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A Caspase-Independent Pathway of MHC Class II Antigen-Mediated Apoptosis of Human B Lymphocytes

Bernard Dréno,* 2*† Vincent Blancheteau,2* David H. Burgess, † Renée Fauchet, † Dominique J. Charron,* and Nuala A. Mooney3*

MHC class II molecules have a crucial role in thymic selection and in generating Ag-specific T cell responses. There is extensive evidence for second messenger generation via MHC class II molecules, which can lead to apoptosis of B lymphocytes. We have examined HLA class II-mediated apoptosis in both normal and tumoral human B lymphocytes. Phosphatidylinerine exposure and DNA fragmentation were observed in B cells within 24 h of stimulation via HLA class II. In marked comparison with Fas, the cell-permeable and irreversible caspase inhibitors zVAD-fmk and DEVD-fmk failed to inhibit HLA-DR-mediated apoptosis. No direct activation of caspase 3 was detected, and cleavage of pro-caspase 3 was not observed. Cleavage of poly(ADP-ribose) polymerase was detected via Fas but not via HLA class II. Although phosphatidylinositol-3-kinase has been implicated in HLA class I-mediated apoptosis, neither wortmannin nor LY294002 affected HLA class II-mediated apoptosis. CD95-sensitive cells were used to reveal that death occurred independently of CD95-CD95 ligand interactions. Overall, these data reveal a pathway of HLA-DR-mediated apoptosis that neither requires nor involves caspases. Moreover, it is phosphatidylinositol-3-kinase independent and Fas/CD95 independent. This pathway of HLA class II-mediated apoptosis could have an important role in the regulation of APC populations or in the control of malignant B lymphocyte proliferations. The Journal of Immunology, 1999, 163: 4115–4124.

Major histocompatibility complex class II Ags are constitutively expressed in APCs and have a crucial role in generating Ag-specific T cell responses. They can also be considered as activation Ags, because their expression is enhanced by activation of B lymphocytes and de novo expression follows activation of various cell types, including T lymphocytes and fibroblasts. There is extensive evidence for the transmission of signals via MHC class II Ags, leading to the generation of cAMP and nuclear translocation of the serine/threonine kinase protein kinase C (PKC) in the mouse (1). Furthermore, priming of murine B lymphocytes via murine IgM in the presence of IL-4 permits an intracellular calcium flux via MHC class II Ags (2). In human B lymphocytes, stimulation via HLA-DR leads to activation of PKC, tyrosine kinases, phospholipase C, and an intracellular calcium flux (3–5). The intracytoplasmic region of the HLA-DR β-chain is critical for the translocation of PKCβ and PKCβII via the HLA-DR molecule (6). The cytoplasmic domain of the I-Aβ is equally critical for the elevation of cAMP in murine B lymphocytes (7). The protein-tyrosine phosphatase CD45 has recently been shown to have a major role in MHC class II-mediated signals (8).

Apoptosis of B lymphocytes occurs by both Fas/CD95-dependent and -independent mechanisms (9). CD95 ligand (CD95L) is a member of the TNF family that induces apoptosis by binding to CD95. Although the role of CD95 has been most clearly demonstrated in T lymphocytes, one of the first CD95 mAbs was raised against a human B lymphoblast cell line, and the sensitivity of B lymphocytes to Fas was most strikingly shown by the regression of human B-lymphoid tumors grown in nude mice (10).

We have previously described HLA class II-mediated B lymphocyte apoptosis (11). Briefly, induction of apoptosis via DR, DQ, and DP was compared, and significant apoptosis was observed via HLA-DR. Our initial study characterized HLA-DR-mediated apoptosis on the basis of morphologic (cell and nuclear shrinkage in association with enhanced propidium iodide uptake in up to 60% of the population) and biochemical (requirement for cytoskeletal integrity, endonucleases, and serine-threonine phosphatases) criteria. The pattern of DNA fragmentation indicated random DNA fragmentation and differed from either the oligosom al DNA fragmentation typical of apoptosis via death receptors or the extremely limited DNA damage observed during necrosis. Small, dense B lymphocytes became more susceptible to HLA-DR-mediated apoptosis after activation with a phorbol ester.

We have also described a HLA-DR-mediated pathway of apoptosis involving Fas/CD95-Fas/CD95L interactions (12). Induction of CD95-CD95L in HLA-DR-mediated regulation of hematopoiesis has also been demonstrated in human marrow cells (13). Interaction of the HLA-DR signaling pathway with the Fas signaling pathway was also indicated by the increased sensitivity of B Lymphocytes.
lymphocytes to Fas-mediated death after a previous signal via HLA-DR (12, 14)

Some of the morphologic characteristics of apoptosis have been conserved throughout evolution, and activation of caspases, which are homologues of the Caenorhabditis elegans ced-3 molecule (15), play a prominent role in many well-characterized programmed cell death pathways (15). The Fas-mediated pathway of cell death involves activation of the caspase cascade by recruitment of an adaptor molecule, Fas-associated protein with death domain (FADD). Caspases are cysteinyi aspartate-specific proteinases, are synthesized as proenzymes, and are activated by proteolytic cleavage (16). Nonetheless, there is increasing evidence that programmed cell death can occur in a caspase-independent manner; e.g., CTL-mediated cytotoxicity was not blocked in the presence of caspase inhibitors, although DNA fragmentation was inhibited (17). In another system, caspase inhibition actually led to enhanced necrosis of fibrosarcoma cells treated with TNF (18). Expression of the IFN-inducible promyelocytic leukemia gene induces programmed cell death in the absence of caspase activation (19). It has recently been shown that oligomerization of the adaptor molecule FADD can lead to cell death in the absence of caspase activation (20). These reports provide strong evidence for the existence of a cell death mechanism that is caspase independent.

The present study examines the potential role of caspases in an in vitro model reproducing signals mediated via HLA-DR during Ag presentation. Caspase-independent HLA-DR-mediated apoptosis in human B cells of both normal and tumor origin is demonstrated by comparison with caspase-dependent Fas-mediated apoptosis. CD95 and its ligand are not required, and inhibition of phosphatidylinositol-3-kinase (PI3K) does not alter induction of apoptosis. These data lead us to suggest a direct pathway of B lymphocyte apoptosis via HLA-DR that is conserved in malignant cells and could therefore have a role in regulating the size of lymphocyte populations.

### Materials and Methods

#### Cells

Characteristics of all of the cells used in this study are summarized in Table I.

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| Nalm-6 | 100       | 0/0  | 100 | 100| 100 | 0    |      |      |      |      |
| Raji   | 100       | 0/0  | 100 | 100| 100 | 0    |      |      |      |      |
| RJ     | 100       | 0/0  | 100 | 100| 100 | 0    |      |      |      |      |
| Ca     | 100       | NT   | 100 | 100| NT  | 0    |      |      |      |      |
| Jurkat | 0         | 0/0  | 0   | 100| 100 | 100  | 0    |      |      |      |

* The proportion of B lymphocytes is indicated by the percentage of cells expressing CD19. CLL, B-CLL; FL, follicular lymphoma; MCL, mantle cell lymphoma.

** Notes: **
- cyIgM.M.
- NT, not tested.

#### Antibodies

The L227 (25) HLA class II and the L243 (26) HLA-DR mAbs were purified from ascitic fluid. W6/32 is a monomorphic HLA class I mAb (27). We have previously reported that L227 and L243 induce equivalent degrees of B lymphocyte apoptosis (11). All stimulations were conducted at a concentration of 5 μg/ml (per 0.5 × 10^6 cells) for 24 h unless otherwise indicated. Two agonistic anti-CD95 mAbs were used to induce apoptosis and are derived from clones CH11 and 7C11 (2 g/ml; Immunotech, Marseille, France). Comparison of these two Abs failed to reveal any difference in the level of apoptosis induced by one or other. Anti-human IgM was purchased from Capell (Birmingham, AL). The goat anti-mouse cross-linking Ab was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

#### Apoptosis detection

Induction of cell death was assessed by the following criteria: binding of annexin V FITC (to detect exposure of phosphatidylserine (PS)), propidium iodide (PI) staining of dead cells (live cells actively exclude PI), and MTT uptake (to detect metabolic activity).
and binding of FITC-dUTP in the presence of TdT (to detect DNA strand breaks).

Annexin V binding

Cells (5 × 10^6) were washed in PBS, and 100 μl of FITC-conjugated annexin V and PI (5 μg/ml) in a calcium-containing buffer were added according to the manufacturer’s instructions (Boehringer Mannheim, Meylan, France). After incubation for 10 min at room temperature, 400 μl of calcium-containing buffer was added, and the samples were immediately analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems (BDIS), San Jose, CA). The excitation source was an argon ion laser emitting at 488 nm. Electronic compensation of the instrument was required to exclude overlapping of the two emission spectra. Multivariate data were collected in list mode, stored, and analyzed with CellQuest software (BDIS). PI was added to the samples to discriminate necrotic events (annexin V–, PI–) from apoptotic events (annexin V+, PI–; annexin V+, PI+). The results are expressed as percentage of specific apoptosis according to the following formula: % specific apoptosis = 100 × [(% apoptotic “test” cells − % of apoptotic “isotype control” cells)/100 − % apoptotic “isotype control” cells)].

DNA fragmentation assay

DNA fragmentation was detected by the TUNEL method using an in situ cell death detection assay (Boehringer Mannheim) according to the manufacturer’s instructions. Briefly, 10^6 B lymphocytes were stimulated via HLA-DR and were harvested 18 h later by washing twice in PBS. After centrifugation, cell pellets were resuspended in 300 μl of PBS containing 4% paraformaldehyde. Fixation was conducted for 30 min at room temperature, and cells were again washed twice in PBS. Permeabilization was conducted with 0.1% Triton X-100 and 0.1% sodium citrate in PBS. Cells were again washed twice in PBS and resuspended in 50 μl of FITC-12-dUTP staining mixture (FITC-dUTP and unlabeled dNTP at optimized concentrations and ratios, 200 mM potassium cacodylate, 25 mM Tris-HCL, 1 mM CoCl2, and 0.25 mg/ml BSA, pH 6.6). Incubation was conducted for 1 h at 37°C before washing cells twice in PBS. Fixation of FITC-dUTP was detected on a FACScan flow cytometer (BDIS).

Coculture assay of “effector” and “target” cells

Apoptosis was detected by flow cytometry after annexin V-FITC labeling. A dual fluorescence assay was used in some experiments involving effector (1 × 10^6 cells) and target cells (1 × 10^6 cells) (e.g., Raji vs R2J2.2.5 (28), Raji vs Jurkat). To detect apoptosis of different cell populations by flow cytometry, the target cells were labeled using the orange fluorescent vital dye PKH26 (Sigma, St. Quentin Fallavier, France). This linker is an aliphatic reporter molecule and is incorporated by the cell membrane. Cell labeling was performed at a concentration of 2 μM for 3 min at room temperature.

PS exposure on the outer leaflet of apoptotic cell membranes was detected by annexin V-FITC binding in a calcium-containing incubation buffer according to the manufacturer’s instructions (Boehringer Mannheim). Briefly, unlabeled B effector cells and orange dye-labeled target cells were harvested after 36 h of culture and resuspended in 100 μl of annexin V-FITC labeling solution.

To study the potential role of soluble molecules produced by HLA class II-stimulated effector cells, the supernatants of cultures (1 × 10^6 cells) were resuspended and added to target Jurkat (1 × 10^6) cells for 36 h. Annexin V-FITC fixation was determined after a 5-min incubation at room temperature. Double labeling with PKH26 and annexin V-FITC was detected using a FACScan flow cytometer (BDIS) as described above.

Specific apoptosis was quantified as follows: [(annexin V test Ab culture − annexin V isotype control culture)/100 − (annexin V isotype control culture)] × 100.

One typical result representative of at least three experiments is shown for the B cell lines tested.

Caspase inhibitors

Acetyl-Asp(Ome)-Glu(Ome)-Val-Asp(Ome)-CH2F (DEVD-fmk) and Z-Val-Ala-Asp(Ome)-CH2F (zVAD-fmk) were from France Biochem (Meudon, France). DEVD-fmk is a cell-permeable and irreversible inhibitor of caspase 3, whereas zVAD-fmk inhibits processing of caspases 2, 3, 6, and 7. Cells were preincubated with the inhibitors for 3 h before adding anti-HLA class II mAb. A range of doses from 10 to 300 μM were tested for each inhibitor.

Measurement of caspase 3 activity

An assay of cleavage of the fluorogenic specific tetrapeptide substrate DEVD-AMC was conducted in triplicate as previously described (29). Briefly, cells were stimulated for 20 min, centrifuged at 200 × g for 10 min, and resuspended in phenol red-free RPMI 1640. Cell pellets were snap frozen in liquid nitrogen and stored at −20°C. Frozen pellets were thawed and directly added to a 96-well plate before adding DEVD-AMC substrate buffer. The DEVD-AMC aqueous salt was added in DTT-containing buffer, and the assay was immediately read in a Fluoroskan II plate reader. AMC release was monitored using 355-nm excitation and 460-nm emission wavelengths. Fluorescence units were converted to pmol of AMC. Enzymatic activity is expressed as pmol AMC released/min.

Annexin V-FITC binding was tested in the same samples after 24 h.

Immunoblotting to detect caspase 3 cleavage and poly(ADP-ribose) polymerase (PARP) cleavage

Lysates of cells stimulated either via HLA-DR or via Fas for 8 h were prepared in a lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 4 mM EDTA, 1% Nonidet P-40, 1 mM PMSF, 0.1 mM iodoacetamide, 20 μg/ml aprotinin, 10 μM leupeptin, and 10 μM pepstatin A) and subsequently mixed with Laemmli’s sample buffer and migrated in a 12.5% SDS-PAGE gel. Fifty micrograms of total protein was migrated in each lane before transferring to a nitrocellulose membrane and immunoblotting with an anti-caspase 3 Ab (PharMingen, France) diluted 1:2000.

Analysis of PARP cleavage was conducted on cells treated for 8 h with either HLA-DR mAb or Fas mAb. Lysates were prepared in lysis buffer as above and migrated in a 10% SDS-PAGE gel. Twenty-five micrograms of total cell lysate was loaded in each well, proteins were transferred to nitrocellulose membranes, and the membranes were immunoblotted with an anti-PARP mAb diluted 1:1000 (PharMingen). Detection of proteins was conducted by chemiluminescence (enhanced chemiluminescence system, Amersham, Paris, France).

Effect of inhibition of PI3K

Raji B lymphocytes were pretreated with either LY294002 or Wortmannin (10 μM) for 1 h before stimulating with HLA-DR, Fas mAb, or an isotype control for 18 h as described above. Although a range of inhibitor concentrations was tested (1–50 μM), this concentration of inhibitor has been previously shown to inhibit PI3K in B cells (30, 31). We verified that no change in viability was induced by either inhibitor before addition of the mAb. Specific apoptosis was measured as described above.

Immunophenotype

Immunophenotyping was performed on fresh tumoral cells without fixation using direct immunofluorescence with the following mAbs: FITC anti-CD95 (clone UB2, Immunotech), FITC anti-α and PE anti-λ (Ortho Clinical Diagnostic, Roissy, France), FITC anti-CD19 (clone J4.119, Immunotech), Cy5 anti-HLA-DR (clone Immu 357, Immunowash), FITC anti-CD10 (clone 55, Harlan Seralab, Crawley, U.K.), PE anti-CD5 (clone BL1a, Immunotech), FITC anti-CD23 (clone 9P25, Immunotech), and FITC anti-CD71 (clone YDJ1.2.2, Immunotech). Phenotypic analysis was conducted on a Cytoron flow cytometer (Ortho). Data were collected in list mode, stored, and analyzed using ImmunoCount II software (Ortho).

Statistical analysis

The comparison between variables (apoptosis) was analyzed using the Student’s t test.

Results

Characterization of B lymphocyte apoptosis via HLA-DR

Lymphocyte entry into apoptosis leads to a loss of plasma membrane phospholipid asymmetry and exposure of PS on the extra-cellular side, which can be recognized by phagocytes (32). Annexin V binding to PS has been described as a sensitive means of detecting B cell apoptosis (33). We have previously characterized HLA class II-mediated apoptosis as a process leading to morphologic changes typical of apoptosis, DNA fragmentation, and PI uptake (11). We therefore examined whether or not HLA-DR signaling led to annexin V binding; stimulation via Fas was used as a control throughout. Raji B lymphocytes or the HLA-DR-negative B cells derived from Raji, R2J2.2.5, were incubated with HLA-DR mAb (L243) or with Fas mAb (7C11) for 18 h. Both Raji and...
HLA-DR signaling leads to annexin V binding and PI staining in B lymphocytes. Figure 1a-f shows Annexin V binding (abscissa) vs PI staining (ordinate) of Raji (HLA-DR⁺) and RJ2.2.5 (HLA-DR⁻) B lymphocytes. Either L243 or 7C11 treatment for 18 h led to the appearance of an annexin V high, PI staining population of Raji (b and c), whereas only 7C11 treatment led to the appearance of a similar population in RJ2.2.5 cells (f).

DNA strand breaks were detected by binding of FITC-dUTP in the presence of TdT. Stimulation of Raji via HLA-DR or Fas led to DNA fragmentation (h and i), whereas stimulation of RJ2.2.5 via HLA-DR did not increase dUTP-FITC binding (k); Fas-mediated stimulation of RJ2.2.5 did lead to increased dUTP-FITC binding (l). DNA fragmentation after stimulation with an isotype control for L243 is shown for both Raji and RJ2.2.5 (g and j).
DR-stimulated B cells could act as effectors of Jurkat cell death either by direct induced apoptosis of B-CLL, FL, MCL, and B-PBL, no evidence was found that HLA-IgM-mediated signaling actually decreased apoptosis in almost all cases. Jurkat T from MCL (43.4\% mean ± SD) compared with B-CLL and B lymphocytes from MCL (43.4 ± 17.7). HLA-DR-mediated signaling also induced significant death in purified B lymphocytes from peripheral blood of two normal donors (55% and 71%). The CD95-specific mAb, CH11, was tested in parallel and, as expected, CD95-mediated death was not observed in B-CLL, because only 1–2% of B-CLL express CD95 (personal observation and Ref. 35). However, B cells from two of five cases of CD95-positive FL were sensitive to CD95-mediated death (Table II). Apoptosis via IgM was also examined for comparison. In marked contrast to HLA class II, signaling via IgM actually protected mature B cells from apoptosis (range, +1 to −110) (Table II).

Evidence that HLA class II-mediated apoptosis is not transferable to Fas-sensitive cells

HLA-DR-stimulated B lymphocytes do not induce apoptosis of Jurkat T cells. Because the Fas-negative B-CLL underwent apoptosis as a result of HLA-DR-mediated signals, we set out to determine whether a Fas-independent pathway was actually initiated via HLA class II. Activated murine and human B lymphocytes express Fas ligand (12, 34, 35), so the possibility of a HLA class II-stimulated B cell inducing apoptosis of a Fas-sensitive target cell line had to be explored. The CD95-sensitive Jurkat T cell lymphoma was cocultured with HLA class II-stimulated B lymphocytes (three CLL, two FL, one MCL, and one B lymphocyte from PBL). Although all of the B cells underwent HLA class II-mediated apoptosis, no significant decrease in Jurkat cell viability was detected after coculture at a ratio of 1:1 for 24 h. Jurkat T cells were also cultured with supernatants of HLA class II-activated B lymphocytes and failed to undergo significant cell death (Table II).

HLA class II-stimulated B cells do not induce death of autologous HLA-DR negative B lymphocytes. To test whether or not HLA class II-stimulated B cells could induce apoptosis of autologous B cells, we cocultured Raji and PKH26-labeled RJ2.2.5 lymphocytes in the presence or absence of anti-HLA class II mAb (Fig. 2). Annexin V labeling of both cell lines then allowed us to determine the proportion of cells that had undergone apoptosis in either population, because the PKH26 labeling distinguished the RJ2.2.5 cells. These experiments were undertaken to determine whether the signal via HLA class II on the Raji B lymphocytes rendered them cytotoxic, to exclude the possibility that the signal via HLA class II caused cell death indirectly (e.g., by activating a death receptor). Fig. 2 shows the proportion of annexin V binding cells in both the Raji (Fig. 2, left) and the RJ2.2.5 (Fig. 2, right) cells after coculture. Comparison of Fig. 2, A and panel C, shows that coculture in the presence of an HLA class II mAb led to cell death (significantly different from that induced by an isotype control IgG) only of the HLA class II-expressing B lymphocytes (Raji). In marked contrast, coculture in the presence of an anti-CD95/Fas Ab led to a high level of cell death in either population (Fig. 2B). All coculture experiments were conducted at a ratio of 1:1 for 18 h.

The RJ2.2.5 B cell line is derived from the Raji B lymphoblastoid cells and differs only by the absence of HLA class II molecules because of the loss of expression of CIITA (class II trans-activator) (28).

HLA-DR-mediated apoptosis is caspase independent

The caspases play a central role in the induction of apoptosis mediated by a variety of cell surface molecules. The role of caspase activation in HLA-DR-mediated apoptosis was therefore examined using the following approaches.

\*VAD-fmk and DEVD-fmk did not significantly inhibit HLA-DR-mediated apoptosis of B cells. zVAD-fmk and DEVD-fmk are cell-permeable, irreversible inhibitors of certain caspases;
zVAD-fmk inhibits a broad spectrum of IL-1 converting enzyme-like proteases, whereas DEVD-fmk is specific for caspase 3. Whereas apoptosis of Raji B cells via CD95 was completely inhibited in the presence of zVAD-fmk or DEVD-fmk, HLA-DR-mediated apoptosis was not completely inhibited in the presence of either inhibitor at concentrations up to 300 μM, and complete inhibition of Fas/CD95-mediated apoptosis was consistently detected at concentrations as low as 10 μM (Fig. 3). Further, whereas zVAD-fmk completely inhibited DNA fragmentation in Fas-stimulated Raji B cells, no inhibition of HLA-DR-mediated DNA fragmentation was detected (data not shown). Apoptosis via HLA-DR was also tested in three different cases of B-CLL in the presence of zVAD-fmk or DEVD-fmk. No detectable inhibition was observed (data not shown).

Caspase 3 was not activated by HLA-DR-mediated stimulation of B cells. A fluorometric assay was used to directly assess activation of caspase 3. The Nalm-6 pre-B cell line was used as a positive control for these experiments because CD95-mediated stimulation led to a 5-fold activation of caspase 3 in these cells, and because they express amounts of HLA-caspase 3 similar to those of mature B cells. The mAb CH11 led to activation of caspase 3 within 15 min (from 1.6 to 9.0 pmol/min for an increase in specific apoptosis of 72% via Fas), whereas a HLA-DR mAb failed to stimulate caspase 3 activation (1.6–1.7 pmol/min for an increase in specific apoptosis of 90%) (Table III). Despite the HLA-DR-mediated increases in specific apoptosis in mature B cells (CLL, MCL, and FL), no increase in caspase 3 activity was detected. The absence of caspase 3 activation was not due to a difference in the time course of activation via CD95 vs HLA-DR, because even after stimulation via HLA-DR for up to 24 h, no activation of caspase 3 was observed (data not shown).

HLA-DR-mediated apoptosis of B cells occurs without processing of caspase 3. Caspase 3 activation is characterized by proteolytic processing of the procaspase (32 kDa) to the active form of caspase 3 (17 kDa), which can be detected by Western blotting. Cleavage of the procaspase 3 to the p17 active form was observed.
after stimulation via CD95 (8 h) in both the EBV-transformed normal B cells CA (Fig. 4, lane 3) and the Raji B lymphoma cells (Fig. 4, lane 6), whereas no cleavage was observed following activation via HLA-DR (Fig. 4, lanes 2 and 5). The p17 cleavage product was barely detectable after CD95-mediated apoptosis of Raji. This is likely to be due to secondary necrosis, and activation is suggested by the reduction of the p32 procaspase. Equal quantities of protein were loaded in each lane.

**PARP is not cleaved via HLA-DR stimulation of B cells.** PARP is implicated in conserving the integrity of the genome because of its role in DNA repair. This enzyme has therefore been examined in apoptosis and was identified as a caspase substrate (36). The cleavage of PARP from a 116-kDa to an 85-kDa form can be detected at an early stage in apoptosis. Cleavage of PARP induced via Fas vs HLA-DR was compared in B cells after 4 h of activation. The 85-kDa form was readily detected in lysates from either the normal EBV-transformed B cell line Ca (Fig. 5, lane 2) or from Raji B lymphoma cells (Fig. 5, lane 5), which had been treated with Fas Ab (7C11) but not in lysates from cells that had been stimulated via HLA-DR (Fig. 5, lanes 3 and 6). Equal amounts of protein were loaded in each well.

**PI3K is not implicated in HLA-DR-mediated apoptosis**

Signal transduction via HLA class I Ags initiates a series of biochemical events that, similarly to HLA class II signaling, can lead to either activation or apoptosis. HLA class I Ags have been reported to initiate an apoptotic pathway in lymphocytes that was caspase independent but required the intervention of PI3K (37). A role for PI3K in B lymphocyte apoptosis via the B cell receptor

### Table III. Caspase 3 activity was measured detecting cleavage of a fluorogenic substrate DEVD-AMC and fluorescence units were converted to pmol of AMC

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<th>CD95/Fas</th>
<th>HLA-DR</th>
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<tr>
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<td>Caspase 3 activity</td>
<td>Specific death (%)</td>
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</tr>
<tr>
<td>CLL 3</td>
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<td>6</td>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>MCL 2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>FL 1</td>
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<td>NT</td>
</tr>
<tr>
<td>FL 2</td>
<td>0.5</td>
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</table>

<sup>a</sup> The enzymatic activity is expressed as pmol AMC released/min. Specific death was assessed by annexin V-FITC binding. NT, not tested.

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**FIGURE 3.** HLA-DR-mediated death of human B lymphocytes is not inhibited either by zVAD-fmk or by DEVD-fmk. The role of caspases in HLA-DR-mediated cell death was examined by comparing the percentage inhibition of specific apoptosis of Raji via Fas/CD95 and via HLA-DR in the absence or presence of the cell-permeable, irreversible caspase inhibitors zVAD-fmk or DEVD-fmk. The results of three separate experiments are shown. Fas/CD95-mediated apoptosis was induced using 7C11 and was completely inhibited by the presence of 10 μM zVAD-fmk or 10 μM DEVD-fmk, whereas HLA-DR-mediated apoptosis was induced using L243 and was not completely inhibited even in concentrations of 300 μM of either inhibitor. Incubation with either Ab was conducted for 18 h, and preincubation with caspase inhibitors was conducted for 3 h.

**FIGURE 4.** HLA-DR-mediated death of human B lymphocytes occurs without processing of procaspase 3. Activation of caspase 3 is mediated via cleavage of the p32 procaspase form to a p17 active form. The activation of caspase 3 via HLA-DR was examined in an EBV-transformed normal B cell line (Ca) and the Raji B cell lymphoma (R), and cleavage was detected by Western blotting of whole-cell lysates. B lymphocytes were stimulated either via Fas or via HLA-DR for 8 h before preparing cell lysates. An equal quantity of total protein (50 μg) was loaded in each well. CD95/Fas-mediated apoptosis (7C11 mAb) induced proteolytic cleavage of the p32 proform of caspase 3 and the appearance of the p17 active form in the normal B cell line (Ca) (lane 3), whereas HLA-DR (L243 mAb)-mediated apoptosis did not (lane 2). In Raji B cells, cleavage of p32 via CD95/Fas led to a clear decrease of p32 (lane 6), whereas HLA-DR stimulation failed to decrease the amount of p32. Lanes 1–3, Ca (lane 1, nonstimulated; lane 2, HLA-DR stimulated; lane 3, CD95/Fas stimulated); lanes 4–6, Raji (lane 1, nonstimulated; lane 2, HLA-DR stimulated; lane 3, CD95/Fas stimulated). Lane 7, Molecular weight markers.
This term describes a form of cell death morphologies has been extended to include a third, oncosis induced cell death (19). The original notion of two distinct cell death types, apoptosis and necrosis, has been broadened to include a third category, oncosis. This form of cell death is characterized by the formation of membrane blebs. A receptor inducing oncosis has recently been identified on Jurkat T cells, porin, which has previously been shown to inhibit PI3K in B lymphocytes (30), did not affect either HLA-DR or Fas-mediated apoptosis (data not shown).

Discussion

In this report, we describe a form of HLA class II-mediated cell death that is caspase independent. Caspase independence is ascribed on the basis of four results: 1) absence of apoptosis in the presence of caspase inhibitors; 2) absence of activation of caspase 3; 3) absence of processing of procaspase 3; and 4) absence of cleavage of PARP, which is a substrate for caspases 3, 7, and 9.

HLA-DR-mediated cell death occurred in the presence of cell-permeable caspase inhibitors, including zVAD-fmk, which has broad specificity (38). Parallel positive control experiments examined cell death via Fas/CD95 confirmed the activation and/or inhibition of certain caspases. The conclusion that death is caspase independent does of course rely on the efficacy of the inhibitors used, and we cannot therefore formally discount the possibility of involvement of unknown caspases that are insensitive to these inhibitors. Challenging the current dogma concerning the role of caspases in programmed cell death, a number of situations in which caspase-independent cell death occurs have been recently described. DNA fragmentation of target cells by CTL was inhibited in the presence of caspase inhibitors, although cell lysis still occurred (17). The overexpression of the proapoptotic member of the Bcl-2 family, Bax, induced certain features of apoptosis despite the presence of caspase inhibitors (although certain characteristics, such as DNA fragmentation, cleavage of PARP, and late stages of chromatin condensation, were blocked) (39). The broad-spectrum caspase inhibitor z-VAD-fmk inhibited PARP cleavage and generation of hypodiploid cells in CD2-mediated death of activated human peripheral T lymphocytes but failed to inhibit other features (40). Moreover, a caspase-independent cell death was described in promyelocytic leukaemia, in which not only did caspase inhibitors fail to inhibit cell death, but zVAD actually accelerated IFN-α-induced cell death (19). The original notion of two distinct cell death morphologies has been extended to include a third, oncosis (reviewed in Ref. 41). This term describes a form of cell death characterized by swelling, increased membrane permeability, and the formation of membrane blebs. A receptor inducing oncosis has recently been identified on Jurkat T cells, porin, although this receptor was not expressed in human PBLs nor in a number of T and B cell lines (42). It is clear that the idea of a ubiquitous cellular death pathway has evolved.

The absence of any effect of either wortmannin or LY294002, each of which is a specific inhibitor of PI3K, on HLA-DR-mediated apoptosis does not support the idea that PI3K is involved in HLA-DR-mediated death. This contrasts with the PI3K-dependent caspase-independent pathway of HLA class I-mediated apoptosis (37).

We have previously described a HLA-DR-mediated signaling pathway for apoptosis (quantified by counting hypodiploid nuclei) in activated human B lymphocytes that involved Fas/CD95 and its ligand (12), and HLA-DR mAbs inhibit hematopoiesis via an apoptotic mechanism that can be inhibited with a Fas mAb (13).
Apoptosis of mature human B cells can be triggered via different membrane receptors and involves both Fas-dependent and Fas-independent mechanisms. The constitutive expression of Fas on mature human B cell is restricted to populations that are post-antigenic stimulation (e.g., germinal center and memory B cells). Further, it has been shown that B cell activation is a prerequisite for Fas-mediated killing of human B cells as exemplified by CD40 signals leading to acquired susceptibility of B cells to the Fas pathway (43, 44). BCR-mediated killing of centrocytes in the germinal center constitutes a Fas/Fas ligand-independent mechanism of apoptosis of mature B lymphocytes (45). It could be hypothesized that the role of Fas-mediated apoptosis of mature B cells could be in the preservation of the fine specificity of T cell-dependent humoral responses (46). Differences in the signaling pathways of the BCR and of Fas in two B lymphoma cell lines were recently attributed to activation of the p38 mitogen-activated protein kinase via the BCR but not via Fas (47).

The results of this study demonstrate that a HLA class II-mediated cell death pathway is conserved in malignant and in normal B lymphocytes and that limitation of B lymphocyte populations subsequent to Ag presentation is therefore a possibility. HLA class II-mediated apoptosis has been previously observed in activated human B lymphocytes in contrast to studies in the mouse describing significant MHC class II-mediated apoptosis in resting B lymphocytes only. Data from the murine model led to the interpretation that MHC class II-mediated apoptosis was a mechanism of preventing nonspecific B cell activation, e.g., in the absence of appropriate Ag (48). The results of the current study support this notion, because mature human B lymphocytes underwent cell death in the absence of preactivation. It is quite possible that different pathways of B cell apoptosis are employed depending on whether or not preactivation has occurred. Apoptosis of resting B cells could provide a first line of defense against nonspecific B cell triggering (e.g., in the presence of high local concentrations of Ag), whereas cell death of activated B lymphocytes could be a consequence of successful and appropriate Ag presentation and a means of eliminating the possibility of further interaction with the T cell. An antitumoral effect of a HLA-DR mAb has been described (49), although neither normal human PBMC nor DR-transgenic mouse spleen were susceptible to the HLA-DR Ab tested; these data therefore contrast with other studies of MHC class II-mediated death in normal B lymphocytes (14, 49). The discrepancy could simply be due to the different techniques used to detect cell death.

Ab stimulation of HLA class II Ags provides a model for some of the signals generated in the B lymphocyte during Ag presentation leading to helper T cell activation. The importance of this caspase-independent pathway is underlined by its conservation in B lymphocytes from different origins. From a clinical point of view, the use of HLA-DR mAbs could be relevant for the development of therapeutic strategies for lymphomas expressing HLA class II Ags. We detected less HLA-DR-mediated apoptosis of B lymphocytes only. Data from the murine model led to the interpretation that MHC class II-mediated apoptosis was a mechanism of preventing nonspecific B cell activation, e.g., in the absence of appropriate Ag (48).

References


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