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Lupus IgG V_H4.34 Antibodies Bind to a 220-kDa Glycoform of CD45/B220 on the Surface of Human B Lymphocytes[1]

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Anti-lymphocyte autoantibodies are a well-recognized component of the autoimmune repertoire in human systemic lupus erythematosus (SLE) and have been postulated to have pathogenic consequences. Early studies indicated that IgM anti-lymphocyte autoantibodies mainly recognized T cells and identified CD45, a protein tyrosine phosphatase of central significance in the modulation of lymphocyte function, as the main antigenic target on T cells. However, more recent work indicates that lupus autoantibodies can also recognize B cells and that CD45 may also represent their antigenic target. In particular, IgM Abs encoded by V_H4.34 appear to have special tropism for B cells, and strong, but indirect evidence suggests that they may recognize a B cell-specific CD45 isoform. Because V_H4.34 Abs are greatly expanded in SLE, in the present study we investigated the antigenic reactivity of lupus sera V_H4.34 IgG Abs and addressed their contribution to the anti-lymphocyte autoantibody repertoire in this disease. Our biochemical studies conclusively demonstrate that lupus IgG V_H4.34 Abs target a developmentally regulated B220-specific glycoform of CD45, and more specifically, an *N*-linked *N*-acetyllactosamine determinant preferentially expressed on naive B cells that is sterically masked by sialic acid on B220-positive memory B cells. Strikingly, our data also indicate that this reactivity in SLE sera is restricted to V_H4.34 Abs and can be eliminated by depleting these Abs. Overall, our data indicate that V_H4.34 Abs represent a major component of the lupus IgG autoantibody repertoire and suggest that the carbohydrate moiety they recognize may act as a selecting Ag in SLE. ***The Journal of Immunology,*** **2004, 172: 4298–4307.**

Systemic lupus erythematosus (SLE)[4] is characterized by increased levels of serum autoantibodies directed against multiple self Ags, including determinants expressed on the surface of lymphocytes (1, 2). Indeed, it is well established that most patients with SLE develop IgM anti-lymphocyte autoantibodies (ALA) at some point in the course of the disease (1, 2). The best-characterized ALA are cold-reactive anti-T cell IgM Abs with lymphocytotoxic activity whose serum levels correlate with the degree of global peripheral lymphopenia and disease activity (3). Yet, warm-reactive IgG ALA have also been reported in SLE (4). Although ALA are most likely heterogeneous in terms of antigenic reactivity, IgM ALA appear to preferentially recognize different isoforms of CD45, a transmembrane protein tyrosine phosphatase expressed in the surface of both B and T cells that plays a central role in lymphocyte homeostasis (5, 6).

SLE anti-CD45 Abs have been reported to recognize nonsialylated carbohydrate determinants in the highly *O*-glycosylated polymorphic domains of CD45 isoforms expressed by T cells (7–9). By and large, these Abs appear to preferentially bind T cells, but not B cells, suggesting that they recognize a T cell-specific CD45 glycoform (4, 7, 10, 11). Yet, the information summarized above needs to be reconciled with the observation that at least a subset of lupus autoantibodies has the ability to bind B cells possibly by recognizing a B cell-specific CD45 isoform (12–14). Such autoantibodies, termed V_H4.34 Abs, owing to their expression of surface Ig encoded by the V_H4.34 gene segment, are intrinsically autoreactive by virtue of their almost universal, and largely L chain-independent, recognition of the *N*-acetyllactosamine (NAL) antigenic determinant of the I/i blood group Ag (15–17). Strikingly, V_H4.34 Abs make up the vast majority of pathogenic IgM anti-i cold agglutinin, and the V_H4.34 gene segment seems to be mandatory for the generation of such autoantibodies (18, 19). Of note, NAL is also expressed on a 220-kDa CD45 B cell-specific isoform, which has been postulated to represent the antigenic target of V_H4.34 IgM Abs derived either from patients with Wiskott-Aldrich syndrome or monoclonal cold agglutinin disease (13, 14, 20–22). However, in these studies, the V_H4.34 Abs used failed to immunoprecipitate CD45, and therefore the actual nature of their antigenic target in B cells remains to be formally established.

Despite the abundance of V_H4.34 B cells in normal individuals, V_H4.34 Abs are virtually undetectable in healthy sera due to strict censoring of V_H4.34 B cells (23, 24). However, circulating V_H4.34 Abs are highly expressed in patients with SLE in whom they constitute a substantial fraction of anti-DNA Abs and highly correlate with overall disease activity, kidney, and CNS involvement (25–28). We have reported that censoring of V_H4.34 B cells in healthy subjects is largely achieved by exclusion from participating in productive germinal center reactions (24). Our studies also show that this censoring mechanism is faulty in patients with SLE in whom V_H4.34 B cells frequently form mature germinal centers and are abundantly expressed in the IgG memory and plasma cell repertoire (29). However, the actual antigenic reactivity of V_H4.34 IgG Abs in SLE sera remains to be determined with a recent study suggesting that such Abs may not represent a major B cell-binding species (27).

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[4] Abbreviations used in this paper: SLE, systemic lupus erythematosus; ALA, anti-lymphocyte autoantibody; $AmSO_4$, ammonium sulfate, LCA, leukocyte common Ag; NAL, *N*-acetyllactosamine; PTPase, phosphotyrosine phosphatase.

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In the present study, we have analyzed the contribution of IgG V_H4.34 Abs to the anti-lymphocyte repertoire in SLE and conclusively established the molecular basis for the reactivity of these Abs with human B cells. We demonstrate that V_H4.34 IgG Abs target a developmentally regulated B220-specific glycoform of CD45, and more specifically, an *N*-linked NAL determinant preferentially expressed on naive B cells. Strikingly, our data also indicate that the reactivity of SLE sera with this CD45 glycoform is dependent on V_H4.34 Abs and can be eliminated by depleting these Abs. To the best of our knowledge, our results also represent the first quantitative analysis of the abundance of V_H4.34 Abs in the SLE IgG repertoire. Our findings indicate that V_H4.34 Abs constitute a large fraction (10–50%) of all IgG in active SLE patients. The implications of our results regarding the antigenic selection and possible pathogenic roles of these autoantibodies in SLE are discussed.

Materials and Methods

Human samples

Peripheral blood (PBL) and tonsil samples were obtained from healthy donors, according to protocols approved by the University of Rochester Medical Center (URMC) Institutional Review Board. Tonsils were obtained as excess tissue from elective tonsillectomies from otherwise healthy patients aged 2–10 years. Only PBL was obtained from SLE patients. Patients were randomly selected from the URMC Lupus Clinic on the basis of their willingness to participate in the study if they had a clinical diagnosis of SLE, fulfilled ≥4 American College of Rheumatology criteria for the classification of SLE (30, 31), and had been only treated with antimalarials and/or low-dose prednisone (<10 mg/day) for at least 4 wk previous to venipuncture. Patients were classified as having nephritis based on the presence of an active urinary sediment, proteinuria ≥1000 mg/24 h, and/or a history of nephritis documented by kidney biopsy.

ELISA for detection of serum V_H4.34-encoded Abs

ELISA plates (Nunc, Naperville, CA) were coated with V_H4.34-specific anti-idiotypic mAb 9G4 (kindly provided by F. Stevenson, Tenovus Research Laboratories, Southampton, U.K.), or its isotype control (rat IgG2a; Sigma-Aldrich, St. Louis, MO), at 2 μg/ml and incubated for 1 h at 37°C (32). Plates were blocked with 2% nonfat dry milk/2% BSA for 1 h at 37°C, and then washed with 0.1% Tween 20 in PBS. Sera were serially diluted in HBSS (Life Technologies, Carlsbad, CA) and incubated for 30 min at 37°C. Plates were washed, then incubated with alkaline phosphatase-conjugated goat anti-human IgG (1/2000 dilution; BioSource International, Camarillo, CA) at 37°C for 1 h. After washing, plates were developed using the *p*NPP substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD), according to manufacturer's instructions, and OD at 405 nm was read on a microplate reader (model 3550-UV; Bio-Rad, Hercules, CA). Serum concentrations were determined using a V_H4.34 IgG standard represented by a V_H4.34 IgG mAb established in our laboratory by EBV immortalization of SLE PBL B cells. V_H4.34 IgG levels were corrected with respect to total serum IgG for all samples analyzed. The amount of total IgG in serum samples was determined by isotype-specific capture ELISA using goat anti-human IgG (5 μg/ml; Kirkegaard & Perry Laboratories) as the coating Ab and a human IgG standard (Sigma-Aldrich, St. Louis, MO) for quantitation.

B cell isolation

All protocols were conducted, as previously described, in our laboratory (24). Briefly, mononuclear cells were isolated from heparinized peripheral blood (PBL) by gradient centrifugation at 4°C using Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden). PBL B cells were obtained through magnetic positive selection using CD19 microbeads (MACS; Miltenyi Biotec, Auburn, CA) with a final purity of >98% $CD19^+$ as determined by FACS. Tonsillar cell suspensions were generated by mincing tissue in RPMI 1640 medium containing 10% FBS (Life Technologies), followed by one round of T cell depletion using 2-aminoethylisothiouronium bromide-SRBC (Colorado Serum, Denver, CO) and Ficoll-Paque centrifugation. The resulting cells (>97–99% $CD19^+$) were used directly for phenotypic analysis via flow cytometry.

Naive and memory B cell isolation

For naive cell purification, 10^8 tonsillar B cells were labeled with anti-CD27 PE for 30 min at 4°C. After removing unbound Abs by washing three times in staining buffer (1× PBS, 1% BSA), cells were resuspended in degassed binding buffer (1× PBS, 2 mM EDTA, 0.5% BSA), incubated for 15 min at 4°C with anti-PE microbeads, and then negatively selected using an anti-CD27 MACS column (Miltenyi Biotec). When necessary, fractions were run over a second column to achieve >98% purity. The $CD27^-$ fraction thus obtained was labeled with IgD FITC, washed, incubated with anti-FITC microbeads, and passed over a MACS column for positive selection. To obtain memory B cells, fractions were initially depleted of IgD^+ cells, followed by magnetic positive selection for $CD27^+$, as described above. The purity of the naive and memory fractions was verified by FACS.

Multiparameter FACS analysis

Single cell suspensions (10^6/sample) were labeled at 4°C for 30 min with predetermined optimal concentrations of fluorophore-conjugated mAbs, and pair-matched isotype controls, in combinations outlined in each figure legend. The following Abs were used: anti-CD19 allophycocyanin (SJ25C1), anti-CD27 PE (L128), streptavidin-PerCP, and rat IgG2a FITC (isotype control for 9G4) (BD Biosciences, San Jose, CA); biotinylated anti-IgD and anti-IgD FITC (IA6-2; BD PharMingen, Los Angeles, CA); and anti-CD45R/B220 allophycocyanin (RA3-6B2; eBioscience, San Diego, CA). V_H4.34 Abs were detected with the rat anti-idiotypic mAb 9G4. Control V_H3 Igs were detected with the avian anti-idiotypic mAb LJ26 (kindly provided by G. Silverman, University of California at San Diego, La Jolla, CA) (33). For indirect staining, cells were washed three times in staining buffer before incubation with secondary Abs. All samples were analyzed via a FACSCalibur flow cytometer using CellQuest software (BD Biosciences). In total, 50,000–100,000 events, gated for live B cells based on forward and side scattering, were collected for each sample. Statistical significance was assessed using nonparametric Mann-Whitney *U* test with the GraphPad Prism software (GraphPad, San Diego, CA).

Detection of V_H4.34 Ab binding to B cells in vitro

Tonsillar B cells were incubated in heat-inactivated sera at 4°C for 30 min. Cells were washed three times in staining buffer, labeled with appropriate fluorophore-conjugated mAbs (and isotype-matched controls), then analyzed via FACS. For blocking experiments, sera were preincubated with 50 μg of unlabeled 9G4 (or rIgG2a, isotype control) for 60 min at 4°C with constant rocking before binding reactions.

V_H4.34 Ig depletion and purification by affinity chromatography

SLE sera were fractionated by ammonium sulfate ($AmSO_4$) precipitation, dialyzed, and applied to an affinity column of either agarose-9G4 or agarose-rIgG2a isotype control (Aminolink Immobilization Kit; Pierce-Endogen, Rockford, IL). After washing extensively with PBS, bound V_H4.34 Ig was eluted in 0.1 M glycine (pH 2.7). Positive fractions, as determined by absorbance at 280 nm, were pooled, neutralized with 1 M Tris (pH 9.5), and dialyzed against 1× PBS. V_H4.34-specific binding of depleted sera and eluates was determined by incubation and FACS, as previously described.

Immunoprecipitation of CD45

Purified B cell fractions (5×10^7 cells/ml) were lysed at room temperature with occasional vortexing in mammalian protection extraction reagent buffer (Pierce-Endogen) supplemented with 150 mM NaCl, 0.1 mM PMSF, and protease inhibitors (Sigma-Aldrich). Extracts were then cleared by ultracentrifugation. Lysates (1×10^7 cells/reaction) were precleared with protein A/G-Sepharose (Pierce-Endogen), then incubated with either anti-CD45/leukocyte common Ag (LCA) (F10-89-4), anti-CD45RA (F8-11-13), anti-CD45R/B220 (RA3-6B2), or anti-CD45RO (UCHL1) (Southern Biotechnology Associates, Birmingham, AL), or isotype control Ab for 2 h with constant rotation at 4°C. Protein A/G-Sepharose beads were added, and incubations were continued for another 18 h at 4°C. Immune complexes were washed five times in lysis buffer, resolved by SDS-PAGE on 7% gels, then electroblotted to nitrocellulose membrane (1 h at 100 V). To specifically determine the binding of serum IgG to CD45, blots were probed with the appropriate dilutions of whole sera (either SLE derived or healthy control) or 9G4 affinity column-purified V_H4.34 Ab fractions, followed by goat anti-human IgG HRP (Sigma-Aldrich). Blots were developed using an ECL Plus detection kit (Amersham Pharmacia Biotech) for autoradiography with BIOMAX film (Eastman Kodak, Rochester, NY), according to the manufacturers' instructions. Alternatively, precleared lysates were incubated with either whole sera, V_H4.34-depleted sera, or 9G4 affinity column eluates for 2 h at 4°C. Protein A/G-Sepharose was added,

and the incubation continued for 18 h. Immune complexes were resolved and blotted, as described above. Blots were probed with either anti-CD45/LCA or anti-CD45R/B220, followed by anti-mouse IgG HRP or anti-rat IgG HRP (Southern Biotechnology Associates), respectively, and developed, as previously described.

Glycosidase treatment

Immunoprecipitated CD45R/B220 was eluted from protein A/G beads under denaturing conditions (0.1% SDS, 0.5% 2-ME) by heating at 100°C for 3 min, then digested with either endo-β-galactosidase (Sigma-Aldrich), *N*-glycanase, *O*-glycanase, or neuraminidase (Glyko, Novato, CA) alone, or in combination, according to the manufacturer's instructions. Deglycosylated and control samples (minus enzyme) were resolved by SDS-PAGE on 7% gels and transferred by electroblotting to nitrocellulose membrane. Immunoblotting with purified SLE V_H4.34 Abs was performed, as previously described. Blots were probed with anti-CD45/LCA mAb in parallel to verify glycosidic digestion.

Results

Identification of IgG V_H4.34 Abs in SLE sera

Serum levels of V_H4.34 Abs (whether IgM or IgG) have been consistently characterized in several reports as very low to undetectable in normal donors (23, 25–28). In contrast, elevated serum levels of IgG V_H4.34 Abs have been highly associated with global disease activity in patients with SLE and with the presence of lupus nephritis and neuropsychiatric lupus (26–28). Therefore, we first sought to identify patients with elevated serum V_H4.34 IgG Ab levels using 9G4 in a capture ELISA. Of 22 SLE patients analyzed, 16 subjects (72%) had significantly elevated Ab titers (defined as values greater than 3 SD over the mean observed in healthy sera). Consistent with previous studies, healthy controls had very low levels of serum IgG V_H4.34 Abs (Fig. 1). We then classified the SLE patients into high and low V_H4.34 IgG Ab cohorts (SLE^{high} and SLE^{low}, respectively) using an arbitrary cutoff point of 0.5 mg/ml, which represented a 4-fold increase over the normal mean. By this definition, 12 patients (55%) belonged in the SLE^{high} cohort and 10 patients in the SLE^{low} cohort (Fig. 1*A*). To assess the relative contribution of V_H4.34 Abs to the SLE IgG Ab repertoire, we also determined the ratio of V_H4.34 IgG to total IgG. As shown in Fig. 1*B*, the same 16 patients classified as having significantly elevated total levels of IgG V_H4.34 Abs were also identified as having relatively increased values of IgG V_H4.34 Abs (again defined as >3 SD over the normal mean). Of note, V_H4.34 Abs contributed a remarkably high fraction (9–45%) of total IgG in SLE^{high} patients.

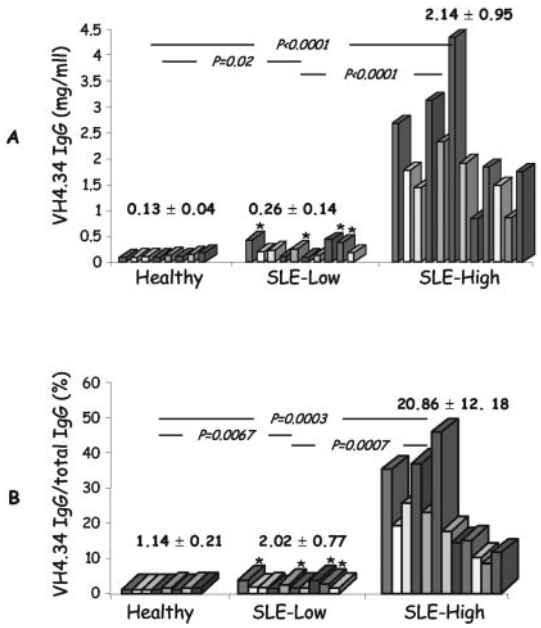

FIGURE 1. Determination of serum levels of V_H4.34 IgG Abs. Serum samples were obtained from SLE patients and normal controls and assayed by capture ELISA using the V_H4.34-specific 9G4 mAb. Results are presented as total levels of V_H4.34 IgG (*A*) or as the relative level of V_H4.34 IgG compared with total IgG (*B*). The values shown for each group represent the mean ± SD. Patients with total levels greater than 3 SD above the normal mean were classified as V_H4.34^{high}, and the remainder SLE patients were classified as SLE^{low}. Four patients in the SLE^{low} cohort had significantly increased total and relative levels of IgG V_H4.34 Abs as compared with normal values (denoted with an asterisk).

Consistent with published observations, only one-third of V_H4.34^{high} patients (5 of 12) also expressed elevated levels of serum IgM V_H4.34 Abs (data not shown) (27). Also in keeping with previous reports, this group had significantly higher anti-dsDNA titers (detected by ELISA, $p = 0.02$), and lower C3 levels ($p = 0.006$) than the V_H4.34^{low} cohort (data not shown). High serum V_H4.34 Abs also correlated with the presence of nephritis (10 of 12 and 0 of 8, respectively, $p = 0.0001$). As shown in Table I, SLE^{high} patients were also characterized by significant lymphopenia affecting the naive B cell subset.

SLE V_H4.34 Abs preferentially bind autologous naive B cells in vivo

We have previously shown that in normal subjects, V_H4.34 B cells represent up to 10% of all naive B cells, but only ~1% of memory B cells (24). Therefore, it is rather remarkable that in V_H4.34^{high} SLE patients, a large fraction of their naive B cells (mean percentage ± SD: 63.3 ± 39.8) stains positive for the V_H4.34-specific 9G4 Ab when analyzed by FACS directly ex vivo (Fig. 2*A* and Table II). In contrast, a significantly smaller fraction (9.6 ± 8.1%) of memory B cells was 9G4$^+$ in these patients. The corresponding values observed in our V_H4.34^{low} cohort were indistinguishable from healthy controls, as determined in this study and in our previous studies (Table II) (24). As opposed to V_H4.34 cells, no significant differences in the relative frequency of control B cells expressing V_H3-encoded Abs were observed between the different cohorts. To determine whether these results reflected the presence of an unlikely high number of V_H4.34 B cells in the SLE repertoire or rather diverse B cells painted by absorbed serum V_H4.34 Abs, we repeated the 9G4-staining experiments after extensive washing in PBS, followed by incubation in complement-inactivated FCS at 37°C for 60 min, a protocol previously used by others to elute cytotrophic Abs (27, 35). After elution, the number of naive B cells that stained positive with 9G4 returned to values close to those observed in healthy donors and in V_H4.34^{low} patients (Fig. 2). These results indicate that in VH4.34^{high} SLE patients, the vast majority of 9G4$^+$ naive B cells represent cells bearing exogenously bound V_H4.34 Abs.

SLE V_H4.34 Abs preferentially bind healthy naive B cells in vitro

Ex vivo studies were expanded by determining the ability of SLE-derived V_H4.34 Abs to stain normal tonsil B cells in vitro (Fig. 3). Thus, incubation with V_H4.34^{high} sera resulted in the staining of a very large percentage of naive B cells (72.0 ± 15.6%) as compared with V_H4.34^{low} sera (13.8 ± 4.9%) or normal sera (7.8 ± 1.2%). Binding was concentration dependent as serial serum dilution gradually eliminated reactivity of V_H4.34^{high} sera (data not shown). In contrast, incubation with V_H4.34^{high} sera produced only a modest staining of memory B cells as compared with V_H4.34^{low} or normal sera (10.0 ± 2.7, 2.0 ± 0.8, and 1.2 ± 0.5%, respectively). It should be noted that preincubation of target B cells

Table I. *Peripheral blood B cell subsets in SLE and healthy controls*[a]

PBL Source	Total Cell Count/μl			Relative Percentage of CD19$^+$ B Cells		
	Naive	IgD$^-$ Memory	IgD$^+$ Memory	Naive	IgD$^-$ Memory	IgD$^+$ Memory
SLE^{high} $n = 12$	12.7 ± 9.2[b]	9.1 ± 5.3	2.3 ± 2.6	43.3 ± 19.7[c]	33.9 ± 14.6[d]	8.2 ± 7.1
SLE^{low} $n = 10$	55.7 ± 28.4	11.5 ± 9.7	3.2 ± 2.2	73.8 ± 17.1	16.2 ± 11.9	6.1 ± 6.0
Normals $n = 8$	ND	ND	ND	64.8 ± 6.80	18.2 ± 20.0	13.0 ± 3.9

[a] *CD19$^+$ PBL B cells were fractionated into naive (IgD$^+$/CD27$^-$) and memory (IgD$^+$/CD27$^+$ and IgD$^-$/CD27$^+$) via multiparameter FACS. For each group, the values shown represent the mean ± SD.
[b] $p = 0.003$ between SLE^{high} and SLE^{low}.
[c] $p = 0.01$ between SLE^{high} and SLE^{low}.
[d] $p < 0.05$ between SLE ^{high} and SLE ^{low} or normals.

with unlabeled 9G4 Ab completely blocked V_H4.34 Ab binding in a dose-dependent fashion (data not shown). As opposed to V_H4.34, the relative frequency of B cells stained with anti-V_H3 Abs was unaffected by incubation with any sera analyzed (Fig. 3, *A–D, right panel*). The later result strongly suggests that V_H3 Abs expressed in SLE sera do not bind B cells and that in contrast, V_H4.34 Abs seem to contribute the majority of anti-B cell Abs in SLE sera. To confirm that V_H4.34 Abs were indeed responsible for the B cell binding observed, V_H4.34^{high} sera were preabsorbed on 9G4 affinity columns before assaying for B cell binding. In each case, the V_H4.34-depleted fraction was devoid of binding activity, while the V_H4.34-enriched fraction recovered in the eluate possessed the same binding characteristics as the original sera (Fig. 4). In contrast, fractions absorbed on either rIgG2a (9G4 isotype control) or LJ26 columns showed no loss in binding activity (data not shown).

V_H4–34 Abs bind specifically to an N*-linked carbohydrate moiety of CD45R/B220, a unique glycoform of CD45*

Previous studies have suggested that at least some V_H4.34 mAbs may cross-react with an isoform of CD45 expressed on the surface of B cells (21). Albeit this reactivity was not formally demonstrated, the expression of NAL oligosaccharides in CD45 and the frequent presence of anti-CD45 autoantibodies in SLE sera lend credence to this hypothesis (9, 20, 22). To identify the antigenic target(s) of anti-B cell V_H4.34 Abs, sera from SLE patients and healthy controls were initially examined by immunoblotting for reactivity to CD45 fractions purified from bulk tonsil B cell lysates by immunoprecipitation using LCA, a pan-CD45 mAb (Fig. 5*A*). From the control LCA lane, it is apparent that CD45 expression is quite complex, involving a large group of alternatively spliced and glycosidically modified species ranging in size from 180 to 220 kDa. Consistent with our FACS results, CD45 reactivity was strictly limited to V_H4.34^{high} sera (Fig. 5*A*). All V_H4.34^{high} sera demonstrated binding to a 220-kDa CD45 species, as determined by probing in parallel with anti-CD45/LCA (Fig. 5*A*, LCA lane). This interaction was also confirmed by LCA Western blot following precipitation of CD45 with $AmSO_4$-fractionated V_H4.34^{high} sera (Fig. 5*B*). However, consistent with previous reports, individual sera displayed a significant degree of variability with regard to reactivity toward other isoforms of CD45, in particular an isoform at ~180 kDa (10, 36). Because in the experiments depicted in Fig. 5*A* the final detection step was performed with anti-human IgG Abs, our results establish that SLE V_H4.34 IgG Abs bind B cell-derived CD45.

Strikingly, recognition of the CD45 220-kDa isoform appeared to be solely dependent on the presence of V_H4.34 Abs, as V_H4.34^{high} sera, depleted of V_H4.34 Abs by prior absorption on 9G4 affinity columns, lost this activity while retaining their ability to bind other species of CD45 (Fig. 6*A*, *lanes 5* and *6*). By contrast, serum fractions purified on either rat IgG2a as an isotype control Ab for 9G4 (Fig. 6A, *lane 3*) or LJ26 (data not shown) columns were devoid of CD45 reactivity. Purified V_H4.34 Abs were further examined by immunoblotting for reactivity with isoform-specific isolates of CD45. As shown in Fig. 6*B*, the ~220-kDa band detected by V_H4.34 Abs following precipitation by LCA was eliminated by prior extract depletion with either CD45RA or B220 (RA3-6B2) Abs (*lanes 4* and *5*, respectively), but not a CD45RO-restricted mAb that specifically recognizes the smaller 180-kDa isoform (*lane 3*). V_H4.34 reactivity was also abolished if extracts were preabsorbed with LCA before precipitation with anti-CD45R/B220, thus confirming that the 220-kDa protein recognized represents a CD45R full-length isoform bearing the B220 epitope (*lanes 6* and *7*).

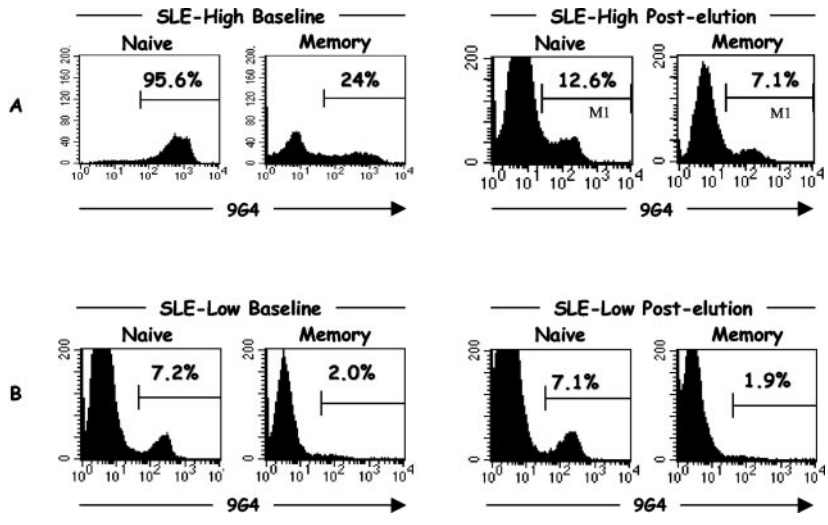

FIGURE 2. Most naive 9G4$^+$ B cells in SLE^{high} patients represent cells with bound V_H4.34 Abs. Peripheral blood CD19$^+$ B cells were purified from SLE patients and analyzed by FACS to determine the frequency of 9G4$^+$ B cells in the naive and memory subsets. The experiments were conducted directly ex vivo (baseline) or after elution of exogenously acquired Abs. Representative examples obtained with SLE^{high} (*A*) and SLE^{low} (*B*) patients are shown.

Table II. *In vivo frequency of V_H4.34 and V_H3 PBL B cells in SLE and healthy controls*[a]

PBL Source	Mean Percentage of V_H4.34 Cells in Each Subset			Mean Percentage of V_H3 Cells in Each Subset		
	Total	Naive	Memory	Total	Naive	Memory
$V_H4.34^{high}$ $n = 12$	32.2 ± 22.7[b]	63.3 ± 39.8[c]	9.6 ± 8.1[d]	28.5 ± 7.0	32.7 ± 7.0	22.8 ± 5.8
$V_H4.34^{low}$ $n = 10$	5.2 ± 1.8	6.3 ± 2.4	2.0 ± 1.3	31.4 ± 4.0	33.9 ± 3.5	24.8 ± 5.2
Healthy $n = 8$	4.4 ± 1.2	5.7 ± 1.8	1.1 ± 0.2	28.6 ± 2.2	30.2 ± 2.7	24.5 ± 3.0

[a] $CD19^+$ PBL B cells were fractionated into naive ($IgD^+/CD27^-$) and memory B cells (IgD^+ and $IgD^-/CD27^+$, and the frequency of V_H4.34 (9G4) and V_H3 (LJ26) cells was determined via multiparameter FACS analysis. For each group, values represent the mean ± SD.

[b] $p < 0.001$ between corresponding values when $V_H4.34^{high}$ and $V_H4.34^{low}$ or healthy are compared.

[c] $p < 0.0001$ between $V_H4.34^{high}$ and $V_H4.34^{low}$ or healthy.

[d] $p < 0.01$ between $V_H4.34^{high}$ and $V_H4.34^{low}$ or healthy.

We further investigated whether V_H4.34 Abs bound to different B cell fractions segregated according to their expression of B220 using a B220 glycoform-specific mAb, RA3-6B2 (37). As shown in Fig. 7, V_H4.34 Abs bind to >90% of $B220^+$ naive B cells, whereas no significant staining of $B220^-$ B cells was observed. However, V_H4.34 Abs only recognize a rather small fraction (~10%) of $B220^+$ memory B cells. Together, the above experiments strongly suggest that while V_H4.34 Abs recognize a full-length CD45R isoform that contains the B220 epitope, the determinant recognized by these Abs is distinct from B220 and is not expressed or exposed in memory B cells.

To further characterize the reactive ligand, CD45R/B220 fractions, immunoprecipitated from either naive or memory B cells, were deglycosylated, and the ability of V_H4.34 Abs to bind these modified fractions was examined via immunoblot. Although binding to naive samples was unaffected by *O*-glycanase, reactivity was completely removed following digestion with *N*-glycanase, an enzyme, which releases *N*-linked sugars (Fig. 8*A*, *lanes 5* and *3*, respectively). Treatment with endo-β-galactosidase (an enzyme that specifically cleaves the β1–4 linkage of NAL) also abolished V_H4.34 recognition (*lane 4*). This is in accordance with previous reports of V_H4.34 Abs displaying sensitivity to endo-β-galactosidase (38). Removal of *O*-linked sugars with *O*-glycanase in naive B cells did not eliminate binding of V_H4.34 Abs, but resulted in a decreased molecular mass of the Ag recognized. This finding is consistent with the fact that multiple sites of *O*-linked glycosylation are encoded by exons A, B, and C present in the larger CD45RA isoform, and indicates that the epitope recognized by V_H4.34 Abs is not created by *O*-linked glycosylation (39). Interestingly, removal of sialic acid by neuraminidase treatment restored the ability of V_H4.34 Abs to recognize the 220-kDa CD45 isoform in memory B cells, and this reactivity was not altered by

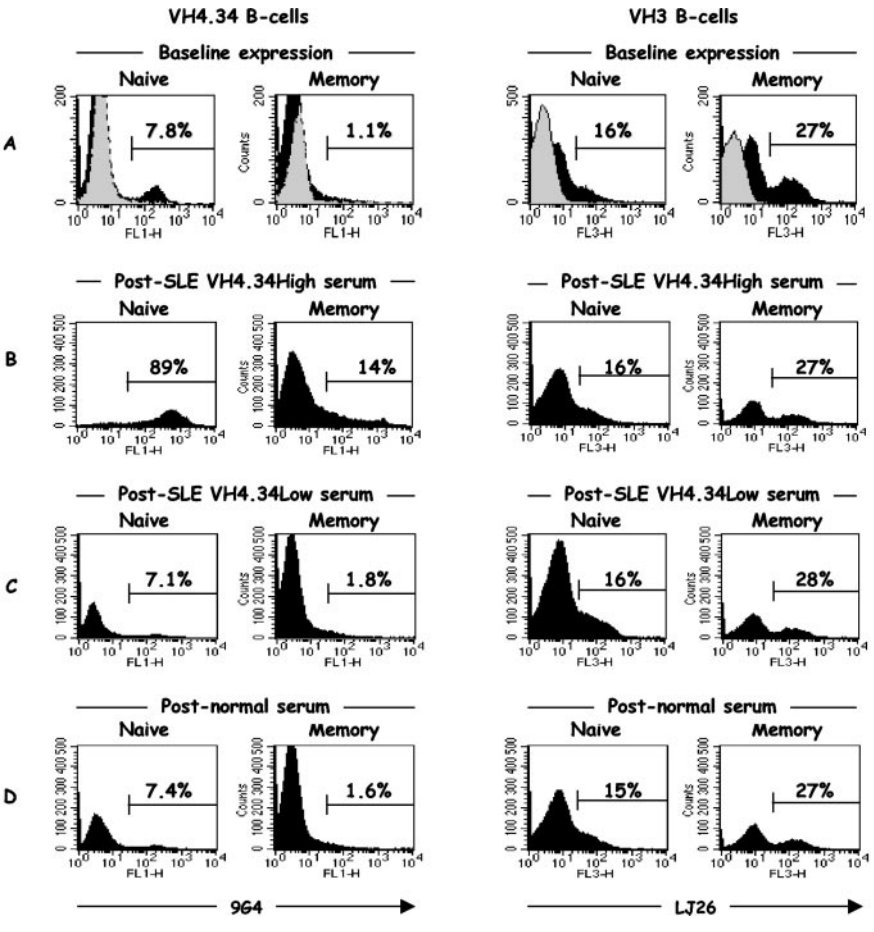

FIGURE 3. FACS analysis for the detection of either V_H4.34 (9G4) or V_H3 (LJ26) Abs on the surface of human tonsil B cells at baseline (*A*) and following incubation (4°C × 30 min) with sera derived from $V_H4.34^{high}$ (*B*), $V_H4.34^{low}$ (*C*) SLE patients, or healthy controls (*D*). Following incubation, cells were stained with either 9G4 or LJ26 mAbs in combination with CD19, CD27, and IgD. $CD19^+$ cells were fractionated into naive ($IgD^+/CD27^-$) and memory ($CD27^+$) compartments by FACS. Histograms depict the frequency of 9G4 and LJ26 cells within each subset. For each baseline plot, isotype Ab controls for 9G4 and LJ26 are superimposed in gray.

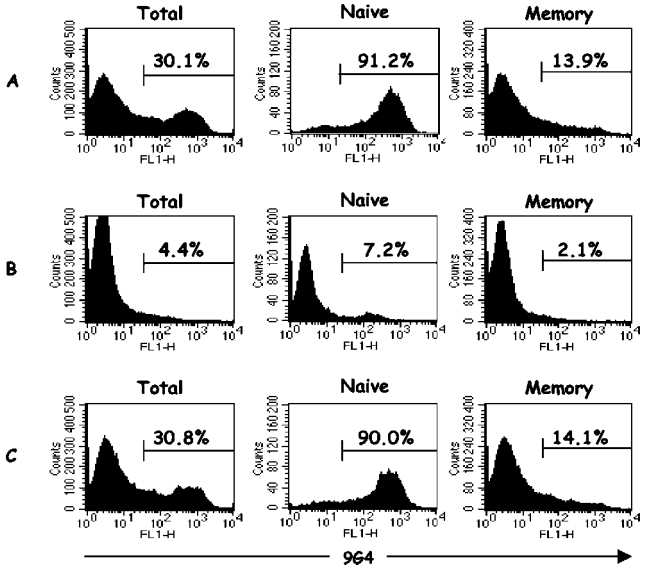

FIGURE 4. Binding of SLE $V_H4.34^{high}$ sera to tonsil B cells is $V_H4.34$ dependent. Purified tonsil B cells were incubated at 4°C for 30 min with $V_H4.34^{high}$ sera (*A*), $V_H4.34$-depleted fractions (*B*), or 9G4 affinity-purified fractions (*C*). Staining and FACS analysis for $V_H4.34$ detection were performed, as previously described in Fig. 3. In each case, histograms depict the frequency of 9G4 cells within the total population or each subset.

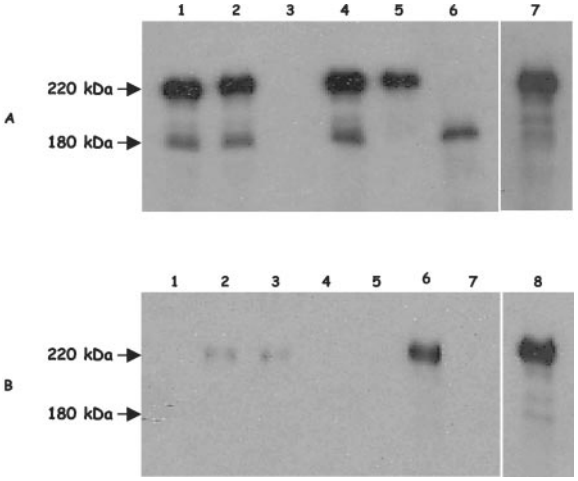

FIGURE 6. Binding of SLE Abs to CD45R/B220 is due to the presence of $V_H4.34$ Abs. *A*, Total B cell lysates were precipitated with $V_H4.34^{high}$ sera (*lane 1*), $AmSO_4$-fractionated $V_H4.34^{high}$ (*lane 2*), and the following fractions derived from $V_H4.34^{high}$ sera: rIgG2a affinity column (9G4 isotype)-purified (*lane 3*), rIgG2a column-depleted (*lane 4*), 9G4 affinity column-purified (*lane 5*), and 9G4 column-depleted $V_H4.34^{high}$ (*lane 6*), or LCA (*lane 7*). Samples were separated via SDS-PAGE and probed with LCA. For *B*, total B cell lysates were precipitated with either a mouse IgG CD45 isotype control (*lane 1*), LCA (*lane 2*), LCA following preadsorption of cell extracts with either CD45RO (*lane 3*), CD45RA (*lane 4*), or CD45R/B220 (*lane 5*), CD45R/B220 (*lane 6*), and CD45R/B220 following preadsorption with LCA (*lane 7*). Samples were separated as described and probed with the $V_H4.34$ Ab fraction recovered from $V_H4.34^{high}$ patient 2, followed by anti-human IgG Abs. *Lane 8*, Positive control for CD45. The weaker intensity of the bands in *lanes 2* and *3*, as compared with *lanes 6* and *8*, most likely reflects the relative abundance of the B220 isoform precipitated by the pan-CD45 LCA Ab and the B220-specific RA3-6B2 Ab, respectively.

subsequent treatment with *O*-glycanase (Fig. 8*C*, *lanes 1*, *2*, and *5*). As it was the case for naive fractions, $V_H4.34$ binding was completely eliminated by digestion with *N*-glycanase (Fig. 8*C*, *lane 3*).

Discussion

Anti-lymphocyte Abs are part of the autoantibody repertoire in patients with SLE. It had been previously established that at least some of these Abs are directed against *O*-linked carbohydrate determinants expressed by CD45 on T cells. It has also been shown that $V_H4.34$ IgM Abs, whether mAbs derived from individuals or from patients with Wiskott-Aldrich syndrome or polyclonal Abs

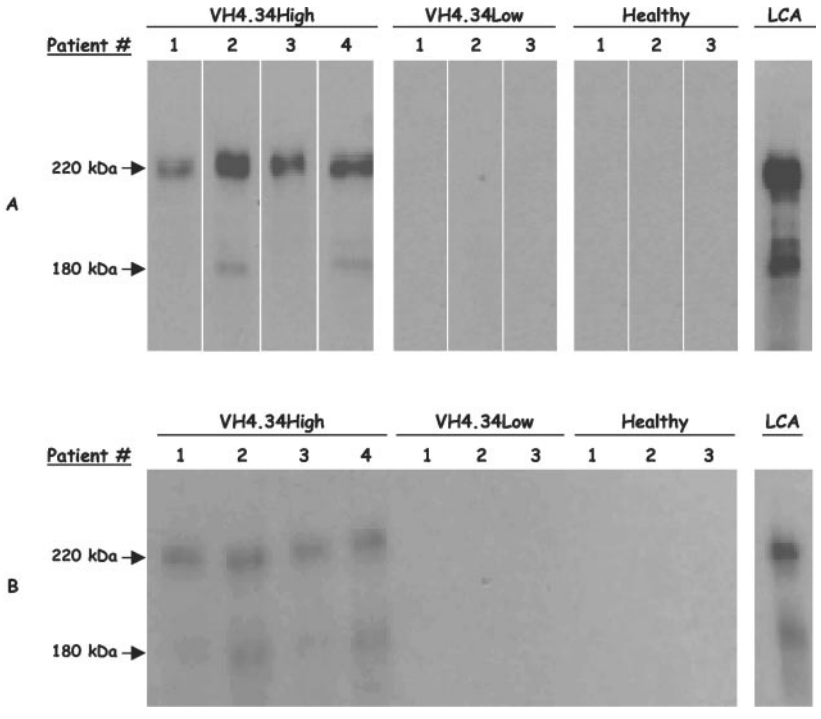

FIGURE 5. CD45 reactivity of serum IgG derived from SLE patients. *A*, CD45 was purified from total tonsil B cells by immunoprecipitation with LCA (a human pan-CD45 mAb), and blotted with SLE $V_H4.34^{high}$ (4 patients), SLE $V_H4.34^{low}$ (3 patients), or healthy control sera (3 subjects), followed by anti-human IgG-HRP. The LCA lane demonstrates probing of CD45 precipitates with LCA. For *B*, proteins precipitated with $AmSO_4$-fractionated sera were immunoblotted with LCA. Sera derived from the same patients were used for both *A* and *B*.

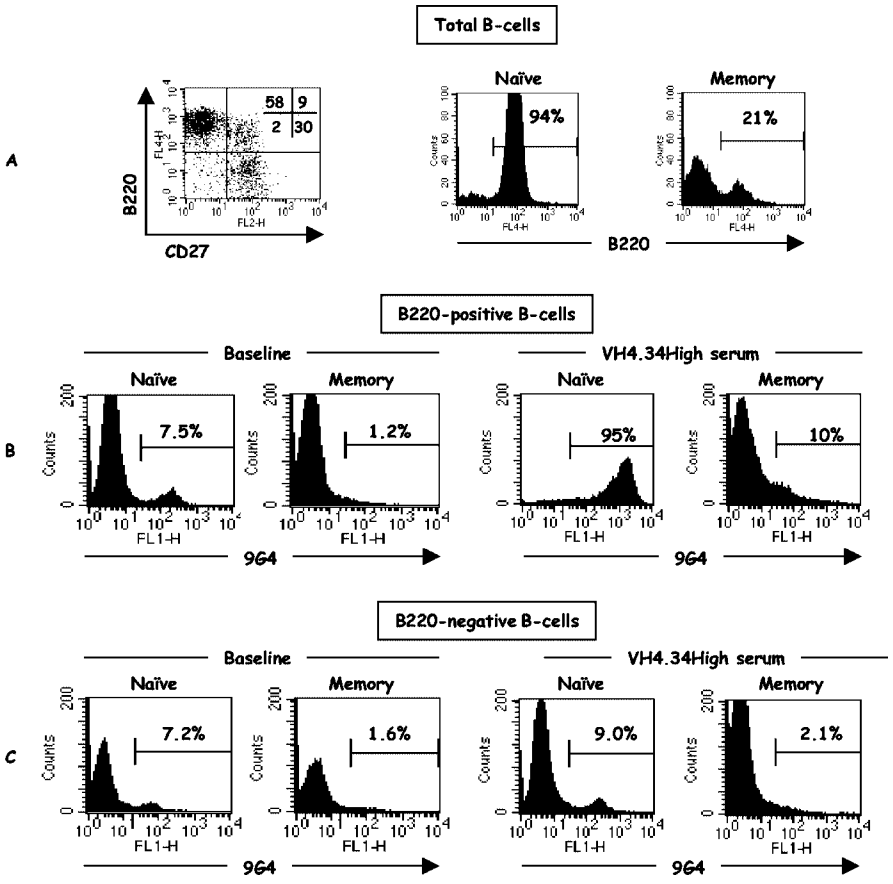

FIGURE 7. V_H4.34 Abs bind to B220$^+$ B cells. *A*, Total CD19$^+$ tonsil B cells were stained with CD45R/B220, IgD, and CD27 mAbs. The majority of B220$^+$ B cells belong to the CD27$^-$ (naive) fraction. Consistently, >90% of naive B cells (IgD$^+$, CD27$^-$) were B220 positive. In contrast, only 20–30% of memory B cells (CD27$^+$) expressed the B220 Ag. For *B* and *C*, B cells were labeled as in *A*, and then stained with 9G4 at baseline or following incubation in V_H4.34^{high} sera. Cells were fractionated on the basis of B220 expression, and then partitioned into naive and memory compartments by FACS. Histograms depict the frequency of 9G4$^+$ cells within each subset. V_H4.34 Abs stained virtually all B220$^+$ naive B cells and a small fraction of B220$^+$ memory B cells, but almost none of the B220$^-$ B cells, whether naive or memory.

obtained from SLE sera, frequently possess anti-lymphocyte reactivity (13, 14, 21, 27, 38). In SLE, in which V_H4.34 Abs are specifically and substantially expanded, these Abs are capable of binding autologous B cells in vivo (27). Our results confirm and expand such observations and demonstrate that SLE V_H4.34 Abs preferentially target naive B cells defined by a conventional CD19$^+$, CD27$^-$, IgD$^+$ surface phenotype (40, 41). This result is consistent with the observation that V_H4.34 IgM mAbs preferentially recognize IgD$^+$ follicular mantle B cells (13). In our studies, both autologous SLE peripheral blood naive B cells and heterologous healthy tonsil naive B cells were equally targeted by these Abs.

This is the first conclusive identification of the antigenic target of anti-lymphocyte V_H4.34 Abs and provides a molecular explanation for their preferential recognition of naive B cells. Previous work had suggested that these Abs might recognize a B cell-restricted isoform of CD45. Such suggestion was based on the observation that B cell binding paralleled the anti-i reactivity typical of IgM V_H4.34 Abs and could be abolished by endo-β-galactosidase, a treatment that degrades the NAL units present in CD45 (13, 14, 20, 22, 27). Yet, the final proof remained elusive as the mAbs used failed to immunoprecipitate this molecule. Using polyclonal SLE V_H4.34 Abs, however, we were able to consistently immunoprecipitate a 220-kDa CD45 species from naive B cells and demonstrate that IgG V_H4.34 Abs strongly bind this antigenic target. Enzymatic treatments designed to modify glycosylation of surface glycoproteins indicate that the differential recognition of CD45 220 kDa in naive vs memory B cells is dependent on the presence in the former cell subset of a CD45 220-kDa glycoform containing an *N*-linked carbohydrate moiety that is masked in memory B cells by the developmentally regulated addition of sialic acid residues (42–44). The chemical nature of the determinant recognized and the fact that 9G4 mAbs block this interaction confirm that the structure recognized is an *N*-linked carbohydrate epitope structurally similar to the NAL determinant of the i Ag.

CD45 is expressed as a complex set of several isoforms ranging in size from 180 to 220 kDa, which are generated by alternative splicing of exons A, B, and C. Human and murine mature T cells express different CD45 isoforms in a pattern that depends on function, differentiation state, and previous antigenic engagement. Thus, naive T cells express the higher molecular mass isoforms containing the A exon (CD45RA, 205–220 kDa), whereas activated memory T cells express the smaller 180-kDa CD45RO isoform, which contains none of the A, B, and C exons (45). CD45/B220 represents a CD45R full-length isoform containing the A, B, and C exons and is specifically defined by the RA3-6B2 Ab (43, 44). Although the majority of murine B cells express B220, this molecule had been previously thought not to be present on human B cells. This notion, however, has been corrected by a report published during the preparation of this manuscript in which the authors demonstrate that B220 is actually expressed by the majority of human naive B cells and that its expression is down-regulated on CD27$^+$ memory B cells (46). In this study, we confirm this report and show that CD45/B220 is expressed in >90% of all human naive B cells, but in only ~20% of memory B cells. Furthermore, we show that while V_H4.34 Abs bind all B220$^+$ naive B cells, they only bind ~10% of B220$^+$ memory B cells. Together, we postulate that a B220 glycoform is recognized by V_H4.34 Abs present in high abundance in SLE sera and that the corresponding epitope is sterically masked by sialylated carbohydrate chains (43, 44).

Our data also show that while SLE sera contain a diversity of anti-lymphocyte and anti-CD45 Abs, anti-CD45R/B220 Abs are essentially restricted to the V_H4.34 Ab fraction. This finding indicates that the remarkable V_H4.34 restriction of the anti-I/i

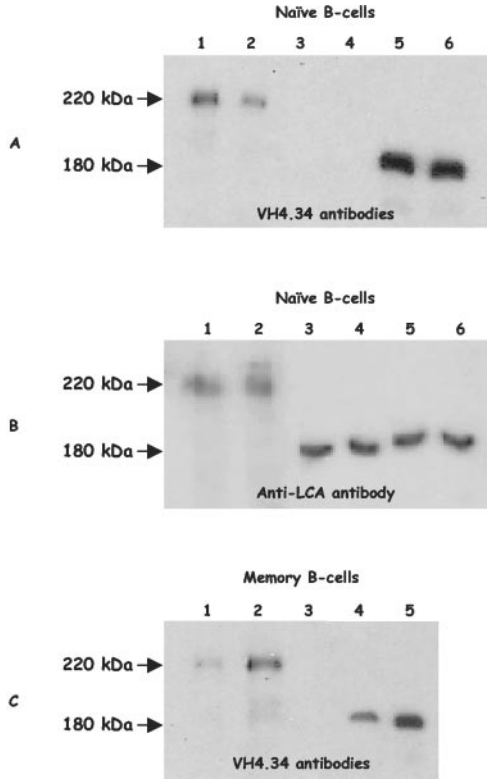

FIGURE 8. The ligand bound by V_H4.34 Abs is an *N*-linked carbohydrate moiety of CD45R/B220. CD45R/B220 was precipitated using the RA3-6B2 mAb from either naive (*A* and *B*) or memory (*C*) B cells, deglycosylated, subject to SDS-PAGE, then probed with either V_H4.34 Abs (*A* and *C*) or CD45/LCA (*B*) to verify digestion. For naive samples, the following digests were performed: no enzyme control (*lane 1*), neuraminidase (*lane 2*), *N*-glycanase (*lane 3*), endo-β-galactosidase (*lane 4*), *O*-glycanase (*lane 5*), neuraminidase, then *O*-glycanase (*lane 6*). For memory fractions: no enzyme control (*lane 1*), neuraminidase (*lane 2*), *N*-glycanase (*lane 3*), *O*-glycanase (*lane 4*), neuraminidase, then *O*-glycanase (*lane 5*).

response is maintained in SLE and suggests that this Ag or similar Ags may play a significant role in the activation and/or selection of a large fraction of the autoimmune IgG Ab repertoire in SLE (18, 19). The pathophysiological significance and the pathogenic implications of this observation are underscored by the magnitude of V_H4.34 serum Ab levels illustrated in this study. Indeed, our data provide a first quantitative appraisal of the magnitude of the V_H4.34 IgG Ab response in SLE. As shown in Fig. 1, V_H4.34 Abs contributed up to 45% of total serum IgG (mean, 21%; range, 9–45%) in SLE^{high} patients (representing >50% of all SLE patients analyzed and a large majority of active patients). Along these lines, it is noteworthy that the I/i Ag may be expressed in oxidized apoptotic cells and that B220 is expressed by preapoptotic T cells (43, 47, 48). These findings may explain our observation that V_H4.34 Abs (both monoclonal and polyclonal) bind apoptotic cells (49). Given the proposed role of autoantigen-bearing apoptotic cells in the pathogenesis of SLE, it is tempting to speculate that apoptotic bodies could contribute to the expansion of V_H4.34 B cells in this disease (50, 51).

At least a subset of V_H4.34 Abs may also bind DNA, and serum V_H4.34 Abs have been shown to make up a substantial fraction of anti-dsDNA Abs in patients with SLE (52). Therefore, it is apparent that V_H4.34 Abs could play a role in the disease process through their participation in this pathogenic Ab response (53). However, it is also plausible that V_H4.34 Abs could exert a pathogenic role through anti-CD45 effects either by enhancing or damping CD45 activity. Indeed, CD45 is a transmembrane phosphotyrosine phosphatase (PTPase) with the ability to modulate Ag receptor-mediated B and T cell responses both positively and negatively through its conventional PTPase activity and a recently described Janus kinase phosphatase activity (54). It has been proposed that the activity of CD45 may be dependent on the balance between monomeric and dimeric forms because dimerization results in inhibition of the PTPase activity of the CD45 cytoplasmic domain and negative regulation of Ag receptor signaling. In turn, the interaction between the extracellular domains of the different CD45 isoforms may determine the extent of homodimerization with the larger isoforms such as B220 being less prone to dimerize (55). It is therefore conceivable that anti-CD45 Abs could interfere with the dimerization process and consequently enhance CD45 function. As demonstrated by the wedge mutation model, unabated CD45 activity may result in polyclonal T and B cell activation and severe autoimmune nephritis with autoantibody production (56, 57). Should V_H4.34 anti-CD45 Abs indeed result in increased CD45 activity, this effect could also help explain the expansion of TCR $\alpha\beta^+$, $CD4/CD8^{double\text{-}negative}$ T cells observed in SLE because these cells have been shown to express the B220 Ag (43, 58). Conversely, V_H4.34 Abs could facilitate cross-linking of the larger CD45 isoforms, thereby enhancing dimerization and silencing CD45 signaling. This effect could also have pathogenic consequences, as demonstrated in murine models of B cell tolerance in which the absence of CD45 activity promotes positive selection and expansion of autoreactive B cells (59).

The reactivity of V_H4.34 Abs and the correlation with lower naive B cell levels described in this work (Table II) suggest that these Abs could also contribute to the naive B cell lymphopenia observed in patients with active SLE (34). Naive lymphopenia could be induced by V_H4.34 Abs whether through their reported lymphocytotoxic activity or by alternative mechanisms (38, 60). Thus, while costimulatory signaling through the Ag receptor and CD45, in particular B220, is essential both for B cell activation and proliferation, ligation of CD45 alone promotes apoptosis of both T and B lymphocytes (61–64). Therefore, V_H4.34 Abs could induce apoptosis of virgin naive B cells upon ligation of CD45 alone while promoting activation and expansion of autoreactive naive B cells actively costimulated by self Ag through the B cell receptor. In turn, lymphocyte apoptosis induced by V_H4.34 Abs would contribute to the availability of exposed intracellular autoantigens that could in turn amplify the autoimmune response possibly through the induction of IFN-α production by PBMC. This mechanism would be consistent with the recently reported ability of lupus IgG-apoptotic cell complexes to activate IFN-α-producing cells, a phenomenon that could bear significant pathogenic potential in SLE, and the ability of IFN-α to induce B cell lymphopenia (51, 65–67). It is also plausible that anti-CD45 V_H4.34 Abs could contribute to naive lymphopenia by inducing naive B cell differentiation and isotype switch, as previously postulated by others on the basis of in vitro experiments (21, 68). This mechanism could also help explain the expansion of peripheral blood plasmablasts observed in patients with active SLE (34, 69).

Additional studies, currently underway in our laboratory, will be required to dissect the mechanisms and the actual consequences of the overexpression of V_H4.34 Abs in SLE.

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