THE LOW-AFFINITY RECEPTOR FOR IgE (CD23) ON B LYMPHOCYTES IS SPATIALLY ASSOCIATED WITH HLA-DR ANTIGENS

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Receptors for the Fc region of IgE (FcεR)[1] have been found on a variety of cell types of hemopoietic origin. High-affinity receptors are expressed on mast cells and basophils, whereas low-affinity receptors (FcεR$_L$) are expressed on T and B lymphocytes, monocytes, and platelets (1–4). FcεR$_L$-bearing lymphocytes play a major role in the regulation of IgE production (5–7). Recently, we reported the generation of a hybridoma secreting mAb 25, which specifically recognizes the 42,000 M_r FcεR$_L$ expressed on human EBV-transformed B cell lines and on a fraction of normal human B cells (8). We and others (9) have demonstrated that the CD23 antigen is an FcεR$_L$ since the CD23 mAbs inhibited the binding of IgE.

After fusion of splenocytes from mice immunized with either RPMI 8866 cells or an enriched FcεR$_L$/CD23 preparation obtained from RPMI 8866 cells, we obtained two mAbs, mAb 135 and 449B4, respectively, which inhibited the binding of IgE to FcεR$_L$/CD23-bearing cells. Interestingly, these antibodies were found to bind to cell lines that do not express the FcεR$_L$/CD23. In this report, it is shown that mAb 135 and 449B4 bind to HLA-DR antigens and that the FcεR$_L$/CD23 is spatially associated with the HLA-DR antigens on the B cell surface.

Materials and Methods

Production of mAbs

mAb 135. BALB/c mice (Iffa Credo, Les Oncins, France) were injected intraperitoneally three times with 5×10^7 RPMI 8866 cells in PBS at 4-wk intervals. 2 d before the fusion, mouse serum samples were collected and were demonstrated to have anti-FcεR$_L$ activity. The binding of IgE to RPMI 8866 cells was inhibited at 1/50,000 mouse antisera dilutions using the assay described below.

Mouse spleen cells were fused with NS-1 myeloma cells (ratio 5:1) using polyethylene

[1] *Abbreviations used in this paper:* DSF, dithio-bis-(succinimidyl propionate); FcεR, receptors for the Fc region of IgE; FcεR$_L$, low-affinity receptors for the Fc region of IgE.

glycol 1000 (Merck-Schuchardt, Darmstadt, Federal Republic of Germany). After incubation overnight at 37°C in a 50-ml flask in complete RPMI 1640 medium as described below, the cell suspension was distributed in 24-well plates in medium containing hypoxanthine-azaserine. 1 wk later, hybridoma supernatants were screened for their ability to inhibit binding of IgE to RPMI 8866 cells. Hybridomas that produced antibodies inhibiting the binding of IgE were cloned and subcloned by limiting dilution.

mAb 449 B4.   mAb 449 B4 was obtained from a mouse immunized with an enriched preparation of FcεR_L/CD23 derived from RPMI 8866 cells. Briefly, RPMI 8866 cells were solubilized in extraction buffer (see below) containing 0.5% NP-40. The cell extract was passed on an IgE-Sepharose immunoabsorbent. The bound fraction was eluted with glycine-HCl buffer, pH 3.0. BALB/c mice were repeatedly immunized by intraperitoneal injections of the fraction included in CFA, and their spleen cells were fused with SP2/O-Ag14 cells (10).

For ascites production, BALB/c mice were injected intraperitoneally with 0.5 ml of pristane (Aldrich Chemical Co., Strasbourg, France). 10 d later, 10^7 hybridoma cells were injected intraperitoneally and ascitic fluid was collected 2–3 wk later. The ascitic fluid was centrifuged at 30,000 g for 30 min at 4°C to remove lipids and cellular debris.

mAbs were isolated from ascitic fluid by affinity chromatography on protein A columns according to manufacturers instructions (Bio-Rad Laboratories, Richmond, CA). In some cases, mAbs were purified from culture supernatants by affinity chromatography on rabbit anti–mouse Ig coupled to Affigel 10 (Bio-Rad Laboratories). The mAbs were eluted with 0.2 M glycine-HCl buffer, pH 2.5, and the pH was further adjusted to 7.2 with 1 M phosphate buffer. The isotype of the mAb was determined by Ouchterlony analysis using polyclonal antibodies specific for mouse Ig classes and subclasses (Zymed Laboratories, San Francisco, CA). Preparation of mAb F(ab')₂ and Fab' fragments, and biotinylation of mAbs were performed according to standard procedures as described previously (8).

Cell Lines

The Burkitt lymphoma lines (Daudi and BJAB) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). These cell lines were cultured in RPMI 1640 (Flow Laboratories, Irvine, Scotland) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine; all were purchased from Flow Laboratories.

Monoclonal Antibodies

The mAb 25 is specific for the FcεR_L on B lymphocytes (8) and precipitates a 42,000 M_r polypeptide from EBV-transformed lymphoblastoid cell lines. The mAbs L243 (anti-HLA-DR) (11) and B7/21 (anti-HLA-DP) were obtained from Becton Dickinson & Co. (Mountain View, CA). The mAb IOT2 (anti-HLA-ABC) was obtained from Immunotech (Luminy, France). The SPV-L3 mAb (anti-HLA-DQ) has been described previously (12). The mAb 1F5 (CD20) was a kind gift of Dr. E. Clark (University of Washington, Seattle, WA).

Analysis with a FACS

Fluorescence analysis was performed with a FACS 440 (Becton Dickinson & Co., Sunnyvale, CA) equipped with a 5 W argon laser running at 488 nm, 0.5 W. Fluorescence parameters were collected using a built-in logarithmic amplifier after gating on the combination of forward light scatter (FLS) and perpendicular light scatter (PLS), which was used to discriminate viable from nonviable cells.

Inhibition of the Binding of IgE to RPMI 8866 Cells.   Hybridoma supernatants were screened for their ability to inhibit the binding of IgE to RPMI 8866 cells as described previously (13) or to inhibit rosette formation of RPMI 8866 cells with IgE-coated erythrocytes (10). Briefly, 5 × 10^5 cells were incubated 30 min at 4°C under gentle agitation with hybridoma supernatants (100 μl), in a 0.2-ml microtiter plate well. After two washes (150 g, 7 min) with PBS, 1% BSA, 0.1% NaN₃, soluble monomeric (IgE PS) was added at a concentration of 10 μg/ml. The plate was then incubated 30 min at 4°C.

Subsequently, cells were washed and incubated with a specific rabbit anti–human IgE antiserum (1/1,000 diluted) for 30 min at 4°C. Cells were then washed and incubated with goat anti–rabbit Ig coupled to fluorescent microspheres (Fluoresbrite Carboxylate, diameter 0.57 μm, ref. 15700; Polysciences, Inc., Warrington, PA) for 30 min at 4°C. Finally, the cell suspension was layered on 3 ml heat-inactivated FCS and centrifuged 15 min at 100 g to remove nonbound beads. The samples were resuspended in 200 μl PBS, BSA, NaN$_3$, and analyzed by flow cytometry as described above. Under these conditions, 45–70% of the cells were fluorescent. mAb 25 (anti FcϵR$_L$/CD23) stains a higher percentage of cells (80–95%) because it has a 1,000-fold higher affinity for the FcϵR$_L$/CD23 than the IgE.

Single-Color Indirect Staining. Single- and double-fluorescence stainings were performed in microtiter plates. 5 × 10^5 cells (in 50 μl) were incubated with 100 μl of hybridoma supernatant or 10 μl of mAb appropriately diluted. After two washes, cells were incubated with fluoresceinated F(ab')$_2$ fragments of goat anti–mouse Ig (Grub, Vienna, Austria) for 30 min at 4°C at a dilution of 1/100. The stained cells were analyzed with the FACS.

Two-Color Immunofluorescence. For double-fluorescence analysis, mononuclear cells were incubated simultaneously with commercially available FITC-conjugated mAbs appropriately diluted and biotinylated mAb 135. The binding of biotinylated antibody was assessed using phycoerythrin-conjugated streptavidin (Becton Dickinson & Co).

Inhibition of the Binding of mAbs. Inhibition of binding was carried out as follows. The cell suspension was incubated with an excess of the first mAb for 30 min at 4°C. After three washes with PBS/BSA, cells were incubated with a second biotinylated mAb (see below). After three washes, the binding of the second antibody was revealed by incubation of the cells with phycoerythrin-conjugated streptavidin. After three washes, cells were analyzed with the FACS.

Human B Lymphocyte Cultures with Human IL-4

B cells were isolated from tonsils obtained from children with chronic tonsillitis. Mononuclear cells were separated on Ficoll/Hypaque. T cells were removed from the mononuclear cells by twice rosetting with 2-aminoethylisothiouronium bromide (AET, Sigma Chemical Co., St. Louis, MO)-treated sheep erythrocytes. Monocytes were depleted by adhering 250 × 10^6 T cell–depleted cells to plastic flasks (Corning Glass Works, Corning, NY) containing 25 ml RPMI 1640 with 10% FCS for 1 h at 37°C. The tonsillar B cell preparations contained: >95% sIg$^+$ cells, as determined by staining with a fluorescein-conjugated F(ab')$_2$ fragment of goat anti–human Ig (Behring werke, Marburg, Federal Republic of Germany); >95% CD20 antigen–positive cells, as determined by staining cells with a mouse anti–human B cell–specific mAb antibody, B1 (Coulter Immunology, Hialeah, FL) and fluorescein-conjugated F(ab')$_2$ goat anti–mouse Ig (Grub); <1% T cells, as determined by the Leu-1 mAb (Becton Dickinson & Co.); <1% monocytes, as determined by the Leu-M3 mAb (Becton Dickinson & Co).

For activation, the purified B cells were cultured at a density of 10^6 cells/ml in Iscove's medium enriched with 50 μg/ml human transferrin, 0.5% BSA, and oleic, linoleic, and palmitic acid (all from Sigma Chemical Co.) as described by Yssel et al. (14). 2% of FCS was added to the medium. Rabbit anti–IgM antibody coupled to beads (Bio-Rad Laboratories) was added at a final concentration of 5 μg/ml. Human rIL-4 (15) was added as a COS-7 transfection supernatant at a final concentration of 1% (80 U/ml). 1 U of IL-4 is defined as the amount resulting in a half maximal [^{3}H]TdR uptake in activated PHA blasts (15).

This lymphokine is able to induce FcϵR$_L$/CD23 on normal human B lymphocytes (16, 17). After 48 h of culture, viable B lymphocytes were separated from the nonviable cells by centrifugation on a Ficoll/Hypaque gradient and were surface labeled as described below.

Cytotoxicity Inhibition Assay

The cytotoxicity inhibition assay was carried out as described elsewhere (18). Briefly, ^{51}Cr-labeled JY cells were incubated with diluted samples of different mAbs. Then effector

cells were added and after 4 h of incubation, supernatants were harvested, counted, and lysis was calculated with the formula: Percent specific lysis = 100 × [(cpm of sample) − (cpm of medium control)]/[(cpm in presence of Triton × 100) − (cpm of medium control)].

The alloreactive cytotoxic IL-2-dependent human T cell clones HY640 and JR-2-10 (19) were used as effector cells. The clones HY640 and JR-2-10 specifically recognized HLA-DQ-1 and HLA-DRW6 antigens, respectively, on JY cells.

Immunoprecipitation and SDS-PAGE

$5 × 10^7$ RPMI 8866 cells or rIL-4-treated B lymphocytes were washed twice with PBS containing 1 mM PMSF (Sigma Chemical Co.) and then resuspended in 1 ml PBS/0.5 mCi ^{125}I-Na (Oris, Saclay, France); 100 μl lactoperoxidase at 5 mg/ml (Calbiochem-Behring Corp., La Jolla, CA), and 50 μl H_2O_2 (0.03% in water) were added. After 3 min of gentle agitation at room temperature, another 100 μl of lactoperoxidase and 50 μl of H_2O_2 were added. The suspension was again agitated for 3 min at room temperature. This step was repeated once again and the cells were finally washed with PBS containing 5 mM KI.

Cells were resuspended at 0°C for 15 min in 300 μl extraction buffer composed of PBS, pH 7.8, with the protease inhibitors: 20 mM iodoacetamide, 1 mM PMSF, 0.01 M benzamidine hydrochloride, 1 μg/ml each of leupeptin, pepstatin, and 100 μg/ml soybean trypsin inhibitor (all from Sigma Chemical Co.) containing either 0.5% NP-40 or 1% digitonin (Aldrich Chemical Co.). Digitonin was prepared as a 2% stock solution: the solid detergent was added to boiling water, which was stirred for 2 min, cooled, allowed to stand at room temperature for 1 wk, and then filtered. The lysates were then centrifuged at 10,000 g for 15 min and the supernatant at 100,000 g for 30 min before preclearing overnight with a 50-μl packed volume of protein A–Sepharose beads (Pharmacia Fine Chemicals, Uppsala, Sweden) precoated with a nonrelated mAb (100 μl of ascites). Immunoprecipitation was performed by incubating the extract for 4 h at room temperature with 50 μl protein A–Sepharose beads precoated with the different mAbs. Immunoprecipitates were washed five times with the extraction buffer and subjected to SDS-PAGE using the method of Laemmli (20). The gels were then fixed, dried, and autoradiographed using X-OMAT AR-05 film (Eastman Kodak, Paris, France) at −70°C.

Protein Elution from SDS-PAGE Gel

The SDS-PAGE gel containing the 42,000 M_r polypeptide isolated by immunoprecipitation with mAb 135 from ^{125}I-labeled and dithio-bis-(succinimidylpropionate) (DSP)-crosslinked RPMI 8866 cell lysates, was sliced into small pieces and a carrier protein, BSA, (Sigma Chemical Co.) was added. The polypeptide was eluted in extraction buffer mentioned above for 20 h at 37°C. After centrifugation, 12,000 rpm for 30 min, the supernatant was then immunoprecipitated with mAb 25 or with normal mouse Ig as control.

Crosslinking

Radiolabeled cells were resuspended at 3.10^7/ml in PBS pH 7-4 containing 1 mM $MgCl_2$, 0.02% sodium azide. The cleavable crosslinker DSP (Pierce Chemical Co., Rockford, IL) was dissolved in DMSO at a concentration of 10 mM and added to the cell suspension at a final concentration of 1 mM. Reaction was allowed to occur for 30 min at 4°C under gentle agitation. After two washes with PBS to remove free DSP, the cell suspension was lysed and immunoprecipitations were carried out as described above.

Results

Certain mAbs Specific for HLA-DR Antigens Partially Inhibit the Binding of IgE to FcεRL/CD23

To obtain mAbs specific for the FcεRL, the supernatants of hybridomas obtained from a mouse immunized with the FcεRL + RPMI 8866 cell line were
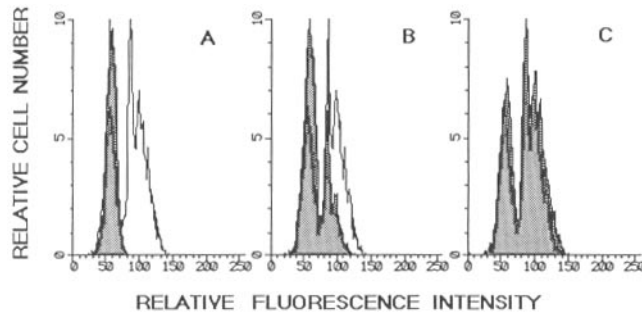
FIGURE 1. mAb 135 inhibits the binding of IgE to RPMI 8866 cells. (A) RPMI 8866 cells were incubated with 10 μg/ml of IgE (open) or without IgE as negative control (shaded), then with polyclonal anti-IgE antiserum and goat anti–rabbit Ig coupled to fluorescent microspheres. (B) RPMI 8866 cells were preincubated with 50 μg/ml of mAb 135 (shaded) or without (open) then reacted subsequently with IgE, polyclonal anti-IgE antiserum and goat anti–rabbit Ig coupled to fluorescent microspheres. (C) RPMI 8866 cells were preincubated with 50 μg/ml of mAb IOT2 (anti HLA-ABC) (shaded) or without (open), then treated as in A and B. Fluorescence was analyzed by flow cytometry. Abscissa, log fluorescence intensity; ordinate, relative number of cells.

screened for their ability to inhibit the binding of IgE to the same cells. The supernatant of one hybridoma, 9P135, inhibited the binding of IgE as determined in an indirect fluorescent microspheres assay. One subclone of this hybridoma was selected that secreted mAb 135 (IgG1) (Fig. 1). Purified mAb 135 and its derived F(ab')$_2$ and Fab' fragments inhibited the binding of IgE to RPMI 8866 cells (Table I), and several other EBV-transformed cell lines (data not shown), in a dose-dependent manner. In contrast with mAb 25 (anti-FcϵR$_L$/CD23), which totally inhibits the binding of IgE to its receptor, mAb 135, even at high concentrations, only partially inhibits IgE binding to the FcϵR$_L$. MAb 135 decreases both the percentage of cells binding IgE and the fluorescence intensity of cells that still bind IgE (Fig. 1). RPMI 8866–reactive nonrelated mAbs such as mAb IOT2 (anti-HLA-ABC) and the CD20 mAbs 1F5 and B1 failed to inhibit IgE binding to RPMI 8866 cells (Table I, and data not shown). mAb 135 was, however, found to bind to FcϵR$_L$/CD23$^-$ cells (e.g., Daudi, BJAB). Immunoprecipitation analysis demonstrated that mAb 135 precipitated two polypeptides of 36,000 and 28,000 M_r (Fig. 2), suggesting that it was specific for the HLA class II antigens. Binding of mAb 135 analyzed by flow cytometry to a large panel of cell lines differentially expressing HLA-DP, -DQ, and -DR antigens suggested that it was binding to HLA-DR antigens (not shown here). The specificity of mAb 135 for HLA-DR antigens was further substantiated by the finding that mAb 135 was able to specifically inhibit the cytotoxicity of the T cell clone JR-2-10 recognizing HLA-DR antigens on the JY target cells, whereas the cytotoxicity of the T cell clone HY640 recognizing HLA-DQ antigens was not affected by mAb 135 (Table II). Another mAb, 449B4, obtained from a mouse immunized with enriched FcϵR$_L$/CD23 preparations, was found to inhibit the binding of soluble IgE to EBV cell lines and inhibit rosette formation of RPMI 8866 cells with IgE-coated ox erythrocytes. Furthermore, mAb 449B4 was found to inhibit the binding of mAb 135 to a variety of cell lines (not shown) and to precipitate two polypeptides with 36,000 and 28,000 M_r, demonstrating that it had the same specificity as mAb 135. The sequence of the 36,000 M_r polypeptide recognized by mAb 449B4 proved to be identical to the sequence of HLA-DRα, and the sequence of the 28,000 M_r polypeptide, also identified by

TABLE I

mAb 135 Inhibits IgE Binding to its Receptor on RPMI 8866 Cells via its Fab Fragments

mAb	FcεR$_L^+$ cells*	Inhibition IgE binding[‡]
	%	%
mAb 135		
50 µg/ml	26	44
20 µg/ml	34.6	22
10 µg/ml	39.6	9
F(ab')₂ mAb 135		
50 µg/ml	29	36
20 µg/ml	36.5	17
Fab' mAb 135		
50 µg/ml	34	24
20 µg/ml	37.7	14
Fc mAb 135	48	0
mAb 25[§]	8.1	91
Nonrelated mAb: C^{+}[∥]	43.1	0
C^{-}[¶]	4.5	

* Cells were incubated with intact mAb 135, F(ab')₂, Fab' or Fc fragments and nonrelated mAb (as control). Then 10 µg/ml of IgE, polyclonal anti IgE antiserum and goat antirabbit Ig coupled to microspheres were successively added.

[‡] Calculated according to the formula: Percent inhibition = 100 × [(assay − C⁻)/(C⁺ − C⁻)] (normalized values of 3 assays).

[§] mAb 25 was used at 10 µg/ml and stained 90% of cells.

[∥] mAb IOT2 (anti HLA ABC) was used at 50 µg/ml and stained 98% of cells.

[¶] IgE was omitted in the microsphere assay procedure.

mAb 449B4, was shown to be homologous to reported sequences of HLA-DRβ (Dr. M. Bond, DNAX Research Institute, personal communication). This demonstrated that certain anti-HLA-DR antibodies are able to partially block the binding of IgE to FcεR$_L$/CD23, suggesting a possible association of HLA-DR antigens with FcεR$_L$/CD23. Not all the anti-HLA-DR antibodies were found to inhibit the binding of IgE. The well-characterized mAb L243 (11) was unable to inhibit the binding of IgE to FcεR$_L$/CD23⁺ cell lines and was also unable to inhibit the binding of mAb 135 (Fig. 3) to the same cell lines, demonstrating that these antibodies are binding to different epitopes of the HLA-DR molecule. An mAb specific for HLA-DQ antigens (12) and an mAb specific for HLA-DP antigens were unable to inhibit the binding of IgE to RPMI 8866 cells.

Anti-HLA-DR antigen mAbs may therefore be divided into two well-defined groups: one group inhibiting the binding of both IgE and mAb 135 and a second group that does not affect the binding of IgE or mAb 135. Nevertheless, both groups of antibodies bound 36,000 and 28,000 M_r proteins expressed on the cell
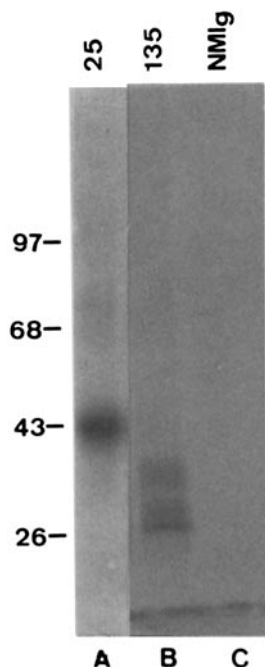
FIGURE 2. Immunoprecipitation of surface proteins of radiolabeled RPMI 8866 cells. RPMI 8866 cells were labeled by surface iodination (Na-^{125}I) with lactoperoxidase and solubilized with NP-40 lysis buffer. After preclearing, cell extracts were incubated with mAb 25 (A), mAb 135 (B), and normal mouse Ig (NMIg) (C). After SDS-PAGE, labeled immunoprecipitated proteins were revealed by autoradiography. Prestained molecular weight standards were coelectrophoresed phosphorylase B (97,000), BSA (68,000), OVA (43,000), and α-chymotrypsinogen (26,000).

TABLE II

The Effect of Antibodies against Class II MHC Antigens on the Lysis of JY Cells by Two CTL Clones

CTL clone	Lysis of the control	Percent lysis in the presence of antibody*									
		SPV-L3 (anti-HLA-DQ)		449B4 (anti-HLA-DR)		L243 (anti-HLA-DR)		mAb 135 (anti-HLA-DR)		mAb 25 (anti-FcεRL)	
		100‡	500	100	500	100	500	100	500	100	500
	%	%		%		%		%		%	
HY-640	39	3	3	40	32	41	39	47	36	39	37
JR-2-10	50	49	46	6	18	5	20	5	13	46	46

^{51}Cr-labeled JY cells were incubated with different mAbs and used as targets in a cytotoxicity assay with class II MHC antigen–specific CTL clone.
* Cytotoxicity was measured at an E/T ratio of 5:1.
‡ Reciprocal dilution of ascites fluid.

surface. Immunoprecipitation experiments excluded the possibility that the anti-HLA-DR mAbs inhibiting the binding of IgE were recognizing an epitope common to both HLA-DR and FcεR$_L$/CD23, since the latter molecule could not be immunoprecipitated by these antibodies.

Taken together, these data demonstrate that certain mAbs directed against HLA-DR antigens have the ability to inhibit partially the binding of IgE to FcεR$_L$/CD23 and suggest a physical interaction between FcεR$_L$/CD23 and HLA DR antigens.

Spatial Association between FcεR$_L$/CD23 and HLA-DR Antigens

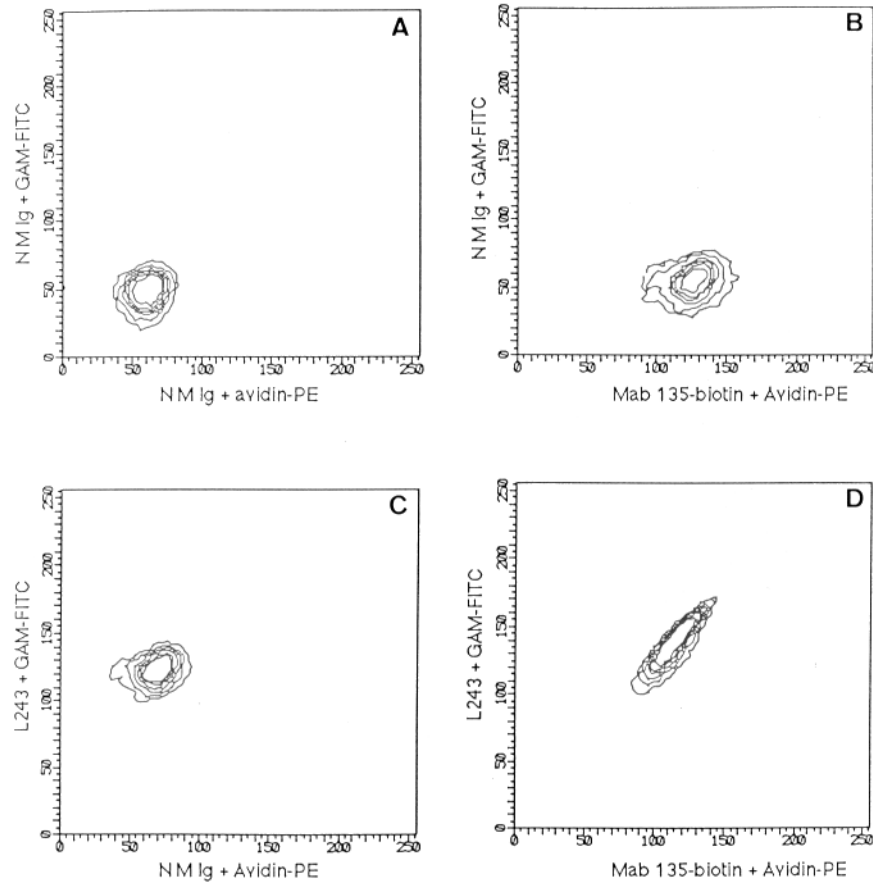*Crosslinking Studies.* One approach to studying the possible physical associa-

FIGURE 3.   Staining of RPMI 8866 cells with the mAbs L243 and mAb 135. RPMI 8866 cells were stained with normal mouse Ig (*NMIg*) and goat anti–mouse coupled to FITC (*A*), with biotinylated mAb 135, revealed with phycoerythrin-streptavidin (*B*), with FITC conjugated L243 (*C*), or cells were double-stained with biotinylated mAb 135 and FITC-conjugated L243. Biotinylated mAb 135 was developed with phycoerythrin-streptavidin (*D*). Samples were analyzed with a FACS 440 with logarithmic amplification.

tion of two cell surface molecules on the cell membrane surface is chemical crosslinking. This approach was used to show the association between the T cell receptor and the CD3 complex (21) and the association between the insulin receptor and the HLA class I antigens (22). To study the possible physical association between FcεR$_L$/CD23 and HLA-DR antigens, ^{125}I-labeled RPMI 8866 cells were crosslinked with DSP and the NP-40 cell lysate was then precipitated with either mAb 135 or mAb 25 (anti-FcεR$_L$/CD23). SDS-gel analysis shows that mAb 135 precipitated, in addition to the expected 36,000 and 28,000 M_r class II MHC polypeptides, a 42,000 M_r polypeptide and, weakly, a 90,000 smear (Fig. 4). The anti-HLA-DR mAb L243 also precipitated these three major polypeptides from the crosslinked cell extract (not shown). To demonstrate that the 42,000 M_r polypeptide was the FcεR$_L$/CD23, the SDS-PAGE gel was sliced in the 40,000–45,000 M_r area and the protein was eluted and subjected to
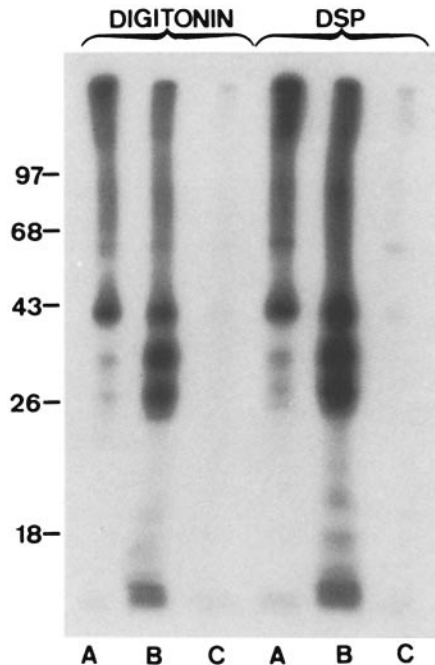
FIGURE 4. $Fc\epsilon R_L$/CD23 and HLA-DR antigens are spatially associated on RPMI 8866 cells. RPMI 8866 cells were labeled by surface iodination with ^{125}I-lactoperoxidase and solubilized with digitonin lysis buffer or treated with DSP and then solubilized with NP-40 lysis buffer. After preclearing, the cell lysates were immunoprecipitated with mAb 25 (A), mAb 135 (B), or normal mouse Ig (C). The precipitates were analyzed in the same conditions as in Fig. 2. Arrows indicate the migration of $Fc\epsilon R_L$ and HLA-DR antigens.

precipitation with the $Fc\epsilon R_L$/CD23-specific mAb 25. Fig. 5 shows that the eluted polypeptide could specifically be precipitated by mAb 25, thus demonstrating that the 42,000 M_r polypeptide coprecipitated with the anti-HLA-DR antibody mAb 135 was indeed $Fc\epsilon R_L$/CD23. The smear around 90,000 M_r was also present and most probably represents an aggregated form of the $Fc\epsilon R_L$/CD23. Conversely, mAb 25 precipitated in addition to the expected 42,000 M_r $Fc\epsilon R_L$/CD23 polypeptide and 90,000 M_r smear, two polypeptides with 36,000 and 28,000 M_r when cell surface components on RPMI 8866 cells were cross-linked (Fig. 4).

Digitonin Cell Extract. The use of the detergent digitonin made it possible to prepare cell extracts in which complex receptors composed of noncovalently linked proteins could be isolated (23). Therefore ^{125}I-labeled RPMI 8866 cells extracts were prepared using digitonin and immunoprecipitated with either mAb 25 or mAb 135. Under these conditions, mAb 25 was able to precipitate, in addition to the expected 42,000 M_r $Fc\epsilon R_L$/CD23, the polypeptides with 36,000 and 28,000 M_r (Fig. 4). Conversely, mAb 135 coprecipitated with the expected 36,000 and 28,000 M_r polypeptides, the 42,000 M_r molecule (Fig. 4). The 90,000 M_r smear was also found under these conditions.

Taken together, these biochemical data demonstrate that the $Fc\epsilon R_L$/CD23 and HLA-DR antigens are spatially associated on the cell surface of EBV-transformed B cell lines.

The IL-4-induced $Fc\epsilon R_L$/CD23 on Normal B Cells Is Associated with HLA-DR Antigens

It has recently been shown that human IL-4 (B cell stimulatory factor 1 [BSF1]) (15) was able to induce a strong expression of $Fc\epsilon R_L$/CD23 on normal B
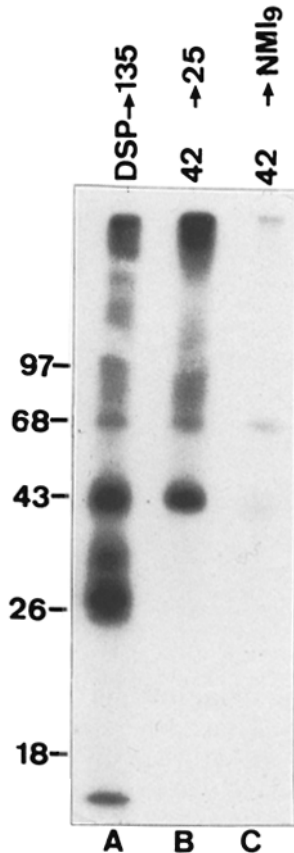
FIGURE 5. The 42,000 M_r molecule precipitated with mAb 135 after DSP crosslinking of labeled RPMI 8866 cells is the FcɛR_L/CD23 antigen. RPMI 8866 cells were cell surface iodinated. After DSP treatment, labeled cells were solubilized with NP-40 lysis buffer. After preclearing, the cell lysates were immunoprecipitated with mAb 135 (A). The 42,000–45,000 M_r polypeptides isolated in A were eluted and immunoprecipitated with mAb 25 (B) and normal mouse Ig (NMIg) as control (C).

lymphocytes (16, 17). We therefore investigated whether the IL-4-induced FcɛR_L/CD23 on normal B lymphocytes was spatially associated with HLA-DR antigens as demonstrated above on the EBV-transformed cell lines. Purified tonsil B cells were cultured for 48 h with rIL-4 (80 U/ml) in the presence of anti-IgM antibody coupled to beads, conditions that were found to be optimal for the induction of FcɛR_L/CD23. As a control, cells were cultured with anti-IgM antibody coupled to beads, which does not induce a significant expression of FcɛR_L/CD23. Viable cells, obtained after centrifugation over Ficoll/Hypaque, were labeled with ^{125}I-lactoperoxidase and crosslinked with DSP. The NP-40 extract was precipitated with mAb 135 or mAb 25. In Fig. 6 it is demonstrated that mAb 135 is able to precipitate the three bands with 42,000, 36,000, and 28,000 M_r from the rIL-4-cultured B cells. Conversely, mAb 25 coprecipitated with the expected 42,000 M_r FcɛR_L/CD23, the 36,000 and 28,000 M_r polypeptides from the rIL-4-cultured B cells. The 90,000 M_r smear was also observed in the IL-4-treated cells. From control cells, which are FcɛR_L/CD23⁻ negative and DR⁺, mAb 135 could only precipitate the 36,000 and 28,000 M_r polypeptides, and mAb 25, as expected, no polypeptide. These results demonstrate that the IL-4-induced FcɛR_L/CD23 on normal B cells is spatially associated with HLA-DR antigens.
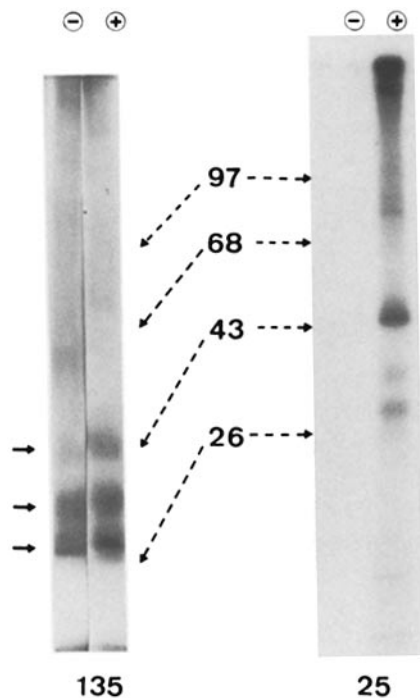
FIGURE 6. The IL-4-induced FcεR$_L$/CD23 on normal human B cells is spatially associated with HLA-DR antigens. IL-4-induced (+) or noninduced (−) purified human B cells were cell surface iodinated. After DSP treatment, labeled cells were solubilized with NP-40 lysis buffer. After preclearing, the cell lysates were immunoprecipitated with mAb 135 (*left*) or with mAb 25 (*right*). The precipitates were analyzed as described in Fig. 2. Arrows indicate the position of FcεR$_L$ and HLA-DR antigens.

Discussion

We have previously described the generation of mAb 25, which specifically recognized the FcεR$_L$/CD23 (8), and blocked the binding of IgE to human B cells. We found two other mAbs (mAbs 135 and 449 B4) that partially inhibited the binding of IgE to the low-affinity receptor for IgE on the EBV-transformed lymphoblastoid cell line RPMI 8866. These antibodies decreased both the percentage of cells binding IgE and the fluorescence intensity of cells that still bind IgE. mAb 135 was obtained from fused splenocytes of a mouse immunized with RPMI 8866 cells, whereas 449 B4 was obtained from fused splenocytes of a mouse immunized with an enriched preparation of FcεR$_L$ derived from RPMI 8866 cells.

Unlike mAb 25, mAbs 135 and 449 B4 were able to stain FcεR$_L$/CD23$^-$ cell lines (Daudi, BJAB). This suggested that these antibodies were binding to either an epitope common to FcεR$_L$/CD23 and to another cell surface molecule or to an antigen not antigenically related to the FcεR$_L$/CD23 but linked to it in such a fashion that the binding of the mAb 135 or mAb 449 B4 would affect the binding of IgE to the FcεR$_L$/CD23.

The partial inhibition of binding of IgE by mAbs 135 and 449B4 and the finding that some FcεR$_L$/CD23 could still be immunoprecipitated by mAb 25 from NP-40 extracts of DSP crosslinked cells precleared with mAb 135 (data not shown) suggest that not all the FcεR$_L$/CD23 were associated with the HLA-DR antigens. Immunofluorescence analysis performed with a panel of cell lines differentially expressing HLA-DR antigens (as well as DP and DQ, not shown here), biochemical analysis (immunoprecipitation of 36,000 and 28,000 M_r

polypeptides), inhibition of cytotoxicity of HLA-DR-specific T cell clones, and protein sequencing of mAb 449B4–recognized antigen (Dr. M. Bond, DNAX Research Institute, personal communication) demonstrated that mAb 135 and 449B4 were specific for HLA-DR antigens. This suggested that the FcεR$_L$/CD23 was somehow associated with HLA-DR antigens. It could be excluded that the mAb 135 and 449B4 were binding to an epitope common to FcεR$_L$/CD23 and HLA-DR since they only precipitated the HLA-DR polypeptides under standard conditions. Interestingly, not all the anti-HLA-DR mAbs were able to inhibit the binding of IgE. For instance, the well-characterized anti-HLA-DR mAb L243 (11) was unable to inhibit the binding of either IgE or mAb 135 to RPMI 8866 cells, suggesting that mAb L243 binds to an epitope of the HLA-DR molecule distant from the site of interaction of HLA-DR molecules with the FcεR$_L$/CD23. To study the possible association of HLA-DR antigens and FcεR$_L$/CD23, ^{125}I-Na-lactoperoxidase-labeled cells were crosslinked with DSP and solubilized with NP-40. The anti-FcεR$_L$/CD23 mAb 25 could precipitate the expected 42,000 M_r polypeptide, a 90,000 M_r smear, and two polypeptides of 36,000 and 28,000 M_r. Conversely, mAb 135 and 449 B4 precipitated the 42,000 M_r polypeptide and the 90,000 M_r smear, in addition to the expected 36,000 M_r and 28,000 M_r polypeptides. The 42,000 M_r polypeptide precipitated by anti-HLA-DR antibodies was found to be the FcεR$_L$/CD23 since it could be precipitated by mAb 25 after solubilization from the SDS-PAGE gel. The smear around 90,000 M_r has been previously suggested to represent an aggregated dimer of the 42,000 M_r band (24). Moreover this 90,000 M_r band, inconstantly found, appeared when the 42,000 M_r eluted material was stored at −20°C for a few days before use (data not shown). When intact noncrosslinked labeled cells were solubilized with Digitonin instead of NP-40, a procedure that maintains the noncovalent association between the different polypeptides of complex receptors (23), mAb 25 could immunoprecipitate the 28,000 and 36,000 M_r polypeptides together with the 42,000 M_r polypeptide. Conversely, the mAb 135 was able to precipitate the 42,000 M_r polypeptide in addition to the 28,000 and 36,000 M_r ones. This suggests that the FcεR$_L$/CD23 is spatially associated with the HLA-DR antigens on the B lymphocyte cell surface. Finally the association of FcεR$_L$/CD23 with HLA DR antigens is further confirmed by the fact that the anti-HLA-DR mAb 449 B4 was obtained after immunizing mice with the partially purified FcεR$_L$/CD23 eluted from an IgE immunoabsorbent. The demonstration of a physical association between FcεR$_L$/CD23 and HLA-DR antigens on the surface of cell lines raises the question as to whether this phenomenon is linked to the transformation induced by EBV. This is particularly relevant since only a fraction of normal B cells express at very low levels the FcεR$_L$/CD23. Recently, it has been demonstrated that human IL-4 (15) is able to induce the FcεR$_L$/CD23 on normal B cells (16, 17). We therefore investigated whether the IL-4-induced FcεR$_L$/CD23 antigen was associated to HLA-DR antigens. Crosslinking experiments demonstrated that the IL-4-induced FcεR$_L$/CD23 on normal B cells is also associated with HLA-DR antigens. This demonstrated that the association of FcεR$_L$/CD23 with HLA-DR antigens is not a consequence of the transformation of B cells by EBV.

Association between Fc receptors for IgG and MHC class II molecules on B

lymphocytes has been reported earlier in rodents, since anti-Ia antibodies inhibited the binding of IgG complexes (25–28). It was further proposed that this inhibition was indeed due to an alteration of the Ia antigens mediated by the binding of the anti-Ia antibody and resulting in the association of the Ia antigens with the Fc receptors, independent of the anti-Ia antibody Fc fragment (29). Thus, although these observations showing interaction of murine MHC class II antigen with FcγR correlate with our observation that human MHC class II antigens are associated with FcεR$_L$, conclusions of these studies differ. It therefore seems of interest to reinvestigate the possible physical association of murine MHC class II antigens with FcγR using the biochemical approach described in our study. Actually, soluble IgG binding factors derived from mouse T cells were found to be absorbed by anti-Ia antisera (30, 31), suggesting either a physical association between IgG binding factors and Ia antigens or the presence of common antigenic determinants on both structures. Actually, the presence of antigenic determinants common to rodent MHC class II antigens and IgE binding factors was recently reported since OX3 mAb was able to recognize an epitope containing an N-linked oligosaccharide common to Ia antigens and 60,000 M_r T cell–derived IgE suppressive factors (32, 33).

Association between other cell surface receptors and HLA antigens has been reported recently. First, an anti-HLA class I mAb was found to effectively inhibit the binding of insulin to insulin receptors (34). Upon crosslinking of cell surface antigens the insulin receptor has been shown to coprecipitate with HLA class I antigens (22, 34, 35). Second, a monoclonal as well as a polyclonal anti-HLA class I antibody have been found to inhibit the binding of epidermal growth factor (EGF) to A431 cells (36). This inhibition required crosslinking of HLA antigens since Fab fragments needed to be crosslinked with an anti–mouse Ig antibody to be effective. This suggests that the mechanisms of inhibition of EGF binding by anti-HLA class I antibodies is different from the mechanisms of inhibition of IgE binding by anti HLA-DR antibodies.

It remains to be determined whether the anti HLA-DR antibodies inhibiting the binding of IgE are specific for the HLA-DR α or HLA-DR β chain. Finally, a further investigation of the possible biological function of the FcεR$_L$/CD23–HLA-DR complex must await an understanding of the role of FcεR$_L$/CD23 itself.

Summary

Two hybridomas that produce the mAbs 135 and 449 B4 were obtained that inhibited the binding of IgE to the FcεR$_L$/CD23 on the EBV-transformed B cell line RPMI 8866. mAb 135 was obtained from a mouse immunized with RPMI 8866 cells, whereas mAb 449B4 was obtained from a mouse immunized with a partially purified preparation of FcεR$_L$/CD23 obtained as the eluate of an IgE immunoabsorbent loaded with a soluble extract of RPMI 8866 cells. These two mAbs bound to FcεR$_L$/CD23$^-$ cell lines and precipitated two polypeptides with 36,000 M_r and 28,000 M_r, which were the HLA-DR α and β chains, respectively.

Immunoprecipitation with mAb 135 of NP-40 lysates from dithio-bis-(succinimidyl propionate) (DSP) crosslinked ^{125}I-labeled RPMI 8866 or normal B cells incubated with rIL-4 showed three polypeptides with 42,000, 36,000, and 28,000 M_r. The 42,000 M_r polypeptide is identical to the FcεR$_L$/CD23 since it could be

precipitated by the anti-FcεR$_L$/CD23 mAb 25 after resolubilization from the SDS-PAGE gel. Immunoprecipitations of the crosslinked cell extracts carried out with the anti-FcεR$_L$/CD23 mAb 25 yielded the same three polypeptides. Furthermore, when RPMI 8866 or rIL-4 preincubated normal B cells were solubilized with a digitonin buffer, which prevents the dissociation of noncovalently linked polypeptide complexes, mAb 135 and mAb 25 precipitated complexes composed of three molecules with 42,000, 36,000, and 28,000 M_r. The well-characterized anti-HLA-DR mAb L243 was unable to block the binding of either IgE or mAb 135 to RPMI 8866 cells, although it could immunoprecipitate the complex (HLA-DR–FcεR$_L$/CD23) from crosslinked cell lysates. Since mAb 135 and L243 were able to both bind the RPMI 8866 cells, it demonstrates that they bind to different epitopes of the HLA-DR complex, the mAb 135 epitope of the HLA-DR molecule being close to the IgE binding site of the FcεR$_L$/CD23. These data demonstrated that the FcεR$_L$/CD23 and HLA-DR antigens are spatially associated on the B cell membrane.

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