Ligation of HLA-DR Molecules on B Cells Induces Enhanced Expression of IgM Heavy Chain Genes in Association with Syk Activation

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We earlier reported that interactions between a T cell clone and monocyte via altered T cell receptor (TCR) ligands, affect monocyte responses to produce IL-12, events which lead to specific up-regulation of interferon-γ production from T cells (1). Thus, signals transmitted to monocytes via HLA molecules are involved in determining immune response patterns. It is highly conceivable that signals transmitted by class II MHC molecules in B cells, in regulating antigen-presenting cell function during cognate T-B cell interactions, are important, for the following reasons: (a) cross-linking class II molecules induces an increase in intracellular calcium and cAMP in mouse or human B cell lines (2–5); (b) class II MHC-mediated signals lead to homotypic aggregation of B cells (6); (c) cross-linking of HLA-DR molecules on B cells induces apoptosis (7); (d) class II MHC molecules without the intracellular domain expressed on B lymphoma cells will not lead to an increase in cAMP and subsequent CD80 up-regulation, when stimulated with a CD28-expressing autoreactive T hybridoma cells (8); (e) cytoplasmic domain mutants of class II MHC abrogate generation of intracellular cAMP (9) and translocation of PKC (10); and (f) cross-linking of HLA-DR molecules expressed on B cells induces phosphorylation of Src family kinases (Lyn, Fgr) (11) and Syk (12). Although functional consequences of such DR-mediated signaling events induced by T cells are largely unknown, these observations do raise the possibility that signaling through class II MHC molecules may affect B cell responses, including Ig secretion, upon TCR-TCR ligand interaction.

A number of protein-tyrosine kinases (PTKs) identified in lymphocytes can be classified into cytoplasmic (e.g. Syk, Btk), membrane-binding (e.g. Lyn, Lck, Fyn), and receptor types (e.g. epidermal growth factor receptor, nerve growth factor receptor). In B cells, a number of PTKs acting downstream of B cell receptors (BCRs) and several Fc receptors lead to activation of B cells. Mechanisms for Syk activation have been extensively examined (13). Src family kinases first phosphorylate Igα/Igβ ITAMs following BCR engagement, and then Syk is recruited to the doubly-phosphorylated ITAM and is activated by Src family kinases. Activated Syk molecules in B cells function in a manner analogous to ZAP-70 in T cells, and play a crucial role in B cell activation (14, 15). Recent studies have also suggested an alternative activating model of Syk that is independent of Src family kinases (16–18).

We now report that DR-generated signals induce IgM production from B cells, in association with Syk activation, but not Src family kinases.

EXPERIMENTAL PROCEDURES

Reagents—HU-4 (anti-HLA-DRB1+DRB5 IgG2a monomorphic; Ref. 19) and L243 (anti-HLA-DRB1+DRB4 IgG2a monomorphic; Ref. 20) were purified from the ascites-form of mAbs, using a Protein A column (Pierce). Mouse IgG and mouse IgG2a were purchased from BioPur AG (Bubendorf, Switzerland) and Biogenesis (Poole, United Kingdom), respectively. F(ab′)2 fragments of L243 and mouse IgG were prepared, using ImmunoPure F(ab′)2 preparation kits (Pierce) with extensive dialysis to remove residual Fc fragments. L243, control mouse IgG, F(ab′)2, and a FITC-conjugated goat anti-mouse IgG (Bubendorf, Switzerland) were used for FACS analysis of HLA-DR- and CD154-positive B cells.

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† The abbreviations used are: TCR, T cell receptor; PTK, protein-tyrosine kinase; ITAM, immunoreceptor tyrosine-based activation motif; BCR, B cell receptor; PKC, protein kinase C; PBMC, peripheral blood mononuclear cell; FMA, phorbol 12-myristate 13-acetate; PAG, polyclonal anti-epidermal growth factor receptor; MBP, myelin basic protein; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; mAb, monoclonal antibody; Ab, antibody; RT, reverse transcription; PCR, polymerase chain reaction; IL, interleukin; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; Fmoc, 9-fluorenylmethoxycarbonyl.
nologies, Inc.) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 1% L-glutamine (Sigma). We cultured the isolated population in RPMI 1640 medium (Life Technologies, Inc., Grand Island, NY), herbimycin A (Life Technologies, Inc.), piceatannol (Sigma), genistein (Calbiochem, La Jolla, CA), 9-fluorenylmethoxycarbonyl (Fmoc) strategy and an automated multiple peptide synthesizer PSSM-8 (Shimadzu Corp., Kyoto, Japan), based on the Fmoc (9-fluorenylmethoxycarbonyl) strategy and was distributed by the 11th International Histocompatibility Workshop (23).

Stimulation of B Cells by Anti-DR mAbs—Anti-DR mAbs (HU4 and H243), mouse IgG2a, and bovine serum albumin were coated onto a 96-well flat-bottomed culture plates for 2 days at 10°C. After cross-linking DR molecules as described above, total cellular RNA was extracted from 10^6 cultured cells with IL-4, 10^5/well) were cultured with IL-4, 10^5/well) were cultured with peptide-pulsed or mock-pulsed B cells prepared from PBMC. Cultured supernatants were assayed for IgM by sandwich ELISA. The peptide-pulsed peripheral B cells were analyzed using an ELISA reader (model 550, Bio-Rad) at 405 nm. The level of cross-reactivity was 833-fold at 37°C, which occurred between IgG1 and IgG3 (data not shown).

In Vitro Culture with Anti-CD154 mAb—PBMC (1.5 × 10^6/well) were cultured with IL-4, 1 µg/ml ionomycin, and 10 ng/ml PMA with neutralizing anti-human CD154 mAb (Ancell, Bayport, MN) or with control mouse IgG, for 8 days to obtain supernatant fluids, or for 3 days to assess proliferative responses. The supernatant fluids of 8-day culture were assayed for IgG1 by sandwich ELISA. The peptide-pulsed peripheral B cells were cultured for 5 days, with emetine-treated T cells in the presence of the anti-CD154 mAb or with control mouse IgG, to determine IgM concentrations of the supernatant fluids.

RT-PCR and Southern Blot Analysis—After cross-linking DR molecules as described above, total cellular RNA was extracted from 10^6 purified B cells by the acid guanidine thiocyanate phenol-chloroform method (TRIzol, Life Technologies, Inc.). The first strand cDNA was synthesized from purified total RNA by reverse transcriptase, using random primers (SuperScript® premplification system, Life Technologies, Inc.). The following oligonucleotides were used as primers: 5'-TGGCGACTGAGCGGACAC-3' (secreted component-origin) and 5'-TTCTCAAGGCCTCCTCCTGTC-3' (membrane component-origin) for PRDI-BF1; 5'-AATCTGAACTGCTGATGCTG-3' (membrane component-origin) and 5'-AAAAACACACACATCTC3'- (Cδ-origin) as a common primer. PRDI-BF1 transcripts were amplified using 5'-CTAAGAAGCCCAA-CAGGAAA-3' and 5'-TGGAGGTTGGAGGATGGA3'- 3'-β-Actin transcripts as a control, were amplified using 5'-CGGGGAATTCGGCGT-GAC-3' and 5'-CTCGTACACTCTGCTTCGTG-3'. One amplification cycle was 95°C for 1 min, 72°C for 1.5 min, and 95°C for 1 min. The final extension cycle was for 7 min at 72°C. Amplification was performed under conditions in which amplified PCR products were visible on an agarose gel under UV wave, so that none of amplified DNA concentrations reached a plateau level. PCR products were separated in a 1.2% agarose gel and transferred onto nylon membrane (Zeta-probe, Bio-Rad), for PRDI-BF1 (kindly provided by Dr. N. Kondo, Gifu University) (28), for PRDI-BF1 (kindly provided by Dr. T. Manatis, Harvard University) (29), or β-actin that were randomly conjugated by dUTP-digoxigenin, using DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Molecular Biochemicals). The hybridization signal was detected with alkaline phosphatase-conjugated anti-digoxigenin Ab and diaminobenzidine. Hybridization signals were analyzed using the public domain Image program (developed at the United States National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov.)

ELISA Assay for Ig Determination—The 96-well plates (ELISA plate H type, Sumito, Bakelitke, Tokyo, Japan) were coated overnight at 4°C with 0.3–0.5 µg/well anti-IgM mAb (JDC-15, Pharmingen), anti-IgG1 mAb (G17-1, Pharmingen), anti-IgG2 mAb (HP-6014, Southern Biotechnologies Associates), anti-IgG3 mAb (G18-3, Pharmingen), anti-IgG4 mAb (G17-4, Pharmingen), anti-IgA1 mAb (G18-1, Pharmingen), or anti-IgM mAb (G18-18, Pharmingen) in PBS, pH 7.2. After washing five times with PBS, the plates were blocked with 1% bovine serum albumin/PBS for 2 h at room temperature. Standard Iggs and culture supernatants were added and incubation run 1 h at room temperature. After washing five times with PBS, biotin-conjugated Abs to IgM (G20-127, Pharmingen), IgG (G18-145, Pharmingen), IgA1/2 (G20-359, Pharmingen), or IgE (A116BN, American Qualex Antibody) were added to each well. After 1 h of incubation, the plates were washed five times with PBS and streptavidin-alkaline phosphatase (Life Technologies, Inc.) was added. After 1 h, the plates were washed five times with PBS and p-nitrophenyl phosphate (KPL Inc.) as a substrate was added, then were analyzed using an ELISA reader (model 550, Bio-Rad) at 405 nm. The level of cross-reactivity was 833-fold at the maximum, which occurred between IgG1 and IgG3 (data not shown) with flow cytometry (Techne, Mountain View, CA).

Immunoblotting—Peripheral B cells and the human B lymphoblastoid cell line LD2B (5 × 10^6) were incubated for 10 min on ice and then pre-incubated either with biotinylated anti-DR mAb (40 µg/200 µl) or with biotinylated mouse IgG (40 µg/200 µl) for 10 min on ice. After washing with ice-cold RPMI 1640, the cells were suspended with 50 µl of 10% FCS/RPMI and cross-linked with 50 µl of avidin (1 mg/ml). After 10 min of incubation at 37°C, ice-cold 100 µl NaNO3/PBS was added, followed by pelleting and lysing in 50 µl of lysing buffer (150 mM NaCl, 20 mM Tris, pH 7.6, 0.5% Nonidet P-40, 2 mM sodium orthovanadate, 1 mM NaF, 5 mM EDTA, plus a protease inhibitor mixture purchased from Sigma). Supernatant fluids of the lysates were electrophoresed on SDS-PAGE gels and transferred to nitrocellulose membrane. After blocking with 10% skim milk, 0.2% Tween 20 in Tris-buffered saline, the membrane was incubated with the anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology, Inc.), washed extensively, and subjected to chemiluminescence detection with peroxidase-conjugated anti-mouse IgG Ab (Amersham Pharmacia Biotech), using an ECL kit (Amersham Pharmacia Biotech). In some experiments, lysates prepared from 10^6 LD2B cells were immunoprecipitated with anti-Syk Ab, and subjected to immunoblot analysis with anti-phosphotyrosine mAb 4G10 or anti-Syk Ab.

In Vitro Immune Complex Kinase Assay—The human B lymphoblastoid cell line LD2B (1 × 10^7) was incubated for 10 min on ice and then pre-incubated either with biotinylated IgGs (40 µg/200 µl) or with biotinylated P(ab)_2 fragments (12 µg/200 µl) for 10 min on ice. After washing with ice-cold RPMI 1640, the cells were suspended with 50 µl of 10%
followed by pelleting and lysing in 400 mM saline, the membrane was incubated with the rabbit anti-Syk Ab, and by DNA staining using propidium iodide.

RESULTS

Cross-linking HLA-DR Molecules on B Cells Induces Increased Production of IgM without Inducing B Cell Proliferation—To test whether signals via class II HLA molecules would affect production of IgGs, we first cross-linked class II HLA molecules on B cells by making use of anti-DR mAb-coated culture plates. The supernatantsfluids of the lysates were pre-cleared with Protein A-agarose beads, then were incubated with a rabbit polyclonal anti-Syk Ab (Santa Cruz Biotechnology, Inc.), using Protein A-agarose beads (Pierce). After shaking for 30 min at 4 °C, the beads were washed four times with lysis buffer. An aliquot of immunoprecipitated proteins was eluted with Laemmli buffer containing 2-mercaptoethanol, for immunoblotting analysis. Residual beads were washed once with kinase buffer (25 mM HEPES, pH 7.4, 0.1% (v/v) Nonidet P-40, 10 mM MgCl2, 3 mM MnCl2, 30 μM Na3VO4; Ref. 30) and were re-suspended in 30 μl of the kinase buffer containing 2 μg (0.11 nmol) of MBP (Sigma), in the presence of either 27.5 nmol of H5S1 peptide or an irrelevant peptide. Reactions were initiated by adding 3.75 g/ml PBS. Purified peripheral B cells were incubated at 5 × 106 cells/well where mAbs are coated, at 37 °C in a CO2 incubator for 3 days (proliferation assay) or for 5 days (IgM determination). HLA type of the B cell donor was DRB1*0101/1201. Mean cpm of triplicate responses ± S.D. is indicated.

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FIG. 1. Cross-linking HLA-DR molecules on B cells induces increased production of IgM (A), without inducing proliferation (B). Mouse IgG2a, anti-DR mAb HU4, anti-DR mAb L243, or bovine serum albumin were coated onto 96-well flat-bottomed culture plates at 10 μg/ml FBS. Purified peripheral B cells were incubated at 5 × 106 cells/well where mAbs are coated, at 37 °C in a CO2 incubator for 3 days (proliferation assay) or for 5 days (IgM determination). HLA type of the B cell donor was DRB1*0101/1201. Mean cpm of triplicate responses ± S.D. is indicated.

FIG. 2. Effect of anti-DR mAb on apoptosis of peripheral B cells. The proportion of B cells that underwent anti-DR-induced apoptosis after 24-h incubation in the presence of soluble mouse Ig (A), soluble anti-DR mAb (B243 (B), coated mouse Ig (C), or coated anti-DR mAb (D), was determined by phosphatidylserine expression on the cell surface using fluorescein isothiocyanate-conjugated annexin V, and by DNA staining using propidium iodide.

B cells were cultured for 3 days in culture plates coated with anti-DR mAbs, in the presence of [3H]thymidine during the final 16 h. As shown in Fig. 1B, no difference was observed between proliferative response induced by anti-DR mAbs and that induced by controls. This was also the case in the 6-day culture experiments (data not shown). Proliferative responses of the same B cell preparation induced by PMA and ionomycexin exhibited 7,164 cpm. To exclude the possibility that the increase in IgM is due to apoptosis of B cells, we cross-linked DR on B cells with the soluble form or solid-phase anti-DR mAbs. Stimulation with soluble-form (Fig. 2B), but not solid-phase mAb (Fig. 2D) and controls (Fig. 2, A and C), induced marked apoptosis. The precise mechanisms for this discrepancy is yet to be determined, but it is conceivable that DR-mediated increase in IgM in the culture supernatants was not due to release of IgM from apoptotic B cells. These observations collectively indicate that increased IgM concentration in culture supernatant fluids cannot be ascribed to B cell proliferation or apoptosis.

Cross-linking HLA-DR Molecules Enhances Both Membrane-type and Secretory-type IgM Heavy Chain Gene Expression—To determine whether signals via DR molecules up-regulate μ chain mRNA, we cross-linked DR molecules on peripheral B cells (1 × 106) with either solid-phase anti-DR mAb (L243) or solid-phase mouse IgG. Due to limitations in the number of purified B cells, we could test only three samples at one time. At 0, 3, and 6 h (Fig. 3A), or 6, 12, and 24 h (Fig. 3B) after the initiation of culture, B cells were analyzed for mRNA expression for μ chains, using RT-PCR and Southern blot analysis. Relative mRNA level was analyzed, using the public domain NIH Image program. When we tested the kinetics, μ chain mRNA increased in a time-dependent fashion (Fig. 3A) and reached maximum at 12 h (Fig. 3B). This increase was not due to the enhanced recovery of mRNA, as evidenced by the presence of an equal amount of β-actin mRNA in each sample. The μ chain mRNA level induced by control mouse IgG at 3, 6, 12, and 24 h was practically the same as that induced by anti-DR mAbs at 0 h (data not shown). To test whether the DR-generated signal induced differenti-
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Fig. 3. Cross-linking DR molecules enhances μ chain mRNA expression. Purified peripheral B cells were incubated in 24-well flat-bottomed culture plates at 1 × 10^6 cells/well where mAbs are coated, at 37 °C in a CO_2 incubator for 0, 3 and 6 h (A) or 6, 12 and 24 h (B). As described under “Experimental Procedures,” RT-PCR and Southern blot analysis were done for membrane-type μ chain (μm), secretory-type μ chains (μs; closed columns), PRDI-BF1 (hatched columns), and β-actin (shaded columns). PRDI-BF1 mRNA expression levels were quantified using NIH Image and represented by relative values compared with those from 0-h membrane-type μ chain, 0-h PRDI-BF1, or 0-h β-actin (A) and 6-h membrane-type μ chain, 6-h PRDI-BF1, or 6-h β-actin (B). HLA type of the B cell donor was DRB1*0101/1201.

Stimulation of B cells to plasma cells, we analyzed PRDI-BF1 transcripts. PRDI-BF1 is a human homologue of Blimp-1, the expression of which is characteristic of late B cells and plasma cells (29, 33, 34). However, as shown in Fig. 3, DR-generated signals up-regulated no mRNA for PRDI-BF1. The presence of PRDI-BF1 transcripts is indicative of the presence of plasma cells in this cell preparation. The experiment was repeated twice with reproducible results. These data suggest that IgM production induced by cross-linking of DR molecules is regulated at the mRNA level and is not associated with B cell differentiation to plasma cells.

Emetine-treated and HLA-DR-restricted T Cells Are Capable of Inducing IgM Production by B Cells—Although earlier observations strongly suggest that the ligation of HLA-DR molecules directly stimulates B cells to produce IgM, the outcome of ligation by mAbs should be affected by epitopes recognized by these mAbs and their affinity. Indeed, anti-HLA-DR mAb HU-4, exerted weaker effects than did L243 (Fig. 1). It is unlikely that HLA-DRB4 molecules recognized by L243 are transmitting the signals, because the B cell donor in Figs. 1 and 3 did not carry DRB4-positive haplotypes. Therefore, we next asked if a similar phenomenon occurs, on natural TCR-peptide-HLA interactions. An HLA-DR-restricted T cell clone was treated with the de novo protein synthesis inhibitor emetine (24), because it is highly likely that T cell membrane proteins or T cell soluble factors newly synthesized after activation by peptide-pulsed B cells, work on B cells. Under conditions where T cells are treated with 90 μg/ml emetine for 1 h, followed by co-culture with peptide-pulsed B cells bearing restriction HLA molecules, T cells produced <25 pg/ml IL-4, whereas non-treated T cells produced 3580 pg/ml IL-4, although cell-surface TCR remains practically the same level (data not shown), indicating that de novo protein synthesis of T cells is abrogated by emetine. A T cell clone BC20.7 (BGGa-specific, DR14-restricted) and B cells purified from PBMC of the donor of BC20.7 were used in subsequent experiments. As shown in Table I, levels of IgM, IgG1, IgG4, IgE, and IgA were detected when mock-pulsed B cells were co-cultured with emetine-treated T cells. However, when B cells were pre-pulsed with the antigenic peptide, marked enhancement of IgM and marginal enhancement of IgA production were observed and such was not the case when peptide-pulsed B cells were cultured in the absence of T cells (data not shown). The experiment was repeated four times with reproducible results.

It seems reasonable to speculate that DR-mediated signals alone can up-regulate IgM production from B cells, based on the following observations made in the current study. (a) Ligation of DR molecules by mAb in the absence of T cells can up-regulate IgM protein and mRNA (Figs. 1 and 3); (b) up-regulation of accessory molecules such as CD154 is not observed in emetine-treated T cells (data not shown); and (c) activated T cells of irrelevant restriction HLA molecules cannot stimulate peptide-pulsed B cells to up-regulate IgM (data not shown). However, we directly confirmed that signals via CD40 molecules are not involved in IgM production from B cells during cognate T-B cell interactions. To determine a saturating concentration of neutralizing anti-human CD154 mAb, we stimulated PBMC with 20 units/ml human recombinant IL-4, 1 μM ionomycin, and 10 ng/ml PMA, with varying concentrations of anti-CD154 mAb. As shown in Fig. 4A, 1–10 μg/ml anti-CD154 mAb inhibited IgE production, whereas 10 μg/ml control mouse IgG did not do so. Because 1 or 10 μg/ml anti-CD154 mAb did not inhibit proliferation of PBMC induced by the same stimuli (Fig. 4B), it is likely that anti-CD154 mAb exerted specific inhibitory effects on PBMC. When we co-cultured peptide-pulsed peripheral B cells and emetine-treated T cells in the presence of 1 μg/ml anti-CD154 mAb, we observed no inhibition of IgM production (Fig. 4C). These findings collectively indicate that: (a) IgM production from B cells is enhanced when HLA-peptide-TCR interaction occurs, and (b) such enhancement occurs without involvement of signaling through either CD40 or FcR molecules.

Piceatannol Inhibits IgM Secretion Induced by DR Ligation—To identify signal transduction molecules involved in DR-mediated IgM production, we co-cultured peptide-pulsed peripheral B cells and emetine-treated T cells in the presence of several kinase inhibitors. These inhibitors were dissolved in Me_2SO and added to culture medium at a final concentration of 0.5%, which did not inhibit DR-mediated IgM production, as shown in Fig. 5. PTK inhibitors genistein and herbimycin A inhibited IgM secretion in a dose-dependent manner, whereas a protein kinase C inhibitor GF109203X (IC_{50} = 20 nM; Ref. 26) did not do so. We next examined the effect of inhibitors specific for individual protein-tyrosine kinase(s). Interestingly, 10 μM Syk kinase inhibitor piceatannol (27) inhibited IgM production, whereas PP2, a selective inhibitor of the Src family of protein-tyrosine kinase (25), failed to do so. Because 500 μM genistein or 100 μM piceatannol did not inhibit anti-DR-induced IL-1β production from monocytes,2 it is likely that the kinase inhibitors at the concentration we used exerted specific inhibitory effect on B cells. The experiment was repeated twice with reproducible results. These observations suggest that Syk kinase but not Src kinase(s) is involved in IgM production induced by ligation DR molecules.

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2 T. Matsuoka, H. Tabata, and S. Matsushita, unpublished observation.
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**TABLE I**

IgM production from B cells induced by a DR-restricted T cell clone

B cells either mock-pulsed or pulsed with BCGap84–100 were cultured with an HLA-DR14 (DRB1*1405)-restricted and emetine-treated T cell clone BC20.7 for 5 days. B cells were purified by, and the T cell clone was established from, a donor carrying DRB1*1405/1502. Mean values of duplicate determinations are indicated. S.D. was less than 25%.

| Peptide       | IgM | IgG1 | IgG2 | IgG3 | IgG4 | IgE | IgA |
|---------------|-----|------|------|------|------|-----|-----|
|               |     |      |      |      |      |     |     |
| −             | 37.5| 124.5|<3.1 |<3.1 |<3.1 |3.13|31.2 |
| +             | 223.5| 96.0 |<3.1 |<3.1 |1.41 |21.2|980  |

![Fig. 4. Signals via CD40 molecules are not involved in IgM production from B cells during cognate T-B cell interactions.](Image 101x405 to 245x624)

**Figs.**

**Fig. 4.** Signals via CD40 molecules are not involved in IgM production from B cells during cognate T-B cell interactions. A and B, PBMC (1.5 × 10⁶ cells/well) were cultured with IL-4, 1 μM ionomycin, and 10 ng/ml PMA with neutralizing anti-human CD154 mAb or with control mouse IgG for 8 days (A; IgG determination) or for 3 days (B; proliferation assay). Mean cpm of triplicate responses ± S.D. is indicated. C, the peptide-pulsed peripheral B cells were cultured for 5 days, with emetine-treated T cells in the presence of anti-CD154 mAb (1 μg/ml) or mAbG (1 μg/ml). ΔIgM was calculated as follows, using mean values of IgM concentration in triplicate cultures: IgM concentration of each culture − IgM concentration of culture with B cells only. T cell and B cell origins are shown in footnotes for Table I.

**Kinase Activity**—To investigate possible protein-tyrosine phosphorylation associated with this event, detergent lysates of peripheral B cells and LD2B cells treated with anti-DR mAb or control mouse IgG, were analyzed. Fig. 6 shows that protein-tyrosine phosphorylation was enhanced by cross-linking of DR molecules on peripheral B cells (Fig. 6A, lane 2 versus lanes 1 and 3). Bands corresponding to proteins with an approximate molecular mass of 65, 70, 110, and 130 kDa were reproducibly hyper-phosphorylated. Likewise, cross-linking of DR molecules on LD2B induced tyrosine-phosphorylation of 65- and 70-kDa proteins (Fig. 6B, lane 2 versus lanes 1 and 3). Furthermore, immunoprecipitation by anti-Syk Ab followed by blotting with anti-phosphotyrosine mAb 4G10, exhibited anti-DR-induced tyrosine phosphorylation of Syk molecules expressed in LD2B cells (Fig. 6C).

To further confirm that Syk is activated directly by HLA-DR, Syk kinase activity was determined by *in vitro* kinase assay, using Syk molecules immunoprecipitated with anti-Syk Ab, and MBP as a substrate. Because a large number of B cells are required for immunoprecipitation followed by *in vitro* Syk kinase assay, we used a B lymphoblastoid cell line LD2B homozygous for DRB1*1501, which secretes IgM in the absence of specific stimuli. LD2B was selected among many B cell lines because (a) anti-DR-induced phosphorylation pattern of LD2B was similar to that of peripheral B cells, including the phosphorylation of 70-kDa protein (Fig. 6A), and (b) LD2B B cells expressed IgM heavy chain genes. Enhancement of IgM production from LD2B B cells after cross-linking DR molecules was only marginal, probably because LD2B cells constitutively showed a 50–80-fold higher IgM secretion than did peripheral B cells, on a single cell basis (data not shown). As shown in Fig. 7A, however, cross-linking of DR molecules by biotinylated anti-DR mAb plus avidin induced marked phosphorylation of MBP (lane 3), whereas incubation of LD2B with biotinylated mouse IgG + avidin, only marginally induced phosphorylation of MBP (lane 2 versus lane 1). Because MBP is not a substrate specific for Syk kinase, and it might be that MBP was phosphorylated by certain kinases co-precipitating with Syk, we also asked if the effect of Syk on MBP would be inhibited competitively by HS1p38–402 peptide, a substrate specific for Syk (22). *In vitro* Syk kinase assay with MBP (2 μg/sample; 0.11 nmol/sample) was done in the presence of either a 250-fold molar excess of the HS1 peptide (27.5 nmol/sample) or an irrelevant peptide carrying two tyrosine residues (EIKYN-GEEYLIL; 27.5 nmol/sample). Indeed, MBP phosphorylation was inhibited by the HS1 peptide, but not by the irrelevant peptide (lanes 4 and 5).

It is also important to note that Syk molecules are associated with FcγR and are activated by cross-linking of the receptor (35, 36). It is therefore conceivable that the increment in Syk kinase activity we observed may be due to cross-reaction of mouse Ig with human FcγR expressed on B cells. To exclude this possibility, we prepared a biotinylated F(ab')₂ fragment of anti-DR mAb L243 or that of control mouse Ig. As shown in Fig. 7B, cross-linking of F(ab')₂ fragment of anti-DR mAb L243 was similar to that of peripheral B cells, including the phosphorylation of 70-kDa protein (Fig. 6A), and (b) LD2B B cells expressed IgM heavy chain genes. Enhancement of IgM production from LD2B B cells after cross-linking DR molecules was only marginal, probably because LD2B cells constitutively showed a 50–80-fold higher IgM secretion than did peripheral B cells, on a single cell basis (data not shown). As shown in Fig. 7A, however, cross-linking of DR molecules by biotinylated anti-DR mAb plus avidin induced marked phosphorylation of MBP (lane 3), whereas incubation of LD2B with biotinylated mouse IgG + avidin, only marginally induced phosphorylation of MBP (lane 2 versus lane 1). Because MBP is not a substrate specific for Syk kinase, and it might be that MBP was phosphorylated by certain kinases co-precipitating with Syk, we also asked if the effect of Syk on MBP would be inhibited competitively by HS1p388–402 peptide, a substrate specific for Syk (22). *In vitro* Syk kinase assay with MBP (2 μg/sample; 0.11 nmol/sample) was done in the presence of either a 250-fold molar excess of the HS1 peptide (27.5 nmol/sample) or an irrelevant peptide carrying two tyrosine residues (EIKYN-GEEYLIL; 27.5 nmol/sample). Indeed, MBP phosphorylation was inhibited by the HS1 peptide, but not by the irrelevant peptide (lanes 4 and 5).
induced phosphorylation of MBP (lane 3), whereas F(ab’)_2 fragment of mouse Ig induced little phosphorylation of MBP (lane 2), compared with a control (avidin only; lane 1). This indicates that Syk phosphorylation is induced by cross-linking DR but not FcγR. These differences in phosphorylation patterns were not due to the enhanced recovery of these kinases, as evidenced by the presence of an equal amount of Syk protein molecule in each sample (Fig. 7, A and B). The experiment was repeated once with reproducible results. These data are consistent with results obtained using the Syk inhibitor piceatannol on IgM production, thereby collectively indicating that HLA-DR molecules on B cells not only present antigenic peptides to T cells, but also up-regulate IgM production, in association with Syk activation and without the involvement of Src kinases.

**DISCUSSION**

It is highly likely that the cognate interaction between T cells and B cells, as mediated by class II MHC molecules, results in the delivery of activation signals to B cells, as evidenced in the current study and as reported by others (2–11, 31, 37–39). Engagement of class II molecules on the THP-1 monocyte cell line with staphylococcal enterotoxin A induced IL-1β and tumor necrosis factor-α (40). Our previous studies demonstrated that certain T cell-monocyte interactions, via altered TCR ligands, affect monocyte responses to produce IL-12 (p70), which leads to specific up-regulation of interferon-γ production from T cells (1). Moreover, we recently observed that cross-linking class II HLA molecules on monocytes induces a wide variety of monokine production (41), which is accompanied by activation of signaling molecules. Thus, class II-mediated signaling events are not specific for B cells and play a crucial role in the activation of antigen-presenting cells, in general.

In this study, we found that ligation of HLA-DR alone is capable of inducing IgM production from peripheral B cells. Conversely, Palacios et al. (39) reported that cross-linking of DR on peripheral B cells with anti-DR Abs induced no Igs (IgM, IgG, and IgA), in the absence of pokeweed mitogen. However, they did not use solid-phase Ab but rather the soluble-form Ab, which was invalid or less efficient for cross-linking in our study (data not shown). In murine systems, IgM production from B cells is achieved, because only cross-linking of class II MHC but also that of membrane IgM (5, 37) or cytokines (31). Possibilities include the following: (a) B cells that have already received signals from BCR in vivo, are responding to the DR engagement in our experimental system; and/or (b) there are essential differences in these aspects between murine and human B cells.

IgM contributes to early defense against microbial infections (42). When B cells are exposed to non-self-antigens, such as those of microbial origin, B cells bearing surface IgM specific for the antigen are capable of concentrating the antigen and present it effectively to T cells. We found that cross-linking DR molecules up-regulates not only secretory-type but also membrane-type μ chains, which may indicate that cross-linking DR molecules leads to more effective antigen presentation. It is also important to note that CD40-generated signals arrest B cell terminal differentiation to produce IgG (43). Although DR-mediated signals appear to up-regulate IgM production in the absence of CD40-CD154 interaction (Fig. 4), further investigation is needed to determine whether or not the generation of signals via CD40 under physiological T-B interactions interferes with IgM production induced by DR-mediated signals. In this study, ligation of DR molecules not only with specific Abs (either solid-phase Abs or soluble Abs), but also with HLA-
peptide-TCR interaction, induced IgM production, suggesting that signals via DR alone are capable of inducing up-regulation of IgM, which may also occur in physiological T-B interactions. In this relation, DR-mismatched transplantation should be one of rare cases, in which massive T-B interaction via DR occurs in vivo. Indeed, when DNA typing of HLA-DR was unavailable, graft-versus-host disease was frequent, and such patients reportedly had deposition of IgM at the dermo-epidermal junction (44).

BCR-Ag-complex is internalized to supply T cell epitopes, and subsequent DR-peptide-TCR interaction results in class switching, which eventually leads to decreased IgM production (45). Indeed, our experimental system did not allow BCR to interact with protein antigens, and T cells were treated with emetine (thereby bearing no class switch pressure). Such a system might have up-regulated IgM to be readily detected. However, because the disappearance of surface IgM at antigen presentation (before class switching) is incomplete, one might speculate that signaling through DR supplies new IgM molecules, for a short and critical period of time for T-B interaction before class switching is initiated. Other factors should also be considered, because even with thymus-independent antigens, IgM production from B cells can be induced (46).

Analysis of syk−/− lymphoid cells showed that the Syk mutation impaired the differentiation of B-lineage cells, apparently by disrupting signaling from the pre-BCR complex, thereby preventing clonal expansion and further maturation of pre-B cells (47, 48). Syk mutation also blocked B cell development in the transition from immature B cells (B220−, IgM−) to mature B cells (B220+, IgM−, IgD−), where up-regulated transcription of μ chains is again taking place. Although physiological roles of Syk in normal mature B cells are not readily determined, it is conceivable that Syk is involved not only in BCR signaling but also in μ chain transcription, occurring at the pre-B cell and at the immature B cell stages.

It was reported that DR-mediated signals lead to apoptosis of human B cells (7). DR-mediated increase in IgM in the culture supernatants in our experimental system was not due to apoptosis of B cells, because (a) the phenomenon was isotype-specific and (b) apoptosis was not observed as shown in Fig. 2, using solid-phase Abs. In Figs. 6 and 7, soluble Abs were used, but short term stimulation (10 min) did not induce apoptosis (data not shown).

Cross-linking of HLA-DR molecules expressed on B cells was reported to induce phosphorylation of Src family kinases (Lyn and Fgr; Ref. 11). PP2 was found to inhibit Src kinases Lck and Fgr (data not shown). But short term stimulation (10 min) did not induce apoptosis of B cells, because (a) the phenomenon was isotype-specific and (b) apoptosis was not observed as shown in Fig. 2, using solid-phase Abs. In Figs. 6 and 7, soluble Abs were used, but short term stimulation (10 min) did not induce apoptosis (data not shown).

REFERENCES

1. Matsusaka, T., Kohrogi, H., Ando, M., Nishimura, Y., and Matsushita, S. (1996) J. Immunol. 157, 4837–4843
2. Cambier, J. C., Newell, M. K., Justement, L. B., McGuire, J. C., Leach, K. L., and Chen, Z. Z. (1987) Nature 327, 629–632
3. Lane, P. J., Mcconnell, P. M., Schieven, G. L., Clark, E. A., and Ledbetter, J. A. (1991) J. Immunol. 144, 3684–3692
4. Mooney, N. A., Grillot-Courvalin, C., Hivroz, C., Ju, L. Y., and Charron, D. (1990) J. Immunol. 145, 2070–2076
5. Bishop, G. A. (1991) J. Immunol. 147, 1107–1114
6. Kansas, G. S., and Tedder, T. F. (1991) J. Immunol. 147, 4094–4102
7. Truman, J. P., Ericson, M. L., Choqueux-Seebold, C. J., Charron, D. J., and Mooney, N. A. (1994) Int. Immunol. 6, 887–896
8. Nahavi, N., Freeman, G. J., Gault, A., Godfrey, D., Nadler, L. M., and Glimcher, L. H. (1992) Nature 360, 268–268
9. Harton, J. A., Van Hagen, A. E., and Bishop, G. A. (1995) Immunity 3, 349–358
10. Rich, T., Lawler, S. E., Lord, J. M., Blanchetteau, V. M., Charron, D. J., and Mooney, N. A. (1997) J. Immunol. 159, 3792–3798
11. Morio, T., Geba, R. S., and Chatila, T. A. (1994) Bar. J. Immunol. 24, 651–658
12. Kiley, S. B., Grosmaire, L. S., Lyman, L., Grifith, J., Schieven, G. L., Maseiwicz, S., Odum, N., and Ledbetter, J. A. (1995) Tissue Antigens 46, 145–154
13. Kurosaki, T. (1999) Annu. Rev. Immunol. 17, 555–592
14. Law, C. L., Siderenko, S., Cheng, A. W. Y., Draves, K. E., Chan, A. C. W., Aedelhoff, S., Disteche, C. M., and Clark, E. A. (1994) J. Biol. Chem. 269, 12310–12319
15. Saez, S. F., Mahajan, S., Rowley, B. R., Kut, S. A., Fargnoli, J., Burkhart, A. L., Tsukada, S., Witte, O. N., and Bolen, J. B. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9524–9528
16. Kolanser, W., Romeo, C., and Seed, B. (1993) Cell 74, 171–183
17. Chiu, D. H., Spits, H., Peyskkens, J. F., Rowley, B. R., Bolen, J. B., and Weiss, A. (1996) EMBO J. 15, 6251–6261
18. Latour, S., Fourmel, M., and Veilllette, A. (1997) Mol. Cell. Biol. 17, 4434–4441
19. Sone, T., Tsukada, K., Hayakawa, K., Nishimura, Y., Takenouchi, T., Aizawa, M., and Sasazuki, T. (1985) J. Immunol. 1288–1298
20. Robbins, P. A., Evans, E. L., Ding, A. H., Warner, N. L., and Brodsky, F. M. (1987) Hum. Immunol. 18, 301–313
21. Matsushita, S., Kobasa, H., and Nishimura, Y. (1997) J. Immunol. 158, 5685–5691
22. Brunati, A. M., Donella-Deana, A., Buzzeno, M., Marin, O., and Pinna, L. A. (1995) FEBS Lett. 367, 149–152
23. Tsuji, K., Aizawa M., and Sasazuki, T. (1992) HLA 1991: Proceedings of the 11th International Histocompatibility Workshop and Conference, Vol. 1, Oxford University Press, Oxford, United Kingdom

24. Weaver, C. T., and Unanue, E. R. (1986) J. Immunol. 137, 3868–3873

25. Hanke, J. H., Gardner, J. P., Dow, R. L., Changelian, P. S., Brissette, W. H., Weringer, E. J., Pullok, B. A., and Connelly, P. A. (1996) J. Biol. Chem. 271, 695–701

26. Toullec, D., Pianetti, P., Coste, H., Bellerose, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Bourrier, E., Loriole, F., Duhamel, L., Charon, D., and Kirilovsky, J. (1996) J. Biol. Chem. 271, 695–701

27. Oliver, J. M., Burg, D. L., Wilson, B. S., McLaughlin, J. L., and Geahlen, R. L. (1994) J. Biol. Chem. 269, 29697–29703

28. Kondo, N., Ozawa, T., Kato, Y., Motoyoshi, F., Kasahara, K., Kameyama, T., and Orii, T. (1992) Clin. Exp. Immunol. 88, 35–40

29. Keller, A. D., and Maniatis, T. (1991) Genes Dev. 5, 868–879

30. Minami, Y., Nakagawa, Y., Kawahara, A., Miyazaki, T., Sada, K., Yamamura, H., and Taniguchi, T. (1995) Immunity 2, 89–100

31. Cambier, J. C., and Lehmann, K. R. (1989) J. Exp. Med. 170, 877–886

32. Mourad, W., Scholl, P., Diaz, A., Geha, R., and Chatila, T. (1989) J. Exp. Med. 170, 2011–2022

33. Turner, C. A., Jr., Mack, D. H., and Davis, M. M. (1994) Cell 77, 297–306

34. Huang, S. (1994) Cell 78, 9

35. Agarwal, A., Salem, P., and Robbins, K. C. (1993) J. Biol. Chem. 268, 15900–15905

36. Chacko, G. W., Duchemin, A. M., Coggeshall, K. M., Osborne, J. M., Brandt, J. T., and Anderson, C. L. (1994) J. Biol. Chem. 269, 32435–32440

37. Hamano, T., Iwasaki, T., Yamasaki, T., Murata, Y., Kakishita, E., and Nagai, K. (1990) J. Immunol. 144, 811–815

38. Harton, J. A., and Bishop, G. A. (1993) J. Immunol. 151, 5282–5289

39. Palacios, R., Martinez-Maza, O., and Guy, K. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 3456–3460

40. Mehdidate, K., Thibodeau, J., Dohlen, M., Kalland, T., Sekaly, R. P., and Mourad, W. (1995) J. Exp. Med. 182, 1573–1577

41. Matsuzaka, T., and Matsushita, S. (1999) FASEB J. 13, A275

42. Boes, M., Prodeus, A. P., Schmidt, T., Carroll, M. C., and Chen, J. (1998) J. Biol. Chem. 273, 2381–2386

43. Randall, T. D., Heath, A. W., Santos-Argumedo, L., Howard, M. C., Weissman, I. L., and Lund, F. E. (1998) Immunity 8, 733–742

44. Tsou, M. S., Storb, R., Jones, E., Weiden, P. L., Shilman, H., Witherspoon, R., Atkinson, K., and Thomas, E. D. (1978) J. Immunol. 120, 1485–1492

45. Markowitz, J. S., Rogers, P. R., Grusby, M. J., Parker, D. C., and Glimcher, L. H. (1993) J. Immunol. 150, 1223–1233

46. Anderson, J., Coutinho, A., Lernhardt, W., and Melchers, F. (1977) Cell 10, 27–34

47. Turner, M., Mee, P. J., Costello, P. S., Williams, O., Price, A. A., Duddy, L. P., Furlong, M. T., Geahlen, R. L., and Tybulewicz, V. L. (1995) Nature 378, 288–292

48. Cheng, A. M., Rowley, B., Pao, W., Hayday, A., Bolen, J. B., and Pawson, T. (1995) Nature 378, 300–306

49. Greer, S. F., Liu, J., Clarke, C. H., and Justement, L. B. (1998) J. Biol. Chem. 273, 11970–11979

50. Sidorenko, S. P., Law, C. L., Chandran, K. A., and Clark, E. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 359–363

51. Aoki, Y., Kim, Y. T., Stillwell, R., Kim, T. J., and Pillai, S. (1995) J. Biol. Chem. 270, 15658–15663

52. Wang, B. H., Lu, Z. X., and Polya, G. M. (1998) Planta Med. 64, 195–199

53. Nelm, K., Keegan, A. D., Zamorans, J., Ryan, J. J., and Paul, W. E. (1999) Annu. Rev. Immunol. 17, 701–738

54. Heldin, C. H., Ostman, A., and Ronnstrand, L. (1998) Biochim. Biophys. Acta 1378, F79–F113

55. Couture, C., Baker, G., Altman, A., and Mustelin, T. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5301–5305

56. Zoller, K. E., MacNeil, I. A., and Brugge, J. S. (1997) J. Immunol. 158, 1650–1659

57. Xu, R., Seger, R., and Pollet, I. (1999) J. Immunol. 163, 1110–1114

58. Kurosaki, T., Takata, M., Yamashita, Y., Inazu, T., Taniguchi, T., Yamamoto, K., and Yamamura, H. (1994) J. Exp. Med. 179, 1725–1729

59. Miller, L. A., Hong, J. J., Kinech, M. S., Harrison, M. L., and Geahlen, R. L. (1999) Eur. J. Immunol. 29, 1426–1434

60. Gao, J., Zoller, K. E., Ginsberg, M. H., Brugge, J. S., and Shattil, S. J. (1997) EMBO J. 16, 6414–6425