Stepwise intraclonal maturation of antibody affinity through somatic hypermutation
(mutagenesis/transfection/recombinant antibody)

CHRISTINE KOCKS* and KLAUS RAJEWSKY

Institute for Genetics, University of Cologne, Weyertal 121, D-5000 Cologne 41, Federal Republic of Germany

Communicated by Leonard A. Herzenberg, August 1, 1988

ABSTRACT Using recombinant DNA techniques, we re-constructed a genealogical tree (Sablitzky, F., Wildner, G. & Rajewsky, K. (1985) EMBO J. 4, 345-350) that connects three clonally related B cells producing somatically mutated antibodies to a progenitor cell expressing a germ line-encoded antibody. The somatic mutants had been isolated from an in vivo immune response. The germ line-encoded progenitor antibody bound the antigen with high affinity. Intraclonal affinity maturation occurred stepwise over a 15-fold range.

Affinity maturation of antibodies in the course of immune responses is a classical phenomenon in immunology (1). In the frame of the clonal selection theory (2), it would reflect the selection and expansion of B-cell clones that existed before immunization and are committed to the production of high-affinity antibodies. However, it recently has become clear that affinity maturation involves a process of intraclonal diversification through somatic hypermutation. This process seems to be set in motion after immunization and leads to the accumulation of memory B cells expressing high-affinity antibodies (for a review, see ref. 3).

A useful approach to study somatic hypermutation in B-cell differentiation pathways is the molecular analysis of antibody genes expressed by clonally related cells (4-8). We present here a direct demonstration of step-wise affinity maturation of a germ line-encoded antibody specificity through somatic hypermutation within a single B-cell clone.

MATERIALS AND METHODS

Variable (V)-Region Gene Cloning from Hybridoma A20/44. A 7.15-kilobase (kb) EcoRI fragment containing the active V- diversity (D)-joining (J) segment (7) was subcloned into pBR328 (pA20/44). A 2.65-kb BamHI/EcoRI fragment containing the V-D-J segment, =0.5-kb 5' region, and the immunoglobulin heavy (H) chain enhancer was isolated from pA20/44. The 5' BamHI site was converted to an EcoRI site by a BamHI/EcoRI adapter (Amersham) (9) and cloned into PUC19 (pA20/44RI). For cloning of the light chain V region (VL), size-enriched EcoRI-digested DNA of cell line A20/44 was ligated to phage arms of Charon 35 (10) and packaged in vitro. Recombinant phages hybridizing to a κ light chain J region (Jκ)-specific probe (a 2.7-kb HindIII fragment derived from the immunoglobulin κ chain locus including Jκ1-5) were isolated and characterized by restriction mapping. From phage P8, which contains the active VL-Jκ and Cκ (κ chain constant region) gene on a 14-kb EcoRI fragment, the VL-Jκ gene was subcloned as an EcoRI/HindIII fragment into PUC19 (pVκA20/44). The Cκ fragment was subcloned as a 4.5-kb HindIII fragment into PUC19 (pCκ). The sequence of the genomic clones containing the active V regions was determined from M13mp18/19 subclones (11, 12) to confirm the mRNA sequence previously described (6). Two positions that represent ambiguities in the mRNA sequence of the heavy chain V region (VH) at position 100 could be determined as two guanosines; the resulting triplet encodes a glycine. Nucleotide 11.3' of Jκ1 is an A→C exchange from the germ line. In position 72 of VH, an ambiguous nucleotide could be resolved as an adenosine, leading to threonine. Nine nucleotides 3' of Jκ in the intron is a G→C exchange from the germ line.

Construction of Expression Vectors. μ chain expression vector: pA20/44μ. The pSV-Vμ1 vector (13) was cut with EcoRI, and the 4.1-kb EcoRI fragment containing the B1-8 V-D-J region was substituted by pSV the 2.65-kb EcoRI fragment containing the A20/44 V-D-J region derived from pA20/44R1.

κ heavy chain expression vectors. The aim of the following construction series was to obtain pyl1gpt, an immunoglobulin κ chain expression vector containing a single EcoRI site for the insertion of V-D-J region genes and all features of a pSV2-gpt vector (14). (i) pGI4A, a 6.6-kb EcoRI fragment containing a genomic κ chain gene from BALB/c mice, was isolated from phage Ch4A.M.lg-γl-3 (15) and cloned into PUC9. (ii) In pGI2AEcoRI, the EcoRI site at the 3' end of the γl chain gene was deleted by a fill-in reaction with the Klenow fragment of DNA polymerase I and subsequent religation. (iii) pyl1gpt, a 6.6-kb EcoRI/SalI fragment containing the γl chain gene, was isolated from pGI2ACEcoRI (the SalI site stems from the PUC9 polyncler) and ligated to a 4.7-kb EcoRI/XhoI vector fragment isolated from pSV-Vμ1 (13) containing eukaryotic expression signals and the bacterial marker gene gpt. (iv) In pA20/44γlpgpt, the V-D-J region of A20/44 was inserted as a 2.65-kb EcoRI fragment derived from pA20/44R1. (v) Mutagenized V-D-J regions were cloned from M13mpK18 vectors as 2.65-kb EcoRI fragments into pyl1gpt or were substituted for the B1-8 V-D-J region in pSV-Vμ1, giving pH65γ1 and pH65μ, which contain the glutamic acid-to-lysine exchange at position 65; pH11γ1 and pH11μ, which contain in addition the arginine-to-lysine exchange at position 11; and pHIP5γ1 and pHIP5μ, which contain in addition the proline-to-alanine exchange at position 105. [pH65, pH11, and pHIP5 all have a 450-base pair (bp) deletion between the HindIII and XbaI site 3' of JHμ. The deletion has no apparent influence on expression.]

κ chain expression vectors. (i) In pκA20/44neo and pκExA20/44neo, the Vκ-J region of A20/44 (2.7-kb HindIII/EcoRI fragment) and Cκ (3-kb HindIII/BamHI fragment) were ligated to BamHI/EcoRI fragments of SV-2neo (16) and SV-2gpt (17). Transfected cell lines containing the pκA20/44neo construct produced less L chain than lines transfected with pκExA20/44. (ii) In pκEx99neo, the A20-44 Vκ-J region in pκEx20/44neo was

Abbreviations: V, variable; D, diversity; J, joining; H chain, antibody heavy chain; L chain, antibody light chain.

*To whom reprint requests should be addressed.
substituted by the mutagenized V\(_{\gamma}\)–J\(_{\gamma}\) region. Direct sequencing (18) of V\(_{\gamma}\) genes in expression vectors pExA2/40, pEx99neo, pH65yI, pH11yI, and pH105yI did not reveal any mutations that would have been introduced by the cloning procedures.

**Mutagenesis.** Oligonucleotide-directed mutagenesis was performed on the VH region (2.65-kb EcoRI fragment) and the V\(_{\gamma}\) region (1.85-kb EcoRI/Sac I fragment containing ≈1-kb 5′ V\(_{\gamma}\) region) in three rounds by using the mutagenized product of one round as template for the next; first V\(_{\gamma}\) position 77 and VH position 65 and then V\(_{\gamma}\) position 74 and VH position 11 were mutagenized by using the EcoK/EcoB restriction system as described by Carter et al. (19). The third round, mutagenizing J\(_{\gamma}\) position 99 and J\(_{H}\) position 105, was performed by the method of Kunkel (20) with the Mutta-Gen kit of Bio-Rad. Plaques containing the desired mutation were identified by screening with the mutagenic primer or directly by screening randomly picked plaques. After each round, the entire coding region of the mutants was sequenced to assure that only the desired amino acid exchange had been introduced. This was done as described by Biggin et al. (12) or by using the Sequenase kit (United States Biochemical, Cleveland, OH).

**Transfection and Selection for Expression of Recombinant Proteins.** Combinations of H and L chain expression vectors were cotransfected into the immunoglobulin-nonproducing myeloma line X63-Ag8.653 (21) by electroporation (22). Cells were grown (23) and selected as described (16). Only neo selection was used, because under double selection for neo and gpt, cells showed retarded growth. Clones producing H and L chains were identified by screening culture supernatants in an avidin-biotin enzyme-linked immunosorbent assay (24). Plates were coated with either 10 μg of antigen (antibody S43.10) per ml, goat anti-mouse IgM, or goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL) and developed with goat anti-mouse κ chain-biotin (Southern Biotechnology Associates).

**Affinity Measurements.** Affinities were determined in a binding inhibition assay (25). Affinities of the recombinant antibodies were determined either directly from cell culture supernatants or after affinity purification, with identical results. As antigen, we used an affinity-purified monovalent Fab fragment of antibody S43.10 [a subclone from the original S43 line (25) that was selected for loss of the immunoglobulin chains of the fusion partner (G. Wildner and K. R., unpublished data)]. I\(^125\)I-labeling was performed on Fab bound to 4-hydroxy-3-nitrophenylacetyle-n-aminono-caproic acid-Sepharose (4). Clones producing antibody and antigen were eluted by an excess of free hapten, which was subsequently removed on a Sephade x G-25 column. The labeled antigen was used at about 0.1 nM. Antibody–Fab complexes were precipitated by adding saturated ammonium sulfate to a final concentration of 44%.

**Purification of Antibodies.** Recombinant antibodies were purified from cell culture supernatant by affinity chromatography: S43.10 antibody (1 mg) was coupled to CNBr-activated Sepharose (1 ml; Pharmacia Fine Chemicals). One liter of filtered cell culture supernatant was slowly applied to a 1-ml column of S43.10-coupled Sepharose overnight at 4°C. The column was washed with 20 ml of phosphate-buffered saline (PBS) and was eluted with 3 ml of 4-hydroxy-3-nitrophenylacetilcaproic acid (3 mM in 0.2 M borate buffer (pH 8.4); 4-hydroxy-3-nitrophenylacetil acid interferes with S43.10-anti-idiotypic binding (unpublished data)). Fractions of 1 ml were dialyzed three times against a 1000-fold excess of PBS and frozen at −70°C. Part of the protein was stabilized by addition of 0.1% bovine serum albumin because of expected low yield. Hybridoma proteins A2/69, A8/4, and A20/44 were purified from ascites fluid by DE-52 ion-exchange chromatography. All proteins appeared pure when analyzed by NaDodSO\(_4\)/PAGE (26, 27). Recombinant IgG proteins had the expected molecular mass of 150 kDa. The IgM recombinants contained predominantly multimeric IgM, although some monomeric IgM was also present [a similar result has been observed earlier (28)].

**RESULTS**

To analyze how somatic hypermutation leads to intracellular affinity maturation, we reconstructed progenitors of clonally related somatic antibody mutants, using recombinant DNA techniques. The three mutants A2/69, A20/44, and A8/4 had been isolated as hybridomas from an anti-idiotypic response against an isogeneic, somatically mutated antibody and turned out to be clonally related upon molecular analysis (6, 7). From the pattern of somatic point mutations in the VH regions of the mutant antibodies, a genealogical tree could be delineated (Fig. 1). The branchpoints of this genealogical tree define three progenitor cells. The initial cell expresses a germ-line combination of VH and VL segments (Fig. 1; see also DISCUSSION). For the experiments presented here, the genealogical tree depicted in Fig. 1. VH and VL genes of hybridoma A20/44 were isolated, mutagenized by oligonucleotide-directed mutagenesis, cloned into H and L chain expression vectors, and, after transfection into an immunoglobulin-nonproducer myeloma, expressed as antibodies.

Affinity determinations of antibodies A2/69, A20/44, and A8/4 and of the reconstructed antibodies 1, 2, and 3 are shown in Fig. 2. Affinities were measured by inhibition of antibody binding to the Fab fragment of the idiotypic antibody against which antibodies A2/69, A20/44, and A8/4 had been raised. The reconstructed germ-line antibody (antibody 1) had a good affinity for the antigen, ≈25 nM. The overall affinity increase from the germ-line antibody to the isolated mutant A2/69 was about 15-fold. Maturation occurred stepwise: the germ-line antibody had a lower affinity than intermediate-stage antibodies 2 and 3; a further affinity increase occurred between these intermediates and the end products A2/69 and A20/44 and between antibody 2 and A8/4, respectively. Although the affinity differences are small, the order of the curves is reproducible. Fig. 2b shows averages calculated for each antibody from values derived from three to seven individual assays. Two steps of affinity maturation are also apparent when sets of curves from independent experiments are compared (Fig. 2c).

Fig. 2b shows that the affinity of A20/44 was not altered by the cloning procedure. The reconstructed antibody has the same affinity as the hybridoma protein derived from the somatic mutant isolated from the mouse.

In the B-cell clone from which hybridomas A2/69, A20/44, and A8/4 are derived, somatic mutation may have occurred both before and after isotype switch (29). Therefore, the recombinant proteins were reconstructed as IgM and IgG, although the results of the binding-inhibition assay should be independent of the antibody class under the conditions used. This was indeed the case (Fig. 2c). Recombinants 1 and 2 yielded virtually identical affinities regardless of antibody class. For unknown reasons, the γ1 chain version of antibody 2 has a slightly higher affinity than the μ chain version of this antibody.

**DISCUSSION**

The progenitor cell of the genealogical tree depicted in Fig. 1 expresses a combination of germ-line VH region genes for the following reasons. The VH gene used by the progenitor (6) has been found to be expressed unmutated in five independently derived hybridomas (205.12, ref. 30; B12B3, R. Dildrop and K. R., unpublished data; 133.25, ref. 31; and 1B15 and 13G5, I. Förster and K. R., unpublished data). The nucleotide sequence of the VH gene expressed by the progenitor was
derived as consensus from three mutated hybridomas and hybridoma As79, whose nucleotide sequence lacks the first 15 codons. In this stretch, however, the consensus sequence is identical to that of the \( V_\alpha \) subgroup 23 (32) and of another \( V_\alpha \) gene whose sequence was partially determined recently (\( V_\alpha \) 10H125, positions 1–59; I. Förster and K.R., unpublished data; the nucleotide sequence is identical to the \( V_\alpha \) consensus in all positions determined). In addition, an identical \( V_\alpha \) gene is expressed by plasmacytoma XRPC-25 (33). Thus, affinity maturation in this B-cell clone can be traced back to a germ line-encoded specificity.

This result was not necessarily expected. With a mutation rate of \( 1 \times 10^{-5} \) per base pair per generation at the pre-B-cell stage (34), somatic mutation might contribute to the diversification of the preimmune repertoire, although this rate is insufficient to account for the accumulation of somatic mutations seen in later stages of an immune response (see model calculations in ref. 29). In the B-cell clone represented in Fig. 1, the alanine-to-proline exchange at position 105 of the \( H \) chain is a good candidate for a mutation that might have been introduced before immunization. A need for the generation of antigen binding specificity by somatic mutation prior to antigenic stimulation would explain why the anti-idiotypic response from which antibodies A2/69, A20/44, and A8/4 were derived was weak and irregular (G. Wildner and K.R., unpublished data). However, the experimental result disproves this notion: the germ-line progenitor (antibody 1) bound the antigen with high affinity. Therefore, the irregularity of the response must have other reasons. The germ-line \( V \) genes expressed by the progenitor cell may be infrequently rearranged. However, \( V_\gamma \) segment 205.12 is a frequently expressed member of the \( V_\gamma \) gene family 1 (35), and the germ line \( V_\gamma \) gene segment has been found expressed in three other independently isolated cell lines (see above). Another possibility is that the germ line-encoded specificity expressed by the progenitor cell is suppressed. Only after the onset of somatic mutation might the clone escape suppression by losing, through somatic mutation, the specificity that was subject to regulation.

The frequency of somatic \( V \) region mutations increases in the antibody population in the course of immunization (for review, see ref. 3). This does not exclude, however, that the antibodies isolated at later stages of the response represent rare somatic mutants that were already present in the preimmune repertoire as a minor fraction. Due to higher affinity, such clones could be preferentially stimulated and overgrow other clones with time. An experiment by Manser and Geffer (36) directly addressed this problem. Anti-idiotypic suppression of a germ-line specificity eliminated the generation of a particular mutant phenotype upon subsequent immunization, indicating that somatic hypermutation occurs predominantly in the antigen-dependent phase of B-cell differentiation. Our result further supports this general concept, since affinity maturation starts from a germ line-encoded antigen-binding specificity.

The present results show stepwise maturation of antibody
affinity through somatic hypermutation within a single B-cell clone. The maturation step between antibody 1 and antibodies 2 and 3 is caused by an alanine-to-proline exchange at position 105 in the complementarity-determining region 3 of the H chain. Another affinity increase occurs between intermediates 2 and 3 and the end products. A mutation in position 49 at the border of the complementarity-determining region 2 of the L chain was independently selected in antibodies A2/69 and A8/4 and therefore may contribute to this increase. The mutation is caused by two different nucleotide exchanges, both of which result in an asparagine-for-lysine substitution.

The genealogical tree delineated in Fig. 1 relates the three somatic mutants isolated to the progenitor of the clone in the simplest way. This does not exclude more complicated patterns. For example, the glycine-to-proline exchange in position 105 of the H chain could have been generated and selected more than once, like the lysine-to-asparagine exchange in position 49 of the L chain. Such variations of genealogy do not affect the main result of our analysis—namely, stepwise affinity maturation from a germ-line encoded specificity.

It is remarkable how small the affinity differences are that can be selected by the system. However, small differences in affinity may lead to profound changes in receptor crosslinking, antigen processing, and antigen presentation—processes known to play a crucial role in B-cell activation (for review, see ref. 37).

In a recent study of affinity maturation of anti-hapten (4-hydroxy-3-nitro-phenylacetyl) antibodies, one particular
amino acid exchange leading to a 10-fold higher affinity was repeatedly found among secondary response antibodies (38) and appeared to be the only somatic mutation available for affinity maturation (23). This seems plausible considering the small number of amino acid residues contacting hapten in hapten binding sites (39). In contrast, antibody complexes with proteins involves contacts between many amino acid residues (40–42). In an antibody–lysozyme complex, for example, 17 residues of the antibody interact with 16 residues of the antigen (40). Amit et al. (40) speculate that somatic mutation might lead to amino acid exchanges permitting additional salt links and hydrogen-bonding, since in the crystallized complex, not all residues in the interface are bonded. In such a situation, most advantageous amino acid exchanges may lead to only small improvements of, rather than drastic changes in, affinity. That is what we have observed in our analysis of intraclonal affinity maturation.

We thank F. Sablitzky for the V_{H} A20/44 gene and T. Honjo for the genomic γ_{H} chain gene, T. Simon for help in vector construction, M. Cramer for technical advice, K. Otto and B. Müller-Hill for synthesizing oligonucleotides, U. Ringiesen for the graphical work, and Á. Bohm and E. Siegmund for typing the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft through Grant SFB 74, the Fazit Foundation, and the Fritz Thyssen Foundation through a stipend to C.K.