Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 11120–11123, December 1991
Immunology

Antibody redesign by chain shuffling from random combinatorial immunoglobulin libraries

(combinatorial immunoglobulin repertoire/catalytic antibodies)

ANGRAY S. KANG*†, TERESA M. JONES*, AND DENNIS R. BURTON*‡

*Department of Molecular Biology, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037; and ‡Department of Molecular Biology and Biotechnology, The Krebs Institute, The University of Sheffield, United Kingdom

Communicated by Richard A. Lerner, August 19, 1991 (received for review June 24, 1991)

ABSTRACT **A number of experiments on the shuffling of heavy and light chains from antibodies of defined specificity for the transition-state analogue hapten nitrophenyl phosphonamidate are described. The experiments report on the promiscuity of heavy and light chains in binding antigen and the feasibility of antibody redesign by this shuffling process. The concepts of incestuous and extraclonal promiscuous association are described. Shuffling opens the possibility of generating panels of antibodies with related specificity but of distinct idiotypic composition that may have significance in the use of human monoclonal antibodies in therapy.**

A major problem in antibody structure and function is to what extent an antigen binding specificity can be generated by various heavy- and light-chain pairs. With very few exceptions (1–5), we have been mostly limited to those pairs provided by nature. However, with the cloning of heavy- and light-chain repertoires in phage (6–9) and the means to generate all or selected heavy- and light-chain repertoires, the problem can now be approached experimentally. Here we describe a number of experiments on shuffling of heavy and light chains from antibodies with specificity for the hapten nitrophenyl phosphonamidate (NPN) that reflect on the promiscuity of heavy and light chains in binding antigen and the feasibility of antibody redesign by this shuffling process.

Access to an extended family of antibodies of similar specificity has important consequences. For instance in therapy, a given monoclonal antibody may induce an anti-idiotypic response that could be avoided by switching to another antibody of similar specificity. Elimination of unwanted cross-reactivity with the maintenance of the desired specificity is another potential advantage of the extended family. The ability to generate various antigen binding combinations may also have implications in, for example, the generation of human autoantibodies such as anti-CD3 and anti-CD4 for immunosuppressive therapy. The autoantibodies may be absent *in vivo* but could be generated from various heavy–light chain combination *in vitro*. Finally, chain shuffling should permit a functional chain to be recombined with a repertoire of complementary chains; e.g., a metallo-light chain (10) could be recombined with a repertoire of heavy chains in the study of antibody catalysis.

METHODS

λ Phage Libraries and Screening. An initial Fab expression library from mice immunized with NPN-keyhole limpet hemocyanin conjugate was constructed as reported (6). The combinatorial library was screened by probing plaque lifts with ^{125}I-labeled antigen, 22 clones were plaque-purified for recombination studies, and the phagemids were excised for Fab expression and sequence determination. Libraries of heavy (γ1) and light (κ) chains were generated from NZB mice and from human bone marrow and peripheral blood lymphocytes (8) for random recombination experiments using the λ vectors.

Standard plaque-lift methods were used in screening. Cells (XL1 Blue) infected with phage were incubated on 150-mm plates for 4 h at 37°C, protein expression was induced by placing nitrocellulose filters soaked in 1 mM isopropyl β-D-thiogalactoside over the cells, and the plates were incubated at 25°C for 8 h. Duplicate filters were obtained during a second incubation under the same conditions. Filters were blocked in a solution of 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h before incubation at 25°C for 1 h with a solution of ^{125}I-labeled BSA-NPN in 1% BSA/PBS. After labeling, filters were washed repeatedly with PBS containing 0.05% Tween 20, before overnight development of autoradiographs.

ELISA. Overnight cultures (10 ml of Luria broth plus carbenicillin at 0.1 mg/ml) of XL1 Blue cells carrying the phagemid were induced to express the Fab by addition of isopropyl β-D-thiogalactoside (5 mM). The cells were maintained at 22°C for a further 24 h prior to pelleting the cells (7000 rpm in a JA-20 rotor for 15 min at 5°C), the supernatants were decanted, and protease inhibitors were added prior to storage (4°C). The bacterial supernates were applied to a microtitration plate coated with BSA-NPN in the presence of various concentrations of NPN hapten. The antibody bound to the microtitration plate was detected by addition of goat anti-mouse κ-chain antibody conjugated to alkaline phosphatase followed by color development. From the hapten inhibition studies apparent affinity constants (a*K*) were determined.

Microtitration plates were coated with BSA-NPN (1 μg/ml, 0.1 ml per well) and blocked with 1% BSA/PBS. Fab supernates (50 μl) were mixed with hapten (50 μl) in PBS/0.05% Tween 20/0.1% BSA and incubated in the wells (4°C, 18 h), the plates were washed with PBS/Tween, and goat anti-mouse κ-chain antibody conjugated to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL) was added to 0.1 ml per well, 1:1000 dilution in PBS/Tween and incubated (25°C, 2 h). The plates were washed as before, substrate was added [0.1 ml of *p*-nitrophenyl phosphate at 1 mg/ml in 0.1 M Tris (pH 9.4) containing 50 mM $MgCl_2$] and incubated (25°C for 30 min), and the absorbance was read at 405 nm. Apparent affinity constants (a*K* values) were determined as the reciprocal of the hapten concentration required to inhibit 50% of the maximal binding in a competitive ELISA. Thus an approximation of the affinity was made that

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: NPN, nitrophenyl phosphonamidate; V_H, variable region of heavy chain; V_L, variable region of light chain; CDR, complementarily-determining region; BSA, bovine serum albumin; a*K*, apparent affinity constant.

†To whom reprint requests should be addressed.

Table 1. a*K* values determined for the Fabs by hapten inhibition ELISA

Clone	I_{50}, nM	a*K*, M^{-1}
1a	380	2.6×10^6
1b	300	3.3×10^6
1d	550	1.8×10^6
1f	100	1.0×10^7
1g	400	2.5×10^6
2a	1000	1.0×10^6
2b	100	1.0×10^7
3a	150	6.7×10^6
4a	800	1.3×10^6
5b	700	1.4×10^6
8a	2000	5.0×10^5
8b	1000	1.0×10^6
9a	100	1.0×10^7
12b	1000	1.0×10^6
12c	3500	2.9×10^5
14a	500	2.6×10^6
14b	500	2.6×10^6
14c	200	5.0×10^6
14d	1000	1.0×10^6
15a	270	3.7×10^6

I_{50}, hapten required for 50% inhibition of maximal binding in competitive ELISA. a*K* = $1/I_{50}$.

permitted the ranking of the binding activities. The complete nucleotide sequence of the variable regions of the heavy and light chains (V_H and V_L, respectively) were determined using Sequenase (United States Biochemical).

Shuffling of Immunoglobulin Chains. The recombinations of heavy and light chains were performed in a manner similar to that used to construct the library. The DNA of the heavy chain was cleaved with *Hin*dIII, dephosphorylated, and then cleaved with *Eco*RI. This process destroyed the right arm, but the left arm containing the heavy-chain sequences remained intact. The DNA of the light chain was cleaved with *Mlu* I, dephosphorylated, and cleaved with *Eco*RI. This process cleaved the left arm of the vector into several pieces, but the right arm containing the light-chain sequences remained intact. The DNAs so prepared could be mixed and ligated. After ligation, only clones that resulted from combinations of a right arm of light-chain-containing clones and a left arm of heavy-chain-containing clones reconstituted a viable phage.

For random shuffling within a defined set of antigen-binding Fabs, *Eco*RI-cleaved DNA could be recombined without recourse to *Hin*dIII or *Mlu* I cleavage.

RESULTS AND DISCUSSION

In this study, we address several pertinent issues relating to the functional diversity and conceptual understanding of combinatorial libraries. The combinatorial approach to generating antibodies (6) is a powerful technique for rapidly generating monovalent Fabs from immunized animals. Moreover, the possibility to recombine a heavy or light chain with a library of light and heavy chains, respectively, opens the way to generate vast numbers of functional antibodies starting with a restricted set. The experimental system employed was based on a combinatorial Fab library in phage λ derived from a mouse immunized with the hapten NPN as described (6).

Initially, 22 NPN antigen binding clones were isolated and characterized. The a*K* determination (Table 1) illustrates that the combinatorial library approach leads to recovery of diverse array of functional molecules with a*K* values in the range 10^6–10^7 M^{-1}, as determined by inhibition ELISA. The a*K* values determined by this method probably underestimate the true binding constant but are useful in allowing the variation in affinity to be demonstrated. The nucleotide sequence of the V_H and V_L regions showed that at least 21 clones were distinct in that they showed more than four base differences in heavy and/or light chains from any other clone. However, a number were clonally related and probably arose by somatic mutation from the same initial B-cell clone. It is estimated that at least four heavy-chain and four light-chain families are utilized based on examination of nucleotide sequences. Typical sequences of complementarity-determining region 3 (CDR3) of heavy and light chains are shown in Table 2.

Three unrelated clones that bound to antigen were chosen and the heavy (Fab)- and light-chain genes from each clone were recombined with the entire immune NPN library of light (1.5×10^6 members) or heavy (1×10^6 members) chains as described in Table 3. The frequencies observed for regenerating antigen binding clones showed why the random com-

Table 2. DNA sequences of CDR3 from heavy- and light-chain-encoding clones

CDR3 chain	Group	Clone	Sequence
Heavy	I	2c	CGG GGA CTT CGG CCA CCT CAC TAC TTT GCC TAC
		8b	T–– ––– ––A ––– –A– ––– ––– ––– ––– CA– –––
		9a	––– ––– ––A ––– NA– ––– ––– ––– ––– ––– –––
		15a	––– ––– ––A ––– –A– ––– –G– ––– ––– ––– –––
	Ib	12c	TC– ––– ––A ––– –A– ––– ––– ––– TAC TTT GCC TAC
	II	4a	GGN ACT CCT ACG GCT ATG GAC TTC
		14d	––G ––– ––– ––– ––– ––– ––– –––
	IIb	2b	––– ––– AGG GCC TCT GCT ATG GAC TAC
	III	14a	CGA AGT GAT ACG GTT GTA GTG GGG GTT TGG TTA
Light	I	2c	CAG CAG TGG AGT AGT AAC CCA CCG TGG ACG
		8b	––– ––C ––– ––– –T– ––– ––– ––– ––N –––
		9a	––– ––– ––– ––– GA– ––– ––– ––– ––– –––
		12c	––– ––– ––– ––– ––– ––– ––– ––– ––– –––
		14a	––– ––C ––– ––– ––– ––– ––– ––– ––– –––
		15a	––– ––– ––– ––– GA– ––– ––– ––– ––– –––
	II	2b	CAA CAT CAT TAT GGT ACT CCG CTC ACG
		4a	––– ––– ––– ––– ––– ––– ––– ––T –––
		14d	––– ––– ––– ––– ––A ––– ––– ––– –––

DNA sequencing was performed by the dideoxynucleotide method using a Sequenase kit (United States Biochemical). Sequences were aligned according to Kabat (11) and CDR3 encoding regions were tabulated. Closely related sequences are grouped together. Dashes indicate identity with the first sequence in the group.

binatorial approach is successful: the occurrence of a suitable partner for a given chain is a relatively common event. Hence, the frequencies of productive heavy–light chain combinations are relatively high and sufficient to produce an overall frequency of antigen binding molecules of the order of 1 in 5000 (6–8) (expressed relative to the number of clones coexpressing heavy and light chains).

To determine whether the high frequencies of generating antigen binding clones might reflect an unexpectedly high promiscuity in heavy–light chain combinations, the 22 NPN antigen binding heavy and light chains were recombined with repertoires of 2×10^6 members of the complementary chains from a nonimmunized NZB mice. This strain, which is used as a model for autoimmune disease, is polyclonally activated but can be considered naive with respect to NPN antigen. No antigen binding molecules were isolated. The 22 NPN heavy chains were then recombined with a κ light chain library of 1×10^5 members prepared from human bone marrow. The 22 NPN light chains were recombined with a library of 5×10^5 members of heavy chains derived from human peripheral blood lymphocytes. Again, no antigen binding molecules were isolated. These results indicate that, for this hapten-BSA conjugate at least, the functional chains are highly discriminating in terms of partners and that the redesign of antibodies through recombination of a somatically mutated chain with a naive partner may be a difficult process.

The results also mean that the original library must be greatly enriched in functional chains (i.e., those originating from antigen-specific clones). This is what might be anticipated given that RNA was source material for the libraries. Indeed, frequencies for functional heavy chain of 1 in 50 have been observed in a mouse anti-hemagglutinin (7) and human anti-tetanus toxoid (M. A. A. Persson and D.R.B., unpublished data) systems. In fact, by its sensitivity to RNA levels, the random combinatorial approach is reporting on the response to antigen.

To determine whether unique combinations of heavy and light chains were required for antigen binding or whether promiscuity in heavy and light chains occurs, the phage λ DNA was prepared from 22 clones that exhibited binding to antigen. The λ DNA was cleaved with *Eco*RI and religated, thus effectively generating 484 (22 × 22) possible combinations of the initial heavy and light chains. The resulting mixture was screened to determine the frequency of generating antigen binding clones. This frequency was some 5-fold greater than anticipated if heavy–light chain combinations were unique; i.e., there was notable promiscuity as shown in Table 4.

Table 3. Frequency of generating a functional binding site if either the heavy or light chain from a binding clone is recombined with an entire immune library of light or heavy chains, respectively

Fab	Chain recombined	Frequency: NPN library V_L	Frequency: NPN library V_H	Frequency: Functional $V_L \times V_H$ site
1	V_H	1:165	—	1:22,605
	V_L	—	1:137	
2	V_H	1:210	—	1:107,100
	V_L	—	1:510	
3	V_H	1:118	—	1:67,260
	V_L	—	1:570	

Three random combinations of heavy and light chains that bound to NPN-BSA antigen were selected as described (6) and recombined with the entire NPN library of light and heavy chains, respectively. For recombination of a single heavy chain with a library of light chains, the λ phage DNA encoding the Fab was cleaved with *Hin*dIII, treated with calf intestinal phosphatase (CIP), followed by digestion with *Eco*RI. The light-chain library was cleaved with *Mlu* I, treated with CIP, and then digested with *Eco*RI. The two were ligated using T4 ligase and packaged using Gigapack Gold II (Stratagene). Experimental conditions were as in ref. 6. The recombination of a single light chain with a library of heavy chains was performed in a similar way. The light chain was digested with *Mlu* I, and the heavy chain library was digested with *Hin*dIII prior to CIP treatment, *Eco*RI digestion, and subsequent ligation. The resultant phage were screened as described in the text.

Examination of sequences of the heavy and light chains of antigen-binding clones suggests that this promiscuity can have different origins. For instance, Table 2 illustrates that heavy chains from a clonal family can pair with different light chains from a complementary family and vice versa. Examples of this are clones 2c, 8b, 9a, and 15a in group I and clones 4a and 14d in group II. These clones are almost certainly somatic variants arising from the same original B-cell clone as determined by nucleotide alignment of complete or partial sequences of both heavy and light chains. (The alternative explanation of mutations arising from the PCR is thought unlikely because of the frequency of mutations observed and their concentration in the CDR regions.) We term this promiscuity incestuous.

A different type of promiscuity is provided by clones 12c and 2b. The sequences of the heavy chains of these clones are similar to those of the groups I and II, respectively, of Table 2 but they have a different clonal origin as indicated by the occurrence of an extra codon in the CDR3 region. These clones then generate antigen binding by combination with light chains belonging to groups I and II, respectively.

A further type of promiscuity is provided by clone 14a. The heavy chain is unique among those studied but the light chain belongs to group I. We term these latter types of promiscuity as extraclonal.

In a sense, one should not be surprised at the phenomenon of incestuous promiscuity. Affinity maturation is a stepwise process and as such the likelihood of effective heterologous chain complementation with chains generated prior to and after somatic mutation would not be greatly diminished. The clonal promiscuity involving closely related sequences might also be anticipated. The promiscuity exemplified by clone 14a is interesting and worthy of a detailed structural analysis to understand its origin.

Finally, 20 nonbinding clones were isolated from the library generated by reshuffling of the 22 NPN antigen binding clones. The heavy and light chains of these combinations were reshuffled again and the frequency of generating antigen binders was determined (Table 4). The occurrence of antigen binding molecules confirms the ability to generate functional combinations from nonfunctional combinations by chain shuffling.

In summary, our results suggest that chain shuffling is an effective route to accessing combinatorial libraries, since once a single antigen binding clone has been identified the family can be readily expanded by shuffling a particular

Table 4. Shuffling of V_H and V_L sequences to generate functional fragments

V_H/V_L combinations shuffled	Total plaque-forming units, no.	Total antigen binding clones, no.	Frequency, %: Obs	Frequency, %: Pred*
NPN binding clones (22)	2900	773	27	5
Nonbinding clones derived from above (20)	2150	240	11	—

Shuffling of the heavy and light chains was achieved by *Eco*RI cleavage of the λ phage DNA encoding the 22 Fabs, followed by ligation, packaging, and screening as described (6).

*The numbers of clones that were subjected to shuffling is shown in parentheses. Obs, observed; Pred, predicted.

heavy and light chain against a library of light and heavy chains, respectively. Moreover, such shuffling would reach to "optimized" pairing of heavy and light chains for antigen recognition. Shuffling could equally well be applied to combinatorial libraries expressed on the surface of M13 phage (9, 12) and in phage λ as described here. Shuffled chains giving rise to functional combinations often reflects the clonal relationship of families. Such incestuous functionality could be used to map events of somatic mutation providing an insight into affinity maturation of both the heavy and light chains to a particular antigen.

We are most grateful to Richard A. Lerner for his continued support and encouragement; to Roger Caothien, Chi Wong, Kim Janda, and Diane Schloeder for assistance and the use of blood drawing services through Scripps Clinic and Research Foundation, Clinical Research Center (Grant M01 RR 00833). D.R.B. is a Jenner Fellow of the Lister Institute of Preventive Medicine. A.S.K. acknowledges the support of the Science and Engineering Research Council Protein Engineering Club.

1. Kranz, D. & Voss, E. D. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 5807–5811.
2. Manjula, B. N., Glaudesmans, C. P. J., Mushinski, E. B. & Potter, M. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 932–936.
3. Streefkerk, D. G., Vrana, M. & Glaudesmans, C. P. J. (1978) *J. Immunol.* **120**, 408–410.
4. Hamel, P. A., Klein, M. H. & Dorrington, K. J. (1988) *Mol. Immunol.* **23**, 503–510.
5. Hamel, P. A., Klein, M. H., Smith-Gill, S. J. & Dorrington, K. J. (1988) *Immunology* **139**, 3012–3020.
6. Huse, W. D., Sastry, L., Iverson, S. A., Kang, A. S., Alting-Mees, M., Burton, D. R., Benkovic, S. J. & Lerner, R. A. (1989) *Science* **246**, 659–662.
7. Caton, A. J. & Koprowski, H. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 6450–6454.
8. Persson, M. A. A., Caothien, R. & Burton, D. R. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 2432–2436.
9. Kang, A. S., Barbas, C. F., Janda, K. D., Benkovic, S. J. & Lerner, R. A. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 4363–4366.
10. Iverson, B. L., Iverson, S. A., Roberts, V. A., Getzoff, E. D., Tainer, J. A., Benkovic, S. J. & Lerner, R. A. (1990) *Science* **249**, 659–662.
11. Kabat, E. A., Wu, T. T., Reed-Miller, M., Perry, H. M. & Gottesman, K. S. (1987) *Sequences of Proteins of Immunological Interest* (U.S. Public Health Serv., Natl. Inst. Health, Bethesda, MD).
12. Barbas, C. F., Kang, A. S., Lerner, R. A. & Benkovic, S. J. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 7978–7982.