Glostrup, Denmark) or tetrameric HLA-A2/M1 on ice. Rabbit anti-human IgG or IgM Abs used for blocking tetramer binding were from DAKO.

B cell isolation

B cells were isolated with the positive or negative selection methods as described above and were incubated with tetrameric HLA-A2/HPV (100 ng/106 cells) on ice for 40 min, washed, and aseptically sorted for PE staining in a FACSVantage SE (BD Biosciences). Cells with PE staining intensity exceeding channel number 10 were de
tected and seeded directly into 1/well in 96-well plates that had been preseeded with EL4.B5 cells (50 000/well). Supernatants from hybridomas were determined by ELISA (11).

Ig determination

IgG and IgM concentrations in B cell supernatants and hybridoma supernatants were determined by ELISA (11). Some supernatants were used for blocking tetramer binding with goat anti-human IgM Abs blocked binding of tetrameric HLA-A2 to anti-HLA-A2 hybridomas ROU2D3 and SN607D8, respectively (data not shown), which indicates that tetramer binds to the hybridomas’ B cell receptor (BCR). Of five hybridomas producing HLA mAbs that did not include HLA-A2 in their specificity patterns, four failed to react with tetrameric HLA-A2. The positive staining of hybridoma BRO11F6, which produces HLA-A11/3 Mab, is not due to interaction of tetrameric with its BCR, since rabbit anti-IgG Abs did not inhibit subsequent binding of the tetrameric HLA-A2. These rabbit anti-IgG Abs showed 50% inhibition of the binding of tetrameric HLA-A2 to hybridoma SN607D8. In addition, the mAb BRO11F6 failed to react with HLA-A2/HA1 monomers bound on streptavidin-coated plates (data not shown). No staining of hybridoma BRO11F6 was observed with streptavidin-PE alone.

Staining of HLA-A2-specific B cells

Three women with HLA-A2 serum Abs and with measurable HLA-A2 BCPF, as determined by limiting dilution culture (11), were tested for the presence of HLA-A2 tetramer-stainable B cells. These women’s B cells were isolated by positive CD19 selection with rabbit anti-human IgM-HRP (Jackson ImmunoResearch Laboratories, West Grove, PA; for IgM-containing supernatants) or goat anti-human IgG-HRP (Jackson ImmunoResearch Laboratories; for IgG), washed, re-
dected with ABTS, and read at 415 nm. Biotin-labeled human mAbs SN66E3 (IgM anti HLA-A2; Table I) and SAL1A6 (IgG anti-RhD, M.E.I.L.F.-V.D and A.M., unpublished data) served as positive controls. As a cutoff value for positivity we used 2× OD caused by culture medium alone.

### Results

#### Hybridoma staining of tetrarmers

As a model system for the interaction of tetrameric MHC class I with alloreactive B cells, tetrameric HLA-A2/M1 was allowed to react with 11 hybridomas. These hybridomas had been rigorously subcloned and were actively producing HLA mAb at the time of testing. Tetrameric HLA-A2/M1 bound to all six HLA-A2 Ab-producing hybridomas (Table I). Examples of two hybridomas are given in Fig. 1. The irrelevant HLA-B17 mAb-producing hybridoma, VN2F1, was not stained by HLA-A2 tetramer (Fig. 1A), although it was surface IgM positive. The anti-HLA-A2 mAb-producing hybridoma ROU2D3 showed a biphasic distribution of tetramer staining (Fig. 1D) and this heterogeneity was also observed when this hybridoma was stained for IgM (Fig. 1C). Spontaneous emergence of negative subclones or asynchrony with respect to cell cycle may underlie this heterogeneity, but this was not further explored. Preincubation with rabbit anti-IgM or anti-IgG Abs blocked binding of tetrameric HLA-A2 to anti-HLA-A2 hybridomas ROU2D3 and SN607D8, respectively (data not shown), which indicates that tetramer binds to the hybridomas’ B cell receptor (BCR). Of five hybridomas producing HLA mAbs that did not include HLA-A2 in their specificity patterns, four failed to react with tetrameric HLA-A2. The positive staining of hybridoma BRO11F6, which produces HLA-A11/3 Mab, is not due to interaction of tetrameric with its BCR, since rabbit anti-IgG Abs did not inhibit subsequent binding of the tetrameric HLA-A2. These rabbit anti-IgG Abs showed 50% inhibition of the binding of tetrameric HLA-A2 to hybridoma SN607D8. In addition, the mAb BRO11F6 failed to react with HLA-A2/HA1 monomers bound on streptavidin-coated plates (data not shown). No staining of hybridoma BRO11F6 was observed with streptavidin-PE alone.

#### Staining of HLA-A2-specific B cells

Three women with HLA-A2 serum Abs and with measurable HLA-A2 BCPF, as determined by limiting dilution culture (11), were tested for the presence of HLA-A2 tetramer-stainable B cells. These women’s B cells were isolated by positive CD19 selection

### Table I. Cell surface characteristics of hybridomas

<table>
<thead>
<tr>
<th>Hybridoma</th>
<th>HLA Specificity</th>
<th>Isotype</th>
<th>Fluorescence Channel 1</th>
<th>Fluorescence Channel 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hybridoma</td>
<td></td>
<td></td>
<td>Control</td>
<td>Anti-IgG-FITC</td>
</tr>
<tr>
<td>JOK3H4</td>
<td>A2</td>
<td>IgM</td>
<td>16'</td>
<td>19</td>
</tr>
<tr>
<td>BVK55C4</td>
<td>A9</td>
<td>IgG</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>SN66E3</td>
<td>A2/A28</td>
<td>IgM</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>SN230G6</td>
<td>A2/B17</td>
<td>IgG</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>OKF23</td>
<td>A3</td>
<td>IgM</td>
<td>15</td>
<td>18</td>
</tr>
<tr>
<td>VN2F1</td>
<td>B17</td>
<td>IgG</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>ROU2D3</td>
<td>A2/B17</td>
<td>IgM</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>HA5C2</td>
<td>A2/A28</td>
<td>IgG</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>SN607D8</td>
<td>A2/A28</td>
<td>IgG</td>
<td>19</td>
<td>62</td>
</tr>
<tr>
<td>VTM1F11</td>
<td>B27/B27/B60</td>
<td>IgG</td>
<td>11</td>
<td>22</td>
</tr>
<tr>
<td>BRO11F6</td>
<td>A11/A3</td>
<td>IgG</td>
<td>14</td>
<td>43</td>
</tr>
</tbody>
</table>

*Mean fluorescence was calculated from fluorescence histograms that were recorded for FITC (channel 1) and PE (channel 2). A ratio (reagent:control) of 1.5 or higher was considered positive. Positive reactions are given in bold italics.*
and stained with tetrameric HLA-A2/M1, HLA-A1/HY, or streptavidin-PE and compared with similarly stained B cell suspensions from three men who had been typed as HLA-A2 positive (Table II). Examples of staining patterns are given in Fig. 2. The proportions of specific tetramer staining of B cells in HLA-A2-immunized women (0.110 ± 0.044%) were not significantly higher than those in unimmunized men (0.083 ± 0.012%), while tetrameric HLA-A1/HY or streptavidin-PE alone caused background staining (means, 0.01 and 0.02%, respectively). Linear regression analysis showed no correlation between HLA-A2 BCPF and the proportions of tetrameric HLA-A2/M1 staining of B cells of these three women. Clearly, the proportion of B cells stainable with tetrameric HLA-A2 was considerably higher than that found producing HLA-A2 Ab in culture, by factors of 18 and 13, for the two women with the highest BCPF.

Culture of tetrameric HLA-A2-sorted cells

In two experiments, B cells from one individual (Table II, donor 1) were isolated by positive or negative isolation, respectively. B cells were stained with tetrameric HLA-A2/HPV, sorted, and cultured. This woman had developed CDC-reactive HLA-A2 Abs due to three pregnancies with HLA-A2-positive children and had a HLA-A2-specific BCPF of 77/10^6 B cells (11) at 20 mo after the third delivery. A total of six HLA-A2 Ab-producing B cells were found in two experiments, when supernatants were tested by CDC against cells of two HLA-A2-positive donors (Table III). Positive selection, followed by tetrameric HLA-A2 staining and subsequent sorting, yielded two HLA-A2-reactive wells, both containing IgM, while negative selection followed by tetrameric HLA-A2 staining and subsequent sorting yielded four HLA-A2-reactive wells, three of which were IgG and one was IgM. Of six HLA-A2 Ab-producing B cell lines, four produced Ab at both sampling days. In the remaining two wells, anti-HLA-A2 Ab could be demonstrated at only one of the sampling days (one at day 9 and one at day 16). The average percentage of CDC on HLA-A2-positive PBLs caused by all positive supernatants was considerable: 73 ± 22%, while CDC against the autologous PBLs was 0%. To test whether cell sorting had produced Ig-positive wells, that were nevertheless CDC negative, all supernatants of experiment 3, and a large fraction of experiment 4, were analyzed by ELISA for IgG and IgM content (Table III). IgM-containing supernatants were found at higher frequencies than IgG supernatants in both experiments. This unequal frequency distribution was also found when unsorted B cells were cultured at 1/well and screened for IgG/IgM production (data not shown).

We suspected that the streptavidin moiety of the tetrameric HLA-A2 complex had been responsible for sorting at least a fraction of B cells producing the Ig-containing, but CDC-negative supernatants. Indeed, of 61 positively selected B cells, sorted with tetrameric HLA-A2, that produced either or both IgG and IgM (but not anti-HLA-A2 Abs), 2 streptavidin-reactive supernatants (1 IgG and 1 IgM) emerged, and 30 negatively selected B cells, when sorted likewise, yielded 1 IgG and 2 IgMs with streptavidin reactivity. The specificity of the majority of Ab-producing B cells remains undefined.

The proportion of HLA-A2 specific within the total unsorted Ig-producing B cells was 179/10^6 as calculated from the HLA-A2-specific B cell BCPF (77/10^6 B cells) and the frequency of total Ig-producing cells (429,000/10^6 B cells when tested by limiting dilution at low B cell density). Tetrameric HLA-A2 sorting permitted the isolation of HLA-A2-specific B cells at high efficiency: upon tetramer sorting, the proportions of HLA-A2-specific B cells within the Ig-producing B cells were 31,700/10^6 and 62,500/10^6 in two experiments (Table III). Thus, we have achieved by tetramer sorting a 176-fold enrichment of HLA-A2-specific B cells in the positively selected cells, and a 349-fold enrichment of HLA-A2 B cells in the negatively selected cells. In contrast, only a proportion of sorted B cells ended up producing Ig (13 and 18% in these two experiments, respectively), which decreased the overall efficiency of the procedure. Further inefficacy may be due to the use of CDC for detection of HLA-A2 Abs: some HLA-A2-binding Abs may have escaped detection due to the inability to activate complement.

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**Table II. Tetrameric HLA-A2 staining of B cells**

<table>
<thead>
<tr>
<th>Donor</th>
<th>HLA-A2 BCPF</th>
<th>Tetramer HLA-A2/M1</th>
<th>Tetramer HLA-A1/HY</th>
<th>Streptavidin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>77</td>
<td>0.14</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>2</td>
<td>119</td>
<td>0.13</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>0.06</td>
<td>0.00</td>
<td>0.02</td>
</tr>
</tbody>
</table>

* B cell purities were 98.6 ± 0.1 % (SD) as determined with anti-CD19 FITC staining of 100,000 cells; B cells (430,000–730,000) were stained with 40–70 ng of MHC class I tetramers or streptavidin-PE.

* HLA-A2 BCPF was determined as in Ref. 11 and expressed as number of cells per 10^6 B cells seeded.

* Results expressed as percentage of cells stained with FL2 intensity higher than channel 90. The number of gated cells ranged from 77,022 to 203,574 (mean, 141,067).

* For comparison, HLA-A2/M1 tetramer staining of B cells of three male individuals (all typed as HLA-A2) was 0.08 ± 0.01%.
FIGURE 2. Tetramer staining of B cells. B cells were isolated by positive selection from an HLA-A2-immunized female (donor 1, A–C) or a male donor (D–F). Then, 7 × 10^6 B cells were incubated with 20 ng of streptavidin PE (A and D) or 70 ng of tetramer HLA-A2/M1 (B and E) or 70 ng of tetramer HLA-A1/HY (C and F), washed, and fixed with paraformaldehyde; 1.5 × 10^5 gated cells were analyzed.

Discussion
Germinal center formation requires the interaction of follicular dendritic cells, Th cells, and naive B cells as well as Ag and results in the production of Ag-specific plasma cell precursors, which further differentiate in the bone marrow, and memory B cells that survey for Ag in the periphery (14). By CD40 ligation and cytokine stimulation, peripheral memory B cells can be driven in vitro to proliferate and secrete Ig (15). The level of Ig secreted by individual B cells in the EL4.B5 system is sufficiently high for detection of specific Ab.

To study the interaction between T cells and B cells at the Ag-specific level, as occurring in the germinal center reaction, requires establishing long-term cloned B cell lines with Ag specificity, besides T cell clones. In this study, we describe anti-HLA Ab-secreting B cells established without immortalization.

Tetrameric recombinant MHC class I complexes are instrumental for Ag-specific T cell analysis (12), bypassing limiting dilution culture. Although tetramers have been used in syngeneic situations, recently the isolation of alloreactive, peptide-selective T cells from alloactivated bulk cultures has been reported (16). The present study further extends the range of applications for tetrameric HLA class I molecules in B cell immunology. Although we have not examined monomeric HLA-A2 for this purpose, the use of the tetrameric form likely is the key to efficient BCR recognition, as was recently demonstrated for the identification in mice of B cells reactive with tetrameric peptides after immunization with peptide conjugates (17). Similar to findings with CTLs, we observed a discrepancy between the proportion of B cells stainable with tetrameric HLA-A2 and the frequency of anti-HLA-A2 Ab-producing B cells. Limiting dilution analysis underestimates the frequency of specific T cell responses by 1–2 logs due to the inability of a proportion of CD8^-tetramer-stainable T cells to proliferate in culture (18), and, likewise, a proportion of tetrameric HLA-A2-stainable B cells might fail in CD40-driven culture. Taking advantage of the relatively high HLA-A2-specific BCPF in a donor, we showed that isolation of B cell clones with alloantibody production is now within reach.

The reactivity of anti-HLA-A2 Abs in serum with tetrameric HLA-A2 has recently been shown by Barnardo et al. (19) and was independent of the peptide incorporated in the tetrameric complex. Our assumption that tetrameric HLA-A2 recognizes HLA-A2-specific BCRs appears to be valid and this recognition is independent of Ab fine specificity (such as the serologically recognized anti-HLA-A2, A2/A28, and A2/B17 specificities), which was demonstrated with HLA-A2-specific hybridomas. However, peptide selectivity by mAb and alloreactive B cells cannot be ruled out (20). Thus, in our affinity isolation procedure, B cells producing certain anti-HLA-A2 alloantibodies may have been specifically enriched or ignored.

Although useful for specific B cell sorting, tetrameric HLA class I appears not to be suitable for direct identification of alloantigen-specific B cells by flow cytometry in unsorted B cell suspensions of HLA-immunized individuals. The interference of background staining caused by streptavidin, which is observed in nonalloimmunized individuals, may be circumvented by immunomagnetic depletion with streptavidin beads before specific staining, but we did not attempt this in the present study. The introduction of a cell-sorting step with tetrameric HLA-A2, followed by CD40-driven culture enabled the isolation of HLA-A2-specific B cells, separate from the streptavidin-reactive B cells.

Table III. HLA-A2 tetramer sorting of B cells

<table>
<thead>
<tr>
<th>Expt.</th>
<th>B Cell Isolation by</th>
<th>No. of Wells Seeded^a</th>
<th>IgG</th>
<th>IgM</th>
<th>totalG+M</th>
<th>IgG HLA-A2-Ab</th>
<th>IgM HLA-A2 Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Positive selection</td>
<td>470</td>
<td>26</td>
<td>39</td>
<td>63^b</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>Negative selection</td>
<td>384</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2^c</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Negative selection</td>
<td>181</td>
<td>11</td>
<td>21</td>
<td>32</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

^a B cells from donor 1 were stained with tetrameric HLA-A2/HPV.

^b Of which two wells were found containing both IgG and IgM.

^c These two HLA-A2 Ab-positive wells tested for IgM and IgG retrospectively.
The availability of HLA-specific B cell lines permits the analysis of their Ig H and L chain genes in terms of V family usage. Such analyses have been reported for specific B cells in other antigenic systems (9), but for allografts these studies have been done on established hybridomas (21, 22). The anti-HLA-A2 repertoire has been studied by phage display (23); however, this technology is unable to distinguish correct pairing of H and L chain genes within single B cells.

In summary, we developed a method for culturing HLA-A2-specific B cells that are isolated by interaction with tetrameric HLA-A2 and that bypasses classic approaches such as EBV transformation and hybridoma formation.

Acknowledgments

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