Adhesion of Human B Cells to Follicular Dendritic Cells Involves both the Lymphocyte Function-associated Antigen 1/Intercellular Adhesion Molecule 1 and Very Late Antigen 4/Vascular Cell Adhesion Molecule 1 Pathways

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Summary

Presentation of antigen in the form of immune complexes to B lymphocytes by follicular dendritic cells (FDC) is considered to be a central step in the generation of memory B cells. During this process, which takes place in the microenvironment of the germinal center, B cells and FDC are in close physical contact. In the present study, we have explored the molecular basis of FDC-B cell interaction by using FDC and B cells derived from human tonsils. We found that FDC express high levels of the adhesion receptors intercellular adhesion molecule 1 (ICAM-1 [CD54]) and vascular cell adhesion molecule 1 (VCAM-1), while the B lymphocytes express lymphocyte function-associated antigen 1 (LFA-1 [CD11a/18]), very late antigen 4 (VLA-4 [CD49d], and CD44. Furthermore, we established that both the LFA1/ICAM-1 and VLA-4/VCAM-1 adhesion pathways are involved in FDC-B lymphocyte binding, and therefore, these pathways might be essential in affinity selection of B cells and in the formation of B memory cells.
sion molecules on FDC and hence a role of both these molecules as ligands for lymphocyte binding to FDC. Indeed, recently it was shown that isolated FDC express ICAM-1 (34). In the present study, we have explored the molecular basis of FDC–B cell binding by analysis of adhesion receptor expression on isolated FDC and by studying FDC–B cell clustering. The results show that both the LFA-1/ICAM-1 and VLA-4/VCAM-1 pathways are involved in adhesion of B cells to FDC.

Materials and Methods

Monoclonal Antibodies. The mAbs used were NKI-P2 (IgG1) reactive with CD44 (35); CLB-LFA-1/1 (IgG1) and CLB-LFA-1/2 (IgG1) specific for the β and α subunits of LFA-1, respectively (17); W6/32 (IgG2a) specific for a nonpolymorphic determinant of HLA-ABC (36); F10.2 and RR/1 (IgG1) specific for ICAM-1 (37); 2G7 (IgG1) and 4B9 (IgG1) specific for VCAM-1 (30, 31); 7A9 (IgG1) specific for ELAM-1 (31), HPl/3 (IgG3) and HP2/1 (IgG1) specific for the α subunit of VLA-4 (38); CLB-CD2 (IgG1); CLB-CD3 (IgG1); CLB-CD45 (IgG1) (CLB, Amsterdam, The Netherlands); and DRC-1 (IgM) reactive with FDC (39).

FDC Isolation. FDC were isolated as described previously (40, 41). In brief, freshly obtained tonsillar tissue was dissected free from surface epithelium, and cubes (1 mm³) of lymphoid tissue were incubated under continuous rotation for 30 min at room temperature in Opti-MEM (Gibco Laboratories, Paisley, UK) containing 15 U/ml collagenase (type XI, Sigma Chemical Co., St. Louis, MO), 0.025 U/ml DNase (Boehringer, Ingelheim, FRG), 0.5% BSA (RIA grade, Sigma Chemical Co.). Next, the cells were centrifuged for 10 min at 600g. The pellet was resuspended in Opti-MEM, and subjected to 1g sedimentation (30 min, 0°C) in a discontinuous BSA gradient consisting of layers of 1.5, 2.5, 5.0, and 7.5% BSA. After centrifugation for 10 min at 600g, T cells were pelleted, resuspended in Opti-MEM (Gibco Laboratories, Paisley, UK) containing 0.025 U/ml DNAse (Boehringer, Ingelheim, FRG), 0.5% BSA (RIA grade; Sigma Chemical Co.) and 1% PHA (W6/32 (IgG2a) specific for an nonpolymorphic determinant of HLA-ABC (36); F10.2 and RR/1 (IgG1) specific for ICAM-1 (37); 2G7 (IgG1) and 4B9 (IgG1) specific for VCAM-1 (30, 31); 7A9 (IgG1) specific for ELAM-1 (31), HPl/3 (IgG3) and HP2/1 (IgG1) specific for the α subunit of VLA-4 (38); CLB-CD2 (IgG1); CLB-CD3 (IgG1); CLB-CD45 (IgG1) (CLB, Amsterdam, The Netherlands); and DRC-1 (IgM) reactive with FDC (39).

Results

Expression of Adhesion Receptors on FDC. In accordance with previous reports (32, 33), immunoperoxidase staining of cryostat sections of human tonsils and lymph nodes with anti-ICAM-1 and anti-VCAM-1 mAbs yielded a typical reticular staining of the germinal centers, which suggested the expression of both molecules on FDC. This staining pattern was not observed with mAbs against the other adhesion receptors studied, i.e., with anti-LFA-1, VLA-4, CD44, and endothelial cell adhesion molecule 1 (ELAM-1), which stained variable combinations of lymphocytes, macrophages, and endothelial cells (data not shown).

To discriminate more rigorously between FDC staining and staining of germinal center lymphocytes, cyto centrifuge preparations of tonsillar FDC isolates were double stained with the FDC-specific mAb DRC1 and mAbs against the above-mentioned adhesion receptors, using a combination of FITC- and TRITC-conjugated isotype-specific second-step antibodies. The FDC isolates contained ~30% FDC, i.e., large DRC1-positive, large bimucolated cells with dendritic processes. The contaminating cells consisted almost entirely of B cells (CD19⁺) and a few T cells (CD3⁺).

FDC–B Cell Adhesion Assay. Isolated FDC preparations were seeded in 96-well flat-bottomed microtiter plates. Per well, 2 x 10⁵ cells in 100 µl Hepes-buffered RPMI 1640 (Gibco Laboratories) containing 10% FCS (HyClone Laboratories, Inc., Logan, UT) were incubated for various times at 37°C in the absence or presence of mAbs. All mAbs were titrated to give optimal inhibitory effects. The bivalent cation requirement was studied by washing the cells in HBSS without calcium and magnesium (Gibco Laboratories) containing 2 mg/ml d-glucose. Next, the cells were plated and 1 mM calcium chloride (Merck, Darmstadt, FRG) or 1 mM Magnesium chloride (Merck) was added. The metabolic requirements for adhesion were studied by adding 0.1% sodium azide (Merck) in combination with 50 mM 2-deoxy-d-glucose (Aldrich Chemical Co., Inc., Milwaukee, WI) to the wells.

FDC–B cell adhesion was measured in a semiquantitative manner, analogous to the method described by Rothlein et al. (37) for quantifying homotypic cell aggregation. This method was modified to express adequately the amount of FDC–B cell clustering. Adhesion was scored as: 0, no cell aggregation observed; 1, <20% of the B cells forming small clusters with FDC; 2, 20–50% of the B cells forming clusters with FDC; 3, 50–80% of the B cells were found in FDC clusters; 4, >80% of the B cells were found in FDC clusters; 5, >90% of the cells were found in large FDC clusters.

Immunohistochemistry. A two-step immunoperoxidase staining was performed on acetone-fixed cryostat sections of tonsils and lymph nodes, as described previously (35). All antibodies were diluted to give optimal staining results. The second-step antibody, a horseradish peroxidase-conjugated rabbit anti-mouse IgG antiserum (DAKO, Glostrup, Denmark) contained 5% normal human serum. Sections were routinely counterstained with haematoxylin.

Detection of adhesion molecules on isolated FDC was performed by immunofluorescence double staining on acetone-fixed cytointerface preparations of isolated FDC. Slides were pre-incubated for 10 min with 10% human pool serum diluted in PBS/BSA (1%). Subsequently, the preparations were incubated for 30 min with the mAbs listed in Table 1, washed, and incubated with 1:20 diluted goat anti-mouse IgG-1 FITC (Southern Biotechnology, Inc., Birmingham, AL), followed by 1:5 diluted DRC1 antibody (DAKO), and with 1:100 diluted tetramethyl rhodamine isothio-cyanate (TRITC)-conjugated goat anti-mouse IgM (Southern Biotechnology, Inc.).

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Figure 1. Expression of adhesion molecules on FDC. Cytocentrifuge preparations of isolated FDC were haematoxilin-eosin stained (A, left) or stained for DRC1 using immunoperoxidase (A, right), or double stained for DRC1 and ICAM-1 (B), VCAM-1 (C), or LFA-1 (D) using immunofluorescence. DRC1 is labeled red (anti-mouse IgM-TRITC), while ICAM-1, VCAM-1, and LFA-1 are labeled green (anti-mouse IgG1-FITC) (x350).
Figure 2. FDC-B lymphocyte adhesion assay. FDC and coisolated B lymphocytes were plated in microtiter plates at $2 \times 10^5$ cells per well. Adhesion between B cells and FDC was scored semiquantitatively on a scale ranging from 0 to 5. Typical adhesion patterns scored as 0 (A), 1 (B), 2 (C), 3 (D), 4 (E), and 5 (F) are shown (×200). Adhesion patterns 0, 1, 2, and 3 were recorded during a 3-h adhesion experiment without addition of mAbs. Adhesion patterns 4 and 5 were only observed after incubation with anti-CD44 for 3 and 24 h, respectively.
Table 1. Expression of Adhesion Molecules and Surface Antigens on FDC and Coisolated B Cells

<table>
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<tr>
<th>Antigen</th>
<th>FDC</th>
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<tr>
<td>LFA-1β (CD18)</td>
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<td>LFA-1α (CD11a)</td>
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<td>VLA-4α (CD49d)</td>
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<td>ICAM-1 (CD54)</td>
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- -, negative; +, weak; + +, strong.

FDC–B Lymphocyte Adhesion. When the FDC isolates were kept at 4°C, no cell cluster formation occurred between the FDC and the coisolated B lymphocytes (Fig. 2A). However, upon incubation at 37°C, progressive spontaneous FDC–B cell clustering was observed. This cluster formation reached its maximum within 2–3 h of incubation. The various phases of this clustering process were scored semiquantitatively on a scale from 0 to 5. Typical patterns are shown in Fig. 2. In the presence of sodium azide and 2-deoxy-D-glucose, which like low temperature (4°C) interferes with metabolic energy-dependent processes, no FDC–B cell adhesion occurred. Furthermore, FDC–B cell clustering required divalent cations since no conjugate formation was observed in the absence of calcium and magnesium. Cluster formation was largely restored by magnesium, however, not by calcium (data not shown).

Adhesion Receptors Involved in FDC–B Cell Aggregation. To determine the adhesion pathway(s) involved in FDC–B cell binding, mAb inhibition studies were performed. mAbs against LFA-1α and LFA-1β blocked FDC–B cell adhesion completely (Fig. 3). Antibodies against ICAM-1 (F10.2 and RR1/1), a major ligand of LFA-1, also inhibited FDC–B cell

Figure 3. mAb inhibition studies of FDC–B lymphocyte adhesion. Cluster formation was measured in the absence or presence of mAb against the indicated adhesion receptors. mAbs used were NKI-P2 (anti-CD44); 2G7 (anti-VCAM-1); HPI/3 (anti-VLA-4α); F10.2 (anti-ICAM-1); CLB-LFA1/1 (anti-LFA-1β); and CLB-LFA1/2 (LFA-1α). All mAbs were added in concentrations to give optimal inhibitory effect. mAbs CLB-CD45 (anti-CD45), CLB-CD2 (anti-CD2), and W6/32 (anti-HLA-ABC) were used as additional controls and gave no inhibitory effect. Shown the mean ± SE of three independent experiments.
adhesion, although inhibition by these mAbs was incomplete (Fig. 3). In addition to mAbs interfering with the LFA-1/ICAM-1 pathway, mAbs HP2/1 and HP1/3 against VLA-4, and mAbs 2G7 and 4B9 against its ligand VCAM-1, also inhibited FDC–B cell aggregation, although inhibition by these mAbs was incomplete (Fig. 3). Together, these results indicated that both the LFA-1/ICAM-1 and VLA-4/VCAM-1 pathways were involved in FDC–B cell adhesion. A series of experiments combining antibodies interfering with the LFA-1/ICAM-1 and VLA-4/VCAM-1 pathways further strengthens this conclusion (Fig. 4). Thus, the level of inhibition of FDC–B cell clustering was consistently higher with anti-ICAM-1 in combination with anti-VCAM-1 than with either of the antibodies alone, while the inhibitory effects of anti-VCAM-1 and anti-VLA-4 were not additive. Anti-VLA-4 antibodies in combination with suboptimal (noninhibitory) concentrations of anti-LFA-1α caused strong additive blocking of FDC–B cell adhesion (Fig. 4).

In contrast to mAbs against molecules involved in the LFA-1/ICAM-1 and VLA-4/VCAM-1 pathways, antibodies against CD44 enhanced FDC–B cell clustering (Fig. 3). Like the spontaneous aggregation of FDC and B cells, this induced aggregation could be inhibited with anti-LFA-1 mAb (data not shown). Antibodies against CD2, CD45, HLA-ABC, and ELAM-1 did not affect FDC–B cell adhesion (Fig. 4).

Discussion

Presentation of antigen in the form of immune complexes to B lymphocytes by FDC is thought to be a central step in affinity selection of B cells and in B cell differentiation into memory cells (1–3). The intimate spatial relationship between FDC and B cells, as found in germinal centers (1,5), suggests that initiation of these differentiation steps requires direct FDC–B cell contact. Our present data show that conjugate formation between isolated FDC and B cells depends on at least two different adhesion pathways, i.e., the LFA-1/ICAM-1 and the VLA-4/VCAM-1 pathway. This finding suggests that, in parallel with the requirements for efficient antigen presentation to T cells by macrophages/dendritic cells (11,12,21–24), antigen presentation to B cells by FDC also is not only depend on antigen-specific receptor interactions but on engagement of adhesion receptors as well. These adhesion molecules may help to establish and maintain direct physical contact during interaction of FDC and B cells.

Determination of the antigenic profile of isolated FDC revealed that these cells express high levels of ICAM-1 and VCAM-1 (Fig. 1, Table 1); in contrast, those antigens were not detectable on the coisolated B lymphocytes. On the other hand, LFA-1, VLA-4α, and CD44 were present on the B cells but absent from FDC (Fig. 1, Table 1). These findings suggested that the leucocyte integrins LFA-1 and VLA-4 might both be involved in B cell–FDC binding, since both their ligands, i.e., ICAM-1 and VCAM-1, were present on the FDC. In addition, the metabolic energy and divalent cation dependence of the FDC–B cell interaction was consistent with a role of integrins. Indeed, the results of our blocking studies showed that clustering of FDC and B lymphocytes involves both the LFA-1/ICAM-1 and VLA-4/VCAM-1 pathways (Fig. 3). Antibodies against LFA-1 strongly inhibited conjugate formation of FDC and B cells, while antibodies against the LFA-1 ligand ICAM-1 also inhibited FDC–B lymphocyte interaction, although these antibodies were less effective. Whether this difference in blocking between LFA-1 and ICAM-1 relates to factors such as antibody avidity or reflects involvement of an additional ligand for LFA-1, possibly ICAM-2 (42), is presently unknown. As expected, blocking via the LFA-1/ICAM-1 and VLA-4/VCAM-1 pathways was additive (Fig. 4), a finding that confirms the independent contribution of the two adhesion pathways to the process of FDC–B cell aggregation.

Our present findings greatly extend recent data by Freedman et al. (43). These authors showed that activated B cells bind preferentially to germinal centers in frozen sections of human tonsils. Furthermore, they showed that binding of a cell line (Nalm-6) to germinal centers was completely blocked by anti-VLA-4 and anti-VCAM-1 antibodies. Although this interaction was suggested to involve binding to FDC in the germinal centers, no direct evidence for involvement of FDC was provided. Moreover, the study of Freedman et al. (43) did not reveal a role of LFA-1/ICAM-1 in lymphocyte binding to germinal centers, while our present study indicates that the LFA-1/ICAM-1 pathway is at least as important in FDC–B cell binding as the VLA-4/VCAM-1 pathway. A putative explanation for this discrepancy is that LFA-1 expression per se is not sufficient for effective FDC binding, but that other factors, e.g., other molecules or the state of activation of LFA-1 (12,19,20,22), could also be important. Since Freedman et al. (43) studied a limited number of cell lines, of which only two were LFA-1 positive, these requirements may not have been met.

Our finding that at least two different pathways participate in binding of B lymphocytes to FDC is consistent with many other studies of lymphocyte-cell interaction, which have almost invariably demonstrated involvement of several, sometimes multiple, interaction pathways (11,12,15,17,18,21,44). For instance, CTL require not only the TCR-CD3 complex and the CD8 molecule to interact with their target cells, but also binding of LFA-1 to ICAM-1 and of CD2 to LFA-3 (11,12,21). Similarly, lymphocyte extravasation at sites of inflammation and lymphocyte recirculation are mediated through a series of interactions between cell surface molecules on lymphocytes and endothelial cells. Receptors and ligands involved are LFA-1/ICAM-1 (15,25,26,44), VLA-4/VCAM-1 (27–32), LAM-1 (LECAM-1, MEL-r14) (15,26,42), and CD44 (44,45,46). The fact that multiple receptors are engaged in these lymphocyte interactions does not only allow for sophisticated fine-tuning of these interactions, but, in addition, may have an important back-up function. This latter point is illustrated by the fact that patients with leucocyte adhesion deficiency, which lack expression of LFA-1, show relatively little evidence of lymphocyte dysfunction (46–48).

In marked contrast to the inhibitory effects of anti-LFA-1, ICAM-1, VLA-4, and VCAM-1 mAbs, anti-CD44 mAbs...
promoted FDC–B lymphocyte cluster formation. This anti-CD44-promoted adhesion was completely blocked by anti-LFA-1 mAbs, suggesting that triggering of CD44 molecules can enhance LFA-1-mediated FDC–B cell adhesion, similar to the CD44-induced homotypic T cell aggregation (49). However, further studies are needed to confirm this notion since CD44 can also mediate adhesion via binding to hyaluronate (46, 50, 51).

In conclusion, we have shown that adhesion of human B cells to FDC in vitro involves both the LFA-1/ICAM-1 and the VLA-4/VCAM-1 pathways. These findings suggest that these adhesion pathways may have an important accessory role in FDC–B cell interaction in vivo, and hence, in the process of affinity maturation of B cells and B cell differentiation into memory cells, which takes place in the germinal center.

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