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Delineation of the Human Systemic Lupus Erythematosus Anti-Smith Antibody Response Using Phage-Display Combinatorial Libraries

Inmaculada del Rincon,* Maria Zeidel,* Elena Rey,† John B. Harley,‡§ Judith A. James,‡ Michael Fischbach,* and Iñaki Sanz²†

The anti-Smith (Sm) autoantibody response is highly specific for systemic lupus erythematosus and is predominantly targeted to the Sm-B/B' and -D1 polypeptides. In all animal species thus far studied, anti-Sm Abs initially recognize proline-rich epitopes in the carboxyl terminus of the Sm-B/B' protein and subsequently to multiple other epitopes in B/B' and D. The absence of appropriate mAbs has limited our understanding of the genetic and structural basis of this autoimmune response. Using phage-display technology and lymphocytes from a systemic lupus erythematosus patient we have generated the first and only panel of human IgG anti-Sm mAbs thus far available. These Abs reproduced to a remarkable extent the serological reactivity of the patient. Epitope mapping and genetic studies revealed that the anti-Sm response is produced by distinct B cell clones with restricted epitope reactivity. All of the Abs in our study were exclusively encoded by different members of the V_{H}4 gene family. On the aggregate, our results demonstrate that combinatorial libraries can recapitulate the immune repertoire of peripheral blood B memory cells and that epitope spreading appears to occur through the sequential recruitment of nonclonally related autoreactive B cell clones. The Journal of Immunology, 2000, 165: 7011–7016.

Abbreviations used in this paper; Sm, Smith; SLE, systemic lupus erythematosus.

Materials and Methods

Patient selection and sample collection

Patient CS was a 52-year-old African-American female with long-standing SLE clinically characterized by World Health Organization type IV glomerulonephritis, photosensitive rash, and symmetrical nonerosive polyarthritis. Serologically the patient had high titer antinuclear Ab with strongly positive anti-dsDNA and anti-Sm Abs. At the time that serum and cell samples were obtained for this study, the patient was being treated only with hydroxychloroquine and nonsteroidal anti-inflammatory drugs. Previously, she had received six courses of monthly i.v. cyclophosphamide followed by 1 additional year of i.v. cyclophosphamide administered every 3 mo. Altogether, cyclophosphamide therapy had been discontinued 10 mo before samples were obtained. At that time, the patient’s blood cell counts...
were normal except for moderate absolute lymphopenia (950 lymphocytes/µl).

Construction of an IgG1κ combinatorial library

A combinatorial library of randomly assorted heavy chains of the IgG1 isotype and κ light chains was constructed as previously described according to established protocols (10). Briefly, after informed consent was obtained, 50 ml of peripheral blood was collected from our patient and mononuclear cells were isolated by Ficoll-Hypaque gradient (Sigma, St. Louis, MO). Total RNA was extracted from 2 × 10^7 cells and 5 µg of RNA was reverse transcribed using RNaseH reverse transcriptase and random hexamer primers (PharMingen, San Diego, CA). The resulting ds-cDNA was used as template for the PCR amplification of the Fd fragment of IgG1 heavy chains and full-length κ light chains. Sense primers were designed to amplify all of the known human V_H and V_κ families (10) and contained appropriate restriction sites (SacI for light chains and XhoI for heavy chains) required for directional cloning in the phagemid pComb3 (kindly provided by D. Burton and C. Barbas, The Scripps Research Foundation) (11, 12). The restriction sites generated by the antisense primers were SpeI for the heavy chain and XhoI for the light chain.

Library screening and generation of soluble Fab fragments

The recombinant library was panned against an affinity-purified preparation of the Sm Ag containing both the B/B' and D polypeptides (ImmunoVision, Springdale, AR). Briefly, individual microtiter wells (Costar, Cambridge, MA) were coated overnight at 4°C with the Sm preparation at 20 µg/ml in 0.1 M sodium bicarbonate (pH 8.6), washed with PBS (pH 7.4), and then blocked with 3% BSA/PBS for 2 h at 37°C. Approximately 10^9 PFU of the recombinant library were applied to each of the blocked wells. Unbound phage was removed by vigorously washing 10 times with PBS/0.05% Tween 20, and the remaining phage was eluted with 50 µl of 0.1 M glycine/HC1 (pH 2.2) and neutralized immediately with 2 M Tris. The eluate was used to reinfest XL-1Blue Escherichia coli cells followed by superinfection with VCS-M13 helper phage. This enrichment process was monitored by comparing the number of phage eluted in each round with the phage plated in the previous round. After four rounds of panning, phagemid DNA was isolated from the rounds with higher enrichment and the sequence encoding the minor coat protein (cpIII) was removed by digesting with SpeI and NheI to produce soluble Fab fragments. After refugation, phagemid DNA was used to transform XL-1 Blue cells. Randomly selected individual colonies were grown in Super Broth with 50 µg/ml carbenicillin and protein production was induced with 1 mM IPTG (Boehringer Mannheim, Indianapolis, IN). The supernatant from these cultures was used without further purification to determine anti-Sm reactivity by conventional ELISA as described below.

Purification of recombinant Fab proteins

Ab Fab fragments were obtained from individual bacterial cultures induced with 1 mM IPTG. The Fab protein was purified from the bacterial pellets by affinity chromatography as previously described (10), and the concentration of purified Fab was estimated by sandwich ELISA using as standard a commercially available IgG Fab preparation of known concentration (Pierce, Rockford, IL).

Anti-Sm ELISAs

Dose-dependent reactivity of the recombinant Fabs with either the Sm-B/B'-D Ag or a panel of irrelevant control Ags was determined by conventional titration ELISA using 1 µg of the corresponding Ag/well of polystyrene 96-well plates (Costar) as previously described (4). Both serum samples and mAbs were tested in duplicate. The ability of either Sm or various control Ags in fluid phase to block the binding of recombinant Abs to immobilized Sm was also tested by ELISA in competitive inhibition assays. Purified Fabs were used at 75% of the concentration previously determined to provide optimum binding in standard titration assays. The relative affinity constant of each Fab was calculated according to Friguet et al. (13) as the concentration of soluble competing Ag providing 50% inhibition of maximum binding to Sm. Competing Ags, including Sm itself, were tested at concentrations ranging from 10^{-6} to 10^{-10} M.

Epitope mapping

Overlapping octapeptides spanning the coding regions of Sm-B' and Sm-D1 were constructed on a solid-phase support system as previously described in detail (4). These overlapping octapeptides were simultaneously synthesized on radiation-derivatized polyethylene pins (Chiron Technologies, Clayton, Victoria, Australia). Positive and negative control pins were synthesized from known antigenic and nonantigenic regions of Sm-B'.

OCTOPEPTIDES TO AMPLIFY HUMAN ANTI-SMITH IgG mAbs

Octopeptides were tested for reactivity with whole patient sera or purified Fab fragments of various mAbs, including Fati-1, F-17, F-14, and F-4. First, octapeptides were blocked with 3% low fat milk in PBS for 1 h at room temperature. Second, primary Ab, either patient sera (1:100 dilution) or purified Fab mAbs (at ~1 ng/well) in 3% milk/PBS with 0.05% Tween 20 (PBST) were allowed to incubate overnight at 4°C. Pins were then washed four times with PBST and then reacted with a 1:1000 dilution of antihuman IgG (Fab')2, raised in a goat, affinity-purified and conjugated to alkaline phosphatase (Pierce) for 2 h at room temperature. p-Nitrophenyl phosphate disodium was used as a substrate for alkaline phosphatase, and plates were read at 405 nm with a MicroELISA Reader (Dynatech, Alexandria, VA). Results for each plate were then standardized by comparison to positive control pins.

After completion of an assay, pins were sonicated for 2 h in sonication buffer (4), washed in hot water, and boiled in methanol for 2 min. Pins were then allowed to air dry and were either stored with desiccant or used for another assay (4).

FIGURE 1. Anti-Sm titration ELISA. Recombinant anti-Sm Fab fragments were tested in conventional ELISA for reactivity against affinity-purified Sm/B'B' as well as irrelevant Ags to determine the level of non-specific binding. Good dose-response curves were obtained with all Abs against Sm whereas no significant binding was detected with control Ags. It should be noted that due to the very low reactivity obtained against BSA, the curves obtained for all four mAbs overlap significantly at the bottom of the figure and cannot be easily told apart.

Octopeptides were tested for reactivity with whole patient sera or purified Fab fragments of various mAbs, including Fati-1, F-17, F-14, and F-4. First, octapeptides were blocked with 3% low fat milk in PBS for 1 h at room temperature. Second, primary Ab, either patient sera (1:100 dilution) or purified Fab mAbs (at ~1 ng/well) in 3% milk/PBS with 0.05% Tween 20 (PBST) were allowed to incubate overnight at 4°C. Pins were then washed four times with PBST and then reacted with a 1:1000 dilution of antihuman IgG (Fab')2, raised in a goat, affinity-purified and conjugated to alkaline phosphatase (Pierce) for 2 h at room temperature. p-Nitrophenyl phosphate disodium was used as a substrate for alkaline phosphatase, and plates were read at 405 nm with a MicroELISA Reader (Dynatech, Alexandria, VA). Results for each plate were then standardized by comparison to positive control pins.

After completion of an assay, pins were sonicated for 2 h in sonication buffer (4), washed in hot water, and boiled in methanol for 2 min. Pins were then allowed to air dry and were either stored with desiccant or used for another assay (4).

FIGURE 2. Anti-Sm competitive inhibition ELISA. Recombinant anti-Sm-B/B' Fabs were tested by ELISA for reactivity against the Sm-B/B' polypeptide after reaching equilibrium in the fluid phase with either the Sm Ag itself or a series of irrelevant Ags including BSA, OVA, lysozyme, and transferrin (representative results are shown for FATI-1 and F-17). Only Sm was able to compete for binding in a significant manner. The relative affinity constant of the Fab fragments, estimated as the molar concentration of competing Ag that produced 50% of binding inhibition (dotted lines) ranged between 1 × 10^8 and 2 × 10^9 M^{-1}.

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HUMAN ANTI-SMITH IgG mAbs
DNA sequencing and analysis

Heavy and light chain nucleotide sequences were determined by automated sequencing of both DNA strands using a Taq fluorescent dideoxy terminator cycle sequencing kit (Perkin-Elmer/Cetus, Norwalk, CT). The sequencing was performed in an Applied Biosystems PRISM 377 automatic sequencer (Perkin-Elmer/Cetus) by the Oligonucleotide and DNA Sequencing Core Laboratory of the University of Rochester. The germline counterparts of the rearranged VH and VK sequences were determined on-line using the V-BASE search program (http://www.mrc-cpe.cam.ac.uk; Medical Research Council Center for Protein Engineering, Cambridge, U.K.), and the degree of similarity between the corresponding sequences was established using the MegAlign program of the DNASTAR sequence analysis software (DNASTAR, Madison, WI). Complementarity-determining regions were assigned according to the definition of Kabat (14). Ag-contact loops were defined according to Chotia et al. (14).

Results

Size and complexity of the combinatorial library

As estimated by the efficiency of transformation of XL1-Blue E. coli competent cells, the IgG1/κ library made by in vitro pairing of the Ab heavy and light chains expressed in the PBMCs of SLE patient CS contained \( \sim 2 \times 10^7 \) primary clones. The representation of all VH families in the library was ensured by the successful PCR amplification obtained with all of the primer combinations described above and confirmed by a combination of dot blot hybridization experiments using VH family-specific oligonucleotide probes and random sequencing of bacterial colonies isolated from consecutive rounds of panning against the Sm Ag (results not shown).

Isolation and characterization of recombinant anti-Sm mAbs

A 20-fold increase in the titer of eluted phage was obtained after three rounds of panning against Sm and an additional 10-fold increase was observed after the fourth round, strongly suggesting a significant enrichment in phage particles bearing specific anti-Sm Abs. To further evaluate these clones, phagemid DNA purified from the last round of panning was modified as described and used to generate E. coli colonies that secreted soluble Fab fragments. Four of 10 randomly picked colonies produced significant binding to Sm and were selected for detailed analysis. The characteristics of the recombinant Abs produced by these bacterial clones constitute the focus of this paper. Fab fragments were purified from large-scale cultures induced with IPTG and used for conventional titration ELISA to determine binding against Sm and a panel of irrelevant control Ags (Fig. 1). All four Fabs demonstrated very high binding against Sm in a dose-dependent fashion, with low background reactivity.
binding against other Ags and low background binding. The binding specificity for the Sm Ag was also confirmed by competitive ELISA in which a set of soluble Ags, including Sm itself, were used to compete against immobilized Sm (Fig. 2). These experiments show that only Sm produced significant inhibition as compared with control Ags. Using the method of Friguet et al. (13), the relative affinity constants of the recombinant anti-Sm Abs was estimated in the range of $10^{-8}$ to $10^{-28}$ M.

Epitope mapping of the Smith-B/B' and -D polypeptides

The fine specificity of the recombinant Fabs was defined by ELISA in which the Abs were tested against all of the possible overlapping octapeptides derived from the amino acid sequence of the Sm-B/B' and -D1 polypeptides (233 and 112 octapeptides, respectively). These assays were also performed with serum samples obtained from patient CS concomitantly with the cells used for the construction of the combinatorial library. The results of these experiments are presented in Figs. 3 and 4. Overall, the recombinant Fabs showed very restricted specificity and reacted mainly with only 1 or 2 of the 345 octapeptides tested (the only exception, mAb F-14, will be discussed below). As a rule, they recognized the same epitopes also recognized by the patient's serum Abs. Thus, mAb FATI-1 only recognized the two major and earliest epitopes of the anti-Sm response, the recurrent PPPGMRPP and the similar and overlapping PPPGMRP sequence with a reactivity of 2-3 SD above the mean with octapeptides 76–78 ($10^{-4}$LDTILVDVEPK$^{28}$). This sequence has partial overlap with the main peak obtained with the patient's serum (octapeptide 79, $10^{-3}$LVDVEPK$^{86}$). F-14 also recognized a carboxyl-terminal epitope which contains the GRG repeat characteristic of this part of the Sm-D molecule (octapeptide 105, $10^{-3}$GRGRGRGR$^{112}$) (16). Interestingly, this mAb displayed some significant degree of cross-reactivity with the region encompassed by overlapping octapeptides 144–151 and 150–157 ($10^{-4}$PQGRGTVA$^{157}$) as well as with multiple epitopes of Sm-B/B'.

Genetic analysis of recombinant anti-Sm autoantibodies

DNA sequencing analysis of the genes encoding the variable regions of both heavy and light chains revealed a remarkable restriction in the use of VH genes. Thus, all four mAbs were encoded by different members of the VH 4 family ($V_{H} 4$-31, $V_{H} 4$-34, $V_{H} 4$-61, and $V_{H} 4$-59). In contrast, the light chains were encoded by genes derived from the VK 1 and VK 3 families. Both heavy and light chain genes displayed characteristics of Ag-selected somatic hypermutation with a degree of similarity with the corresponding germ line sequences ranging from 92 to 98%. A detailed analysis of these sequences will be published elsewhere.

Discussion

The anti-Sm autoantibody response has been well-characterized serologically in several species including mice, rabbits, nonhuman primates, and humans. The pattern of epitope recognition of

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Sm-B/B' is remarkably conserved among all species thus far studied and is consistent with a phenomenon of epitope spreading that starts with the preferential recognition of proline-rich sequences concentrated in the carboxyl terminus (3–5, 17, 18). The complex epitope recognition pattern exhibited by our patient’s serum, therefore, is most likely the result of a process of epitope spreading. The actual size and composition of the initial autoreactive B cell repertoire remains to be determined but at least theoretically, a single B cell recognizing the initial epitope (PPPGRMGP or PPPGM RPP) could be enough to set this process in motion. Subsequently, upon B cell presentation of additional epitopes, the spreading of clones with restricted epitope specificity could be generated by intraclonal evolution due to somatic hypermutation or by the progressive recruitment into the dysregulated B cell repertoire of “new” unrelated autoreactive B cell clones. Up until now the lack of suitable mAbs had precluded an understanding of the genetic and molecular basis of this phenomenon in humans. Our collection of recombinant mAbs closely resembled the one obtained with the patient's polyclonal serum (see Figs. 3 and 4). This fact strongly suggests that the individual heavy and light chains used by these mAbs may not correspond to the ones originally expressed in vivo, and indeed several studies have demonstrated that multiple light chains can be paired with a heavy chain while retaining the original specificity of the Ab (20). This issue could be partly elucidated by H-L chain gene recombination experiments. However, a final answer will only be provided by the isolation and analysis of single anti-Sm Ab-producing B cells or plasma cells.

Only in one case did a mAb react with epitopes not recognized by the patient’s Abs. Thus, F-14 bound the sequence GRGRGRGR of Sm-D, which did not react with CS serum. This serum is exceptional with some anti-DNA reactivity in the patients’ sera. These results suggest that phage-display libraries do not contain specific IgG autoantibodies unless they are significantly represented in the in vivo repertoire. This is important since combinatorial technology can create in vitro artificial pairings of heavy and light chains potentially misrepresenting the antigenic specificity of the actual repertoire. It is entirely possible however that the specific H + L chain combinations found in our Abs may not correspond to the ones originally expressed in vivo, and indeed several studies have demonstrated that multiple light chains can be paired with a heavy chain while retaining the original specificity of the Ab (20). This issue could be partly elucidated by H-L chain gene recombination experiments. However, a final answer will only be provided by the isolation and analysis of single anti-Sm Ab-producing B cells or plasma cells.
cloning and expression would have overcome in vivo anergy. Alterna-
tively, F-14 reactivity might have gone undetected in total serum if
it represented a minor fraction of the patient’s secreted autoantibody
repertoire, perhaps by having been absent from the plasma cell com-
partment at the time of the study.

The genetic restriction encountered in the heavy chain used by
the anti-Sm Abs represents another remarkable feature of our study.
Thus, all of the heavy chains were encoded by genes derived from the
VH4 family including one (F-17) encoded by the VH4-3 gene which is
otherwise found in 100% of pathogenic cold agglu-
tinins and in a large variety of other autoantibodies but not in
conventional, protective Abs (25–35). This restriction suggests
that either a triggering event or an amplification mechanism ap-
ppears to preferentially recruit VH4-encoded anti-Sm autoantibodies
into the pathogenic lupus repertoire. Possible mechanisms that
could explain this observation include a superantigen effect of the
Sm Ag or related Ags and/or an enhanced pathogenic potential of
VH4-encoded autoantibodies. In summary, we report the detailed
characterization of the first panel of human anti-Sm IgG monoclo-
nal autoantibodies ever generated from an SLE patient. Remark-
able aspects of this work include the fact that the Abs were gen-
erated by phage-display technology from PBLs and that the
epitope recognition profile of the recombinant autoantibodies over-
lapped significantly with the in vivo activity of the patients’ Abs.
This suggests that this technology can indeed recapitulate in vitro
the Ab repertoire expressed by recirculating memory B cells and
validates the use of this approach while indicating that the avail-
avility of tissues enriched in memory B cells or plasma cells is not
an absolute requirement for this type of study. The ability to
generate relevant monoclonal autoantibodies with this approach
should allow investigators to define the earliest antigenic epitopes
targeted by autoimmune responses as well as to understand the
genetic and structural basis of pathogenic autoimmune responses
and of epitope spreading.

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