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Characterization of Superantigen-Induced Clonal Deletion with a Novel Clan III-Restricted Avian Monoclonal Antibody: Exploiting Evolutionary Distance to Create Antibodies Specific for a Conserved V_H Region Surface

Stephen P. Cary, James Lee, Raymond Wagenknecht, and Gregg J. Silverman

Evolution of the Ab system has yielded three clans of V_H region genes that are represented in almost every known higher species with an adaptive immune system. These clans are defined by sequence homologies primarily in highly conserved framework (FR) subdomains, which serve a scaffolding function maintaining the conformation of loops responsible for Ag binding. Structural analyses indicate that the V_H FR1 and FR3 form a conserved composite exposed surface, which has been implicated in interactions with B cell superantigen. To directly investigate the expression of clan-defined supraclonal sets, we exploited the evolutionary distance of the chicken immune system and the selection power of phage display, to derive Abs diagnostic for clan III Ig. Using a specially tailored immunization and selection strategy, we created recombinant avian single chain Fv Abs specific for the clan III products, including those from the human V_H3 family, and the analogous murine 7183, S107, J606, X24, and DNA4 families, and binding was competitive with natural B cell superantigen. The archetype, LJ-26, was demonstrated to recognize a clan-specific surface expressed in diverse mammalian, and also the Xenopus and chicken, immune systems. In flow-cytometric studies with LJ-26, we found that treatment of heterozygous T15i transgenic mice with a model B cell superantigen induced a clan III-restricted clonal deletion. These studies demonstrate the utility of a novel recombinant serologic reagent to study the composition of the B cell compartment and also the consequences of B cell superantigen exposure. The Journal of Immunology, 2000, 164: 4730–4741.

Understanding the evolutionary origins of lymphocyte Ag receptors, and discerning the molecular and natural selective pressures for diversification or conservation of the genes that encode them, are questions fundamental to the study of the adaptive immune system. Among the foundations of these studies are compilations of the sequences of Ig V_H regions (1) that have led to an appreciation of their genetic organization. Based on their sharing of greater than 80% DNA sequence homology, the inherited gene segments encoding these V_H regions have been organized into multigene families (2). Moreover, sequence analyses of human and murine Ab genes revealed that sets of V_H gene families share highly conserved features, enabling their clustering into three major subgroups or clans (1, 3). From subsequent investigations of the Ab systems in diverse species, the progenitors of these V_H clans have been postulated to have diverged even before the emergence of the mammalian radiation, as genes representative of the clans first appeared more than 300 million yr ago (4–6).

From efforts to dissect the functional capacities of these Ag receptors, V region sequences were shown to contain three non-contiguous linear intervals of greatest variability, which have been termed hypervariable regions or complementarity-determining regions (CDR) (7). Separating these CDR are intervals termed framework regions (FR) that are highly conserved among the members of a family. In crystallographic analyses that have elucidated the β barrel structure of Abs, the CDR were found to represent loops that are juxtaposed to form the classic Ag binding site (reviewed in Refs. 8 and 9). By contrast, the FR subdomains fold into relatively rigid β strands that maintain the overall Ig structure. However, these FR1 and FR3 subdomains were found to contain sequences that are distinct for each of the three clans. In fact, the nucleotide sequences of these FR subdomains are among the most highly conserved in mammals, with the FR1 and FR3 of clan III members displaying the greatest conservation (3, 10–12).

Competing theories have been presented to explain the maintenance of the clan-specific gene sequences across species and evolutionary boundaries. Tutter and Riblet (10, 11) compared inherited gene sequences and found that the frequencies of both silent and replacement mutations were significantly lower than expected, which was interpreted as indicative of selective conservation of clan III sequences at the nucleotide and not at the amino acid level. Presenting an alternative interpretation based on their own sequence analyses and molecular modeling of V regions, Kirkham

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4 Abbreviations used in this paper: CDR, complementarity-determining region; CLL, chronic lymphocytic leukemia; FR, framework region; HEL, hen egg lysozyme; MSpA, chemically modified SpA that retains Fab-binding activity; pFv, protein Fv; scFv, single chain Fv; SpA, staphylococcal protein A.

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and Schroeder (12) proposed that the FR subdomains did not solely provide structural and supportive scaffolding functions for the juxtaposed CDR sites. Instead, they demonstrated that the V_{H} region FR1 and FR3 subdomains together form a separate conformational surface that is conserved and characteristic for each of the clans, which they postulated might represent an alternative ligand contact site (3, 12).

When originally presented, there was little more than circumstantial evidence to support this alternative binding site hypothesis. However, in recent years, certain microbial (13–15) and endogenous proteins (16–18) have been reported to have special properties, enabling direct interactions with these postulated Ig framework-associated alternative ligand-binding surfaces. Due to the obvious parallels with the activities of known T cell superantigens, many of these proteins have been postulated to represent B cell superantigens. We and others have considered the potential importance of the in vivo activities of a B cell superantigen (19), and speculated that interactions mediated through this Ig FR site might induce large scale alterations in the composition of the B cell or Ab repertoire. While this type of influence might affect all members of a species, possibly at an especially susceptible developmental stage, it is also possible that natural exposure may only occur occasionally in an individual host. However, despite their potential importance, except for our recent report (20), studies of these putative B cell superantigens have been limited to in vitro investigations.

The development of effective methods for monitoring of the consequences of in vivo B cell superantigen exposure poses special conceptual and technical challenges. One of the greatest hurdles is the lack of appropriate V_{H} family-specific serologic markers. We suspect that the unavailability of this type of novel V_{H}-targeted reagent is due to the conservation and ubiquitous expression of the V_{H} region FR1 and FR3 subdomains (reviewed in Refs. 25 and 26). Therefore, to create these reagents, we first raised an Ab response to human monoclonal Ig in this avian host, and to isolate the mAbs of the required fine specificity, we harnessed the power of phage-display expression systems for in vitro clonal selection. By this approach, we were successful in isolating novel V_{H} region-specific single chain V region (scFv) Abs specific for a conformational determinant that is completely restricted to the products of clan III genes. Moreover, in applications using diverse immunochemical formats, studies using these recombinant Abs demonstrated that clan III products are represented in the Ig repertoires of many terrestrial species. Further illustrating the utility of this novel highly specific clan III marker, we performed surveys of murine in vivo immune responses to a prototypic microbial B cell superantigen, which revealed the induction of a V_{H}-restricted supraclonal defect. These studies provide a foundation for investigations into how a natural Ig-binding protein can mold the clonal composition within the B cell compartment.

### Materials and Methods

#### Immunization and immunoassays of polyclonal IgY

A Leghorn chicken was immunized and boosted s.c. in the wing with human clan III Ig. For the immunization regimen, chickens received the native human IgM (V3–23/clan III V_{k}) 18/2 (27) and the human rlgM (V3–23/clan III V_{k}2) Fab, 3-15 (28) (50 μg each), in PBS, pH 7.4 (PBS), emulsified in CFA (Difco Laboratories, Detroit, MI), with a boost after 2 wk of 3-15 (27) and HEA (V3–30/clan III V_{k}3) (50 μg each) in IFA. A second boost of 3-15 and 18/2 (50 μg each) in IFA was delivered 2 wk later. After 4 wk, induced Ab responses were demonstrated by comparisons of anti-Ig-binding activity of pre- and postimmunization IgY from egg yolk (data not shown), which was purified using the Eggstraction Kit (Promega, Madison, WI). Briefly, wells were coated with 3-15 at 5 μg/ml in PBS for 1 h at 37°C, and then blocked in 2% BSA in PBS for 1 h at 37°C. Equivalent concentrations of pre- and postimmunization IgY were added in serial dilutions in 1% BSA/PBS and incubated for 1 h at 37°C. To detect binding, peroxidase-labeled goat anti-IgY (Promega) was added in 1% BSA/PBS for 1 h at 37°C. Plates were washed as above, and tetramethylbenzidine substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added to wells. Plates were read at OD_{450–650} in a microplate reader (Bio-Rad, Hercules, CA).

#### Creation of a chicken scFv Ab phage-display library

Adapting a standard protocol (29), avian bone marrow and spleen were separately harvested into serum-free RPMI and dissociated into single cell mononuclear cell suspensions and then lysed in Tryzol (Life Technologies, Gaithersburg, MD). RNA was isolated by phenol/chloroform isoamyl alcohol (24:25:1) extraction and precipitated in isopropanol at −20°C for 30 min. After centrifugation at 14,000 rpm in a microfuge, the RNA pellet was

<table>
<thead>
<tr>
<th>Round</th>
<th>Target</th>
<th>Inhibitors</th>
<th>No. of Washes*</th>
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<tr>
<td>1</td>
<td>18/2</td>
<td>None</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>18/2 (10 μg/ml)</td>
<td>ODO (V_{H}4/clan II IgM, V_{k}3) (10 μg/ml)</td>
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</tr>
<tr>
<td>3</td>
<td>J606 (mu J606 IgG3) (10 μg/ml)</td>
<td>ODO (50 μg/ml)</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>J606 (ms J606, V_{k}) (10 μg/ml)</td>
<td>ODO (50 μg/ml)</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>J606 (10 μg/ml)</td>
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<td>15</td>
</tr>
<tr>
<td>6</td>
<td>Murine splenocytes</td>
<td>None</td>
<td>10</td>
</tr>
</tbody>
</table>

*With each round of selection, after incubation in the wells coating with selecting Ig, plates were washed with PBS-0.05% Tween 20. To increase the stringency of selection, in certain rounds the number of separate washing steps were increased, as indicated.
DETECTION OF AN INDUCED SUPRACLONAL CLAN III DELETION

from a 2% agarose gel. Overlap product inserts and the pComb3H vector of these PCR reactions were combined, and the 2 min, followed by a final extension step at 72°C for 7 min. The products of each reaction tube, with 30 cycles of 96°C for 30 s, 56°C for 15 s, 72°C for 30 s, and 72°C for 7 min. VH products (4732 bp) and V L products (750 bp) were purified separately with a QiAquick kit (Qiagen, Chatsworth, CA). In 20 tubes for overlap PCR reactions, 100 ng of each of VH, and V L PCR products were included in 2.5 μl of dNTP, and 1 μl each of nested sense primer (CSC-Vk-F, GAGGAGGAGGAGGAGGAGTGGCCTGGCCTGGGTTCCGCCGTGACGTTGGACGAG, and CSCG-B, GAGGAGGAGGAGGAGGAGGAGAAGTTGGAGGTGAGCTCCGGCTGCCGGCTGCCTGGCCTGACGGAG). Overlap PCR reactions for H chain and L chain genes used 1 μl of each primer (CSC-Vk-F, GAGGAGGAGGAGGAGGAGTGGCCTGGCCTGGGTTCCGCCGTGACGTTGGACGAG, and CSCG-B, GAGGAGGAGGAGGAGGAGGAGAAGTTGGAGGTGAGCTCCGGCTGCCGGCTGCCTGGCCTGACGGAG). Overlap product inserts and the pComb3H vector were combined, and the 2 min, followed by a final extension step at 72°C for 7 min. The products of each reaction tube, with 30 cycles of 96°C for 30 s, 56°C for 15 s, 72°C for 30 s, and 72°C for 7 min. The products of these PCR reactions were combined, and the 750-bp band was purified from 2% agarose gel. Appropriate DNA bands were excised and electroeluted (Qiagen), and ligated into a similarly prepared pARA plasmid (gift of C. F. Barbas), a compatible bacterial expression vector employing the arabinose promoter that also fuses peptide tags to the scFv product. After transformation into XL-1 Blue cells, and selection on LB plates with 25 μg/ml of chloramphenicol, individual colonies were picked, and expanded in LB media with 25 μg/ml of chloramphenicol. At an OD600 of 0.5, cultures were induced with 0.5% t-arabinose and grown overnight at 30°C. Protein was purified from cell pellets under native conditions over equilibrated Ni-NTA spin columns (Qiagen), and purified protein was eluted and immediately dialyzed in PBS, pH 8. In preparation for biotinylation, Abs were dialyzed against 0.25 M borate-buffered saline, pH 8.8, then reacted with long linker N-hydroxy-succinimide biotin (Sigma, St. Louis, MO) for 4 h at room temperature. The reaction was stopped with 1 M NH4Cl, and unreacted biotin was removed by dialysis against PBS for several days with four buffer changes. To evaluate individual scFv Abs for binding reactivity with a diverse panel of human and mouse monoclonal Ig, wells were coated and blocked as previously described, and equivalent amounts of scFv protein were loaded in serial dilutions. Binding was detected with a biotinylated anti-hemagglutinin reagent (gift of C. F. Barbas) that recognizes a pARA-encoded C-terminal epitope tag, with detection using HRP-streptavidin. Alternatively, in assays using directly biotinylated scFv Abs, binding was directly detected with HRP-streptavidin. To simplify comparisons with the Fab-binding activity of SpA, we used a chemically modified version (MSPA) that retains Fab specificity, but does not bind Fc (31), which was biotinylated for detection of binding. For numeric comparisons, for each murine Ig the concentration values were determined from binding curves that provided an OD405–600 of 0.5 (i.e., stronger binding is associated with lower values).

For competition studies, a representative labeled scFv, LJ-26, or SpA was preincubated with decreasing amounts of unlabeled LJ-26, SpA (Repligen, Cambridge, MA), or a human Fv-binding protein, termed pFv (18) (gift of J.-P. Bouvet, Hopital Broussais, Paris, France). The concentration of pFv was estimated based on silver stain of polycrylamide gel studies and Western immunoblot analysis (not shown). Inhibition values were determined by interpolation against a standard curve of the labeled scFv without inhibitor.

The reactivities of select scFv clones were further characterized in immunoblot assays by purifying Ig from diverse species (Jackson ImmunoResearch, West Grove, PA) were tested on SDS-PAGE, under reducing and nonreducing conditions, in 4–12% Tris-glycine gels (Novex, La Jolla, CA), and electrotransferred to Immobilon P membranes (Millipore). To assess binding, membranes were blocked in PBS/1% casein and blotted with scFv-biotin or a MSPA-biotin at 2 μg/ml, then washed in 0.05% Tween-20/borate-buffered saline. Reactivity was detected with HRP-streptavidin and chemiluminescent substrate (Amersham, Buckinghamshire, U.K.).

Microfluorometric studies

Samples of human PBMC containing greater than 95% monoclonal B cells from patients with chronic lymphocytic leukemia (CLL) (provided by T. J. Kipps, University of California, San Diego), and normal adult PBMC were stained with anti-CD5 PE (clone UCHT2), anti-CD19 APC (clone HIB19), anti-κ FITC (G20-193), anti-λ FITC (JDC-12), and Li-26 biotin or biotin chicken scFv isotype control and streptavidin-peridinin chlorophyll protein. In studies of BALB/c, C57BL/6 or the AB29 (32), or T1si (33, 34) Ig transgenic mice, certain groups received neonatal treatment with endotoxin-free SpA, or hen egg lysozyme or saline, following a previously described treatment regimen (20). After the last treatment, bone marrow and splenic mononuclear cells were isolated as previously described (20) and evaluated or placed in overnight culture before analysis. Cells were cultured for 4 h at 37°C, and stained using a FITC-conjugated anti-IgM (R3-255), or anti-h IgM (4D6-202), or anti-B220 (RA3-6B2), anti-CD5 PE (clone UCHT2), anti-CD19 APC (clone HIB19), anti-κ FITC (G20-193), anti-λ FITC (JDC-12), and Li-26 biotin or biotin chicken scFv isotype control and streptavidin-peridinin chlorophyll protein. Unless indicated, Abs were obtained from PharMingen (La Jolla, CA). In certain studies, cells were also stained with a PE-labeled MSPA (20, 31). Data were acquired on a four-color FACSCalibur analytic flow cytometer (Becton Dickinson, Mountain View, CA) and analyzed with FlowJo (Tree Star, San Carlos, CA) or CellQuest software (Becton Dickinson).

Results

Selection of avian scFv Abs that bind clan III Ig

The immunization regimen was designed to enhance the response to determinants to human Vκ3 Abs, and the panning strat-
egy was designed to positively select for clones with a cross-species clan III specificity, while clones specific for L chain and constant region determinants should be negatively selected (Table I). Hence, the avian scFv phage-display library was subjected to sequential rounds of selection against human and murine clan III Ig coated onto wells, and during the later rounds clan II Ig inhibitors were also mixed in solution with the phage libraries. For the final round, selection was against murine splenic mononuclear cells. The resulting libraries were subsequently evaluated for Ig-binding activity, which demonstrated that Ig-binding activity rose significantly after the fourth round of panning, and continued to increase for the fifth and sixth rounds (Fig. 1). To assess whether these selected libraries contained an identifiable set of clones with a fine specificity for a clan III-restricted Fab surface, separate aliquots of phage from fifth and sixth rounds were mixed with a soluble competitor, an 11-kDa recombinant form of domain D of SpA (Fig. 1). Studies with domain D were performed because this small monomeric protein expresses the parental clan III Fab-binding specificity (28), which targets a common and conserved conformational site formed by clan III-restricted residues primarily in the β strands of the FR1 and FR3 subdomains (35). Domain D was responsible for significant inhibition of the binding to the clan III Ig of the fifth round scFv-phage library \( p = 0.03 \), one-tailed Student t test, and inhibition was even greater for the sixth round scFv-phage library \( p < 0.0001 \) (Fig. 1B). These data suggest that our panning strategy selected for a subpopulation of scFv-display phage with a fine specificity for a Fab site restricted to clan III Ig.

To characterize the reactivities of individual Ab clones, the sixth round library was transferred into a soluble scFv expression system, and the binding of purified scFv clones was tested by ELISA. Initial screening of 60 clones revealed a remarkable focusing of binding specificities, with representative results for 19 clones illustrated in Fig. 2A. Although the chicken was immunized with human IgM proteins, in these studies each of these soluble scFv also reacted with a murine 7183/clan III IgG1 and a murine J606/clan III IgG3, while these scFvs were uniformly nonreactive with a clan II IgM. These data are consistent with specific interactions with cross-species-conserved VH region site(s).

A representative scFv Ab clone, LJ-26, was chosen for further characterization in assays with a diverse panel of human and murine Ig, in which LJ-26 bound to human Ig (3-15, 18/2, GLO, and MYE) from the VH 3 gene family and to murine Ig from the S107 (EO6) and J606 V_H families, all part of clan III. In contrast, LJ-26 did not recognize Ig from other V_H clans, and binding also did not correlate with H chain isotype or V_L usage (not shown) (Fig. 2B). Significantly, despite the presence of variations in the sequences of these clan
III Abs, and the coassociation of diverse isotypes and L chains (Table II), reactivity with LJ-26 was remarkably homogeneous.

Comparisons of the Fab-binding activities of LJ-26 and SpA

The relative reactivities of SpA and LJ-26 were compared in direct Fab-binding assays with murine IgM and IgG from clans I, II, and III, with results compiled in Table II. Herein, the deduced protein sequences of the VH regions of these monoclonal Ig are grouped by family and clan, and aligned to the human germline V3–23 gene segment, which is believed to commonly encode for Abs in the human repertoire with the strongest Fab-mediated binding activity for SpA (14, 28). Similar to results from studies of SpA with monoclonal human Ig (13, 14, 28, 36) and murine Ig (37, 38), LJ-26 also did not exhibit detectable binding reactivity with any of the monoclonal murine Ig from clan I or II, which are significantly divergent from the V3–23 paragon, especially in key diagnostic

FIGURE 2. Characterization of the Ig-binding specificities of representative soluble recombinant mAbs from the sixth round of selection. In A, within a set of soluble Ab clones, each individual clone is shown to recognize one or both of the murine clan III IgG, but did not bind a human IgM V_{H}4/clan II. In B, the binding reactivities of a representative clone (LJ-26) were tested against a broad panel of human and mouse monoclonal Ig. This Ab displayed strong and near equivalent reactivity with diverse human and murine clan III Ig that was independent of V_{L} region or C region usage. In contrast, there was no reactivity with Ig from the other clans. For these studies, a LJ-26 was directly biotinylated and loaded at a fixed dilution, and binding was detected by ELISA with labeled streptavidin.
### Table II. \( V_H \) regions and binding activity

<table>
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<tr>
<th>( V_H )</th>
<th>Framework 1</th>
<th>CDRI</th>
<th>Framework 2</th>
<th>CDRI2</th>
<th>Framework 3</th>
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<td><strong>Human VH3</strong></td>
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<tr>
<td>1-15</td>
<td>m</td>
<td></td>
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<tr>
<td>18/2</td>
<td>m</td>
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</tr>
<tr>
<td>VH4/7183</td>
<td>g2a</td>
<td>NT</td>
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<tr>
<td>423s,109 m</td>
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<td>2</td>
<td>5</td>
<td>T,Y,E,R</td>
<td>M,R,G</td>
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<tr>
<td>452p,93 m</td>
<td>0.01</td>
<td>4</td>
<td>K</td>
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<td>-</td>
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\( \text{mg/ml} \) represents concentrations required to attain an OD \( 450-650 \) of 0.5, as determined in an ELISA assay. Values \( \leq 100 \text{mg/ml} \) are indicated by - .

a) \( V\beta \) reactivity (shown as \( \mu \text{g/ml} \)) represents concentrations required to attain an OD \( 450-650 \) of 0.5, as determined in an ELISA assay. Values \( \leq 100 \text{mg/ml} \) are indicated by - .

b) \( V\beta \) reactivity (shown as \( \mu \text{g/ml} \)) represents concentrations required to attain an OD \( 450-650 \) of 0.5, as determined in an ELISA assay. Values \( \leq 100 \text{mg/ml} \) are indicated by - .

c) \( V_H \) region sequences are organized by families and clans, with alignment to the human germline gene, \( V3-23 \), which encodes for the best \( SpA \) binders in the human repertoire. Bold residues in \( V3-23 \) are critical to \( SpA \) binding. CDR3 and \( V_L \) region, which have little or no influence on binding activity, are not shown. Relative reactivity scale based on ELISA results. Identical amino acids are indicated (.). X, uncertain deduced amino acid.

d) EO6 is encoded by the germline S107.1 gene (G. Silverman, P. Shaw, and J. Witztum, unpublished observation). NT, not tested. Abs representative from the less commonly expressed murine clan IV/H8-3609, clan II/H9/Gam3.8, and clan III/VH13-3609N, and small human clan IV/H7, clan II/H2, and VH6 families were unavailable for testing.

positions in FR1 and FR3 (3). Most of the clan III IgM specifically interacted with both \( SpA \) and LJ-26, and strong reactivity was exhibited by the human \( VH3 \) and murine S107 and J606 family-encoded Ig. However, for certain mAbs (i.e., 7183-encoded Ig; 452s,11, 363p,16, and 452p,18), the binding activities of LJ-26 and \( SpA \) diverged, as they weakly bound \( SpA \) and were nonreactive with LJ-26. The greatest discordance was for several clan III IgG, including the 7183-encoded 452p,2 and MOPC21, which were all strongly reactive with LJ-26, while they bound \( SpA \) weakly or not at all. Furthermore, the lack of reactivity of MOPC21 with \( SpA \) has been correlated with a replacement mutation at position 57 and a natural variation of a serine at position 82a (38), which are reported to be critical residues for \( SpA \) binding (39 – 41). Hence, the strong reactivity of this avian Ab with MOPC21 may indicate that, while most of the contact surface is similar for \( SpA \) and LJ-26, these particular \( V_H \) positions that are near the limits of the \( SpA \)
In general, while reactivity with SpA varied greatly between different clan III Ig, there was much less of a range of reactivities with LJ-26, which may suggest that the LJ-26 Ab recognizes a much less complex V_{H} region surface.

**LJ-26 competes with a bacterial B cell superantigen and an endogenous human Fab-binding protein for binding to clan III Ig**

Competition studies were performed to directly compare the Fab-binding activity of LJ-26 with the natural clan III-restricted Ig-binding proteins, SpA, and the human gut-associated sialoprotein, pFv (18, 42). In the first type of competition immunoassay, a fixed concentration of labeled LJ-26 was mixed with different concentrations of LJ-26, or an avian scFv isotype control, or pFv, or SpA, and later serial dilutions of LJ-26, or SpA, or pFv or a control scFv were mixed with a fixed concentration of labeled LJ-26 Ab. Percent-inhibition values were derived by interpolation of the OD readings into a standard curve of labeled SpA or LJ-26 without inhibitors, as appropriate. Only the control scFv failed to compete for binding.

**FIGURE 3**. The avian LJ-26 Ab competes with SpA and the human gut-associated Fab-binding protein, protein Fv (pFv), for binding to a clan III Ig. For these studies, plates were coated with 3-15, a human V_{H}3/clan III IgM Fab, and later serial dilutions of LJ-26, or SpA, or pFv or a control scFv were mixed with a fixed concentration of labeled LJ-26 Ab. Percent-inhibition values were derived by interpolation of the OD readings into a standard curve of labeled SpA or LJ-26 without inhibitors, as appropriate. Only the control scFv failed to compete for binding.

**FIGURE 4**. Western blot analysis of reactivity with human and murine Ig. Western analysis of a panel of purified Ig, in the following lanes: 1, polyclonal human adult IgG; 2, polyclonal human adult IgA; 3, polyclonal adult human IgM; 4, a human monoclonal V_{H}3/clan III V_{H}1, 18/2; and 5, a murine monoclonal IgM J558/clan I, M104E. In the top panels, proteins were separated under nonreducing conditions, while in the bottom panels, proteins were separated under reducing conditions. Replicates were developed with A, protein stain, while blots were reacted with B, the LJ-26 Ab, or C, MSPA, a form of SpA with only the Fab-binding specificity.

**FIGURE 5**. Western blot analysis of reactivity with Ig from diverse species. Western analysis was performed on purified Ig after nonreducing PAGE. In the following lanes: 1, polyclonal human adult IgM; 2, polyclonal human adult IgG F(ab')_{2}; 3, rat IgG; 4, rabbit IgG; 5, swine IgG; 6, goat IgG; 7, horse IgG; 8, dog IgG; 9, bovine IgG; 10, sheep IgG; 11, frog Ig; 12, chicken Ig; 13, human IgG Fc; 14, monoclonal human V_{H}4/clan II IgM. These replicates were developed with Coomassie stain (A), a form of SpA with only the Fab-binding specificity (MSPA) (B), LJ-26 (C), or an isotype control chicken scFv (D). Specificity was confirmed by lack of reactivity with IgG Fc or with the V_{H}4/clan II IgM.
SpA that has five domains capable of these Fab-binding interactions (28, 43).

An avian recombinant Ab binds conformational V\textsubscript{H} region-associated determinant

To evaluate the structural requirements for V\textsubscript{H} region recognition, reactivity was assessed in immunoblot analysis with purified human and murine Ig. As shown in Fig. 4, both SpA and LJ-26 reacted only with the nonreduced clan III Ig, while reactivity was abolished under reducing conditions. Hence, the LJ-26 Ab, like the natural clan III V\textsubscript{H}-binding proteins, interacts with a conformational V\textsubscript{H} determinant that is abolished by these reducing conditions.

Based on the above described cross-species reactivity, we postulated that LJ-26 recognizes a clan III-associated determinant that has been conserved during the evolution of the adaptive immune system. To test this hypothesis, we ascertained the immunoblot reactivity of LJ-26 with a panel of purified Ig from diverse species. As illustrated in Fig. 5, LJ-26 specifically reacted with Ig from a wide range of placental mammals, including Rodentia (mouse and rat), Lagomorpha (rabbit), Carnivora (dog), Artiodactyla (pig, cattle, goat, and sheep), and Perissodactyla (horse). In addition, these limited surveys also demonstrated specific reactivity with an amphibian (Xenopus laevis), and unexpectedly also with an avian species, chicken, the host that was the source of the LJ-26 Ab. In general, reactivity with LJ-26 was comparable with the level of Fab-mediated SpA binding (Fig. 5B).

Flow-cytometric analyses of human and murine monoclonal B cells

To evaluate the reactivities of LJ-26 with B cell membrane-associated Ig, a series of microfluorometric assays were performed. In studies of PBMC from patients with chronic lymphocytic leukemia (CLL), which contain essentially monoclonal (i.e., >95%) CD5\textsuperscript{+}CD19\textsuperscript{+}B cell populations, neither LJ-26 nor MSpA bound B cell CLL-expressing clan II Ig (e.g., COR) (Fig. 6A) or clan I Ig (not shown). In contrast, LJ-26 recognized most CLL that express clan III Ig (e.g., GOS and HEC), whereas MSpA bound fewer clan III-expressing CLL. These data document that LJ-26 recognizes a broad range of B cell-associated clan III Ig, displaying strong reactivity even with V\textsubscript{H,3}-expressing B cells with low surface Ig levels.

In studies of PBMC from healthy adults, the reactivities of both MSpA (31) and LJ-26 were found to be restricted to a subset of CD19\textsuperscript{+} cells (i.e., B lymphocytes). Moreover, we found that the same ~25% of CD19\textsuperscript{+} gated mononuclear cells stained with MSpA or LJ-26 (Fig. 6, A and B). Of these CD19-positive populations, ~40% of the LJ-26\textsuperscript{-} and MSpA\textsuperscript{-} populations express \lambda chains, while ~60% of reactive populations express \kappa chains, which is the exact physiologic distribution of these L chains in the
human immune system, indicating that these interactions are independent of B cell L chain usage.

The reactivity of the LJ-26 Ab was also evaluated in transgenic mice expressing defined \( V_H \) and \( V_L \) genes. In studies of AB29 mice, which have an expanded monoclonal B cell set expressing a human \( V_H^{\text{IV}}/\text{clan II} \) \( \mu \) chain paired with a human \( V_{\lambda} \) L chain (32), as expected the splenic mononuclear cells bearing human IgM were not recognized by the LJ-26 Ab (Fig. 7A). However, in these mice, about one-third of the B220\(^+\) splenocytes instead express endogenous murine IgM, and LJ-26 recognized about 7\% of these polyclonal splenic murine IgM-bearing B cells. These findings are consistent studies, in which 4–7\% of C57BL/6 and 6–9\% of BALB/c B220\(^+\)/IgM\(^+\) splenocytes interact with the Fab binding site of SpA (20). (G. Silverman, unpublished observation).

We also studied T15i transgenic knockin mouse, which have B cells expressing a S107/clan III/\( V_H^{\text{T15}} \) rearrangement associated with the IgM\(^a\) allotype (33). In homozygous mice, we found that the great majority of these IgM\(^a\)-reactive B cells were strongly reactive with LJ-26 (Fig. 7B) or the MSpA reagent (not shown), which is equivalent to results with a conventional \( V_H^{\text{T15}} \)-specific idiotypic marker (33). The remainder of the B220/CD45R-reactive cells instead express diverse endogenous \( V_H \) rearrangements of the IgM\(^b\) allele (33, 34), explaining the incomplete reactivity of all mature B cells with the clan III-specific reagents.

Investigations of a murine model of in vivo B cell superantigen-induced clonal defects

To test the hypothesis that natural B cell superantigens can affect the in vivo lymphocyte clonal composition, we investigated the consequences of exposure on heterozygous T15i \( \times \) C57BL/6 F1 mice. Similar to the homozygous T15i mice, in these naive mice, most of the B cells that bear the IgM\(^a\) allotype coexpress the H chain with the \( V_H^{\text{T15}} \) rearrangement paired with diverse L chains (34); hence, there is not a significant population specific for any single conventional Ag. The remainder represent endogenous polyclonal B cells expressing the parental IgM\(^a\) allotype, and hence, any fluctuations in the transgene-associated B cell set are measurable by use of \( V_H^{\text{T15}} \)-reactive and IgM allotype-specific markers.

For these studies, we treated the neonatal heterozygous mice with SpA or a control protein Ag, according to the same regimen recently used in studies demonstrating superantigen-induced suprACLONAL loss in BALB/c mice (20), and we evaluated the representation of peripheral B cells in mice sacrificed 18 h after introduction of the last dose. To ensure that a detected decrease in the representation of \( V_H^{\text{T15}} \)-expressing B cells is due to cellular deletion, and is not due to a reversible down-regulation of membrane-associated Ig, in certain studies these splenocytes were evaluated after 24 h of in vitro incubation in the absence of the immunogens. As illustrated in Fig. 8, compared with groups that received the control treatment, in which 32 \( \pm \) 1.9\% (mean \( \pm \) SEM) of B cells were LJ-26 reactive, after SpA treatment only 3.7 \( \pm \) 1.2\% of B cells were LJ-26 reactive. Although due to gating differences, specific values were somewhat different, 41.1 \( \pm \) 2.3\% of the control-treated B cells expressed the transgene-associated IgM\(^a\) allotype, while in SpA-treated mice the representation of IgM\(^a\)-bearing B cells was reduced to 5.3 \( \pm \) 1.4\%, representing a highly significant 87\% loss (\( p < 0.005 \), one-tailed Student \( t \) test). Also illustrated in Fig. 8, by gating on B220/CD45R-bearing cells, the concordance between IgM\(^a\) and LJ-26 reactivity in these mice is directly demonstrated. Importantly, in the SpA-treated mice, the residual LJ-26-reactive B cells displayed lower levels of reactivity than detected in the control groups.

In studies of freshly isolated cells, similar findings were obtained, with greater than 82\% reductions in the representation of \( V_H^{\text{T15}} \)-expressing B cells, as detected by either LJ-26 or IgM\(^a\) allotype reactivity (not shown). Notably, at this time point following exposure, there were no significant differences detected for the total number of splenocytes isolated from the two treatment groups, nor for the representation of mature B cells in the spleen. Therefore, our findings with the clan-specific marker were in close agreement with findings with the allotype-specific marker, documenting similar levels of B cell superantigen-induced postexposure losses of \( V_H^{\text{T15}} \)-expressing B cells in this peripheral compartment.

**Discussion**

In this study, we describe a successful strategy that marries the special functional capacities of the avian immune system with the power of phage-display expression vectors for the efficient in vitro selection of Ab clones specific for highly conserved structural features of the mammalian immune system. By this approach, we
have created unique Abs that are specific for a highly expressed V_H region surface characteristic of clan III Abs.

To accomplish this goal, the avian immunization regimen consisted of sequential exposures with three monoclonal clan III Ig

that derive from different human V_H^3 gene rearrangements, and that included L chains from diverse human V_L genes. Subsequently, the genetic information for this Ab response was transferred into a suitable phagemid vector, and Abs with desirable specificities were isolated by a strategy in which only the initial round of panning used one of the original immunogens, under relatively low selection stringency. Later, to remove clones with irrelevant specificities, washing stringency was increased and selection was performed in the presence of soluble V_H^4/clan II IgM to prevent carryover of unwanted binders, including those to C region and L chain determinants. Within this strategy, a murine clan III Ig-selecting agent was next substituted and washing stringency was further increased. Both to reinforce selection for cross-species conserved determinants, and to ensure that isolated clones would be suitable for cell-staining studies, the final round was performed using selection upon viable murine splenic B cells.

Specificity analysis using a large number of human and murine samples documented that our strategy resulted in the recovery of clones reactive with the products of diverse genes from structurally related and highly expressed human and murine V_H families. Reactivity was completely restricted to clan III Ig, with demonstrated complete nonreactivity with diverse clan I and clan II Ig. Predictably, reactivity with the representative LJ-26 Ab was much more frequent in the adult human peripheral B cell compartment than in the murine immune system, which is consistent with known species-specific V_H gene family expression patterns (i.e., V_H^3/clan III is dominant in humans, while J558/clan I is dominant in mice).

From investigations of cross-species reactivity, we documented that the LJ-26 binding site, like the SpA binding site, is conserved on diverse mammalian species, activities generally consistent with reported Ab gene usage (discussed in Refs. 4–6 and 12). We also demonstrated specific, but relatively weak, reactivity with sheep and bovine Ig, results seemingly in conflict with recent reports that these species do not have clan III genes (44, 45). However, clan III gene homologues have recently been cloned from sheep (John Reynolds, personal communication). We also found LJ-26 reactivity with frog Ig, which was predicted based on the deduced sequence of *Xenopus* V1 and related families (46). Although chicken V_H genes are also clan III homologues, we did not expect to find such strong LJ-26 reactivity with chicken Ig. It is possible that this represents a neospecificity created by the in vitro combinatorial pairing of the avian V_H and V_L genes from cloning into the phagemid vector. However, because this Ab clone was rescued from a postimmunization response, we interpret this as more likely an indication that strict B cell immune tolerance is not maintained in this avian host.

The immunization and selection methods did not include SpA and pFv; therefore, we were somewhat surprised to discover that binding of the avian anti-clan III scFv was competitive with these clan III-specific natural B cell superantigens. Moreover, in several comparative assays of Abs with diverse V_H regions, the binding specificities of SpA and LJ-26 were generally found to be quite similar. Akin to earlier reports of the specificity of superantigen binding (13, 14, 20, 38), LJ-26 was shown to recognize a conformational determinant that could be destroyed by reducing conditions. These findings may indicate that the clan III-specific V_H surface responsible for the binding of B cell superantigens is highly accessible and perhaps dominant for immune recognition in a nontolerant host.

In studies of a panel of murine mAbs, reactivity of clan III Ig with LJ-26 was somewhat more common than interactions with the Fab binding site of SpA. Notably, LJ-26 recognized a product of the clan III/X24 family, while SpA did not. In addition, LJ-26
often had greater reactivity with clan III IgG, suggesting that so-
matic hypermutation may less commonly have an adverse effect
upon recognition by the LJ-26 Ab. In microfluorometric assays of
human CLL specimens, despite low surface Ig levels, more clan III
CLL were also identified by LJ-26 than by SpA. We also found
that in assays of polyclonal human peripheral B cells from healthy
donors, LJ-26 staining displayed a closer direct correlation with
the level of total surface Ig expression, providing a sharp diagonal.
By comparison, although a very similar proportion of binders was
recognized, there was much greater heterogeneity in the Fab-bind-
ing interactions of SpA. We speculate that the binding of the LJ-26
Ab may be less sensitive than SpA to local or remote mutational
effects, perhaps because it interacts with a smaller surface that is
less diversified in the products of different inherited and somati-
cally mutated clan III genes. Based on the competitive inhibition
studies, it is likely that, in part, these differences also reflect the
higher binding affinity of the LJ-26 Ab.

Based on primary sequence correlation, we predict that the site
on the surface of clan III V\textsubscript{H} region involved in the binding inter-
action of the LJ-26 Ab is a more limited surface than the contact
site responsible for the binding of natural superantigens. Impor-
tantly, inherited polymorphisms and somatic variations at V\textsubscript{H}
positions 57 and 82a do not correlate with differences in reactivity
with the LJ-26 Ab, while these residues can greatly affect SpA-
binding activity (39–41). Although the data are still too limited for
meaningful mapping, it is likely that SpA (35), the LJ-26 contact site is remote from the CDR loops involved in binding of
conventional Ags, and may involve the V\textsubscript{H} \textbeta strands of the FR1
and FR3 subdomains.

To directly evaluate the utility of the LJ-26 Ab for investigations
of repertoire changes induced by B cell superantigen exposure, we
used the T15i system generated by Taki et al. (33, 34), which we
found was well suited to our goals. In part, this is because the
expressed V\textsubscript{H}T15 transgene is paired with diverse endogenous L
chains, which does not create a uniform population with a common
conventional ligand-binding specificity. Hence, the effects induced
by SpA exposure can only be due to unconventional V\textsubscript{H} region-
mediated binding interactions (i.e., superantigen-V\textsubscript{H} framework).
Most importantly, rearrangements of unmuted S107 genes com-
monly encode for B cell receptors with a common unconventional
ligand-binding specificity. Hence, the effects induced by
SpA exposure can only be due to unconventional V\textsubscript{H} region-
mediated binding interactions (i.e., superantigen-V\textsubscript{H} framework).

At a minimum, these studies have validated a strategy for the
creation of novel reagents that are uniquely well suited to the study
of the consequences of B cell superantigen exposure. Reiteration
of this avian Ab-cloning approach is almost certain to provide
reagents with complementary specificities to complete the over-
view of the expressed mammalian V\textsubscript{H} repertoire. Additional re-
agents for identifying further subdivisions of V\textsubscript{H} groupings, and
their V\textsubscript{L} analogues should also be obtainable.

More importantly, we have advanced the hypothesis that Ig
frameworks have functions beyond those of a passive scaffolding,
i.e., rigid structural elements that position the CDR loops to create
composite surfaces capable of binding diverse ligands. The V\textsubscript{H}
surface created by the FR1/3 subdomains has been conserved even
in primitive cartilaginous fish (reviewed in Ref. 47), which we
believe is due to relationship(s) with novel environmental li-
gand(s), or perhaps due to their interactions with special Ig adapter
molecules conveying functions that reiterate themes first appreci-
ated for Fc regions. These studies have also provided the first
evidence that in the suitable host, these conserved surfaces can be
recognized by the immune system. In the future, serologic tools
akin to LJ-26 should advance our understanding of the impact of
poorly understood infectious, inflammatory, and autoimmune dis-
ease processes upon the B cell compartment.

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