Combinatorial antibody libraries from survivors of the Turkish H5N1 avian influenza outbreak reveal virus neutralization strategies

Arun K. Kashyap*, John Steel†, Ahmet F. Oner‡, Michael A. Dillon*, Ryann E. Swale*, Katherine M. Wall*, Kimberly J. Perry*, Aleksandr Faynboym*, Mahmut Ilhan‡, Michael Horowitz*, Lawrence Horowitz*, Peter Palese†, Ramesh R. Bhatt*§, and Richard A. Lerner§¶

*Sea Lane Biotechnologies, 1455 Adams Drive, Menlo Park, CA 94025-1438; †Departments of Microbiology and Medicine, Mount Sinai School of Medicine One Gustave L. Levy Place, New York, NY 10029; ‡Yüzüncü Yil University, Van TR-65200, Turkey; and ¶Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037

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The widespread incidence of H5N1 influenza viruses in bird populations poses risks to human health. Although the virus has not yet adapted for facile transmission between humans, it can cause severe disease and often death. Here we report the generation of combinatorial antibody libraries from the bone marrow of five survivors of the recent H5N1 avian influenza outbreak in Turkey. To date, these libraries have yielded >300 unique antibodies against H5N1 viral antigens. Among these antibodies, we have identified several broadly reactive neutralizing antibodies that could be used for passive immunization against H5N1 virus or as guides for vaccine design. The large number of antibodies obtained from these survivors provide a detailed immunochemical analysis of individual human solutions to virus neutralization in the setting of an actual virulent influenza outbreak. Remarkably, three of these antibodies neutralized both H1 and H5 subtype influenza viruses.

Newly emergent, highly pathogenic influenza virus strains pose a profound threat to man. Three influenza pandemics have occurred within the past 100 years, each with devastating consequences (1). The recent emergence of the H5N1 virus sub-type, although mainly confined at present to avian hosts, has already demonstrated virulence in humans, causing the death of >200 people (2). Therefore, health care officials, researchers, and governments are actively considering their options should a pandemic occur.

One widely considered approach concerns the use of passive immunization either for the prevention of disease or for treatment after exposure to virus (3). The potential for passive immunization against influenza has been evident since the Spanish influenza outbreak nearly a century ago, where the benefits of transfused blood, sera, and blood products reduced the risk of mortality by >50% (3). Recently, the benefits of treatment with convalescent plasma in instances of H5N1 influenza have also been reported (4, 5). Additionally, passive immunization with human and mouse monoclonal antibodies has been reported to protect animals from death, even when administered after H5N1 infection (6).

The most logical source of human antibodies for passive therapy would be patients who have survived infection. With modern combinatorial antibody library technologies, it is now possible to capture the entire immunological history of an individual's response to an infection (7, 8). Because antibody libraries contain the complete record of an individual's response to pathogens, one can recover the repertoire specific to a given agent by using a laboratory process of selective enrichment. Such libraries give archival information about the nature of antibodies made during the infection and allow recovery of potentially therapeutic human monoclonal antibodies. Importantly, antibody recovery is independent of whether an active antibody response is still occurring at the time the sample is taken. Thus, depending on when the libraries are constructed, one may obtain antibodies that are currently being made and/or are part of the individual's immunological history. For infections that may be lethal, such analyses carried out on surviving patients may be particularly important because they chart some of the immunological mechanisms used during a successful host defense in the actual clinical setting of an outbreak.

Typically, when libraries are prepared from individuals who have been infected with a virus, hundreds to thousands of different antibodies are obtained, as opposed to only a few when other methods are used (8). A comparative sequence analysis of these antibodies allows a detailed map of the chemistry of antibody binding. Similarly, a comparison of neutralizing and nonneutralizing antibodies can give important information about the nature of binding interactions that are critical to neutralization.

Here we describe the creation of comprehensive avian influenza antibody libraries made from survivors of infection with an avian influenza virus during a confirmed outbreak. We have used these libraries to obtain large numbers of monoclonal antibodies to the H5N1 avian influenza virus, some of which have broad reactivity and are neutralizing across viral subtypes. Ultimately, combinatorial antibody libraries may hold the key to immunotherapy, such as passive immunization using one or more member antibodies, or they may guide the development of vaccines directed at the antigenic target(s) of the neutralizing antibodies in the library.

Results

The Outbreak and Source of Material. Between December 2005 and January 2006, an outbreak of avian influenza H5N1 occurred in Turkey (9). In total, 12 individuals were infected and only 8 survived. Because bone marrow RNA contains the archived record of all antibodies made by an individual, we selected it as our source material. We obtained bone marrow and serum from six of the Turkish survivors after their recovery and successfully prepared antibody libraries from five of the six bone marrow samples. In the sixth sample, the RNA was degraded.

Serological Analysis. The hemagglutinin (HA) protein is essential for binding the influenza virus to the cell that is being infected and is generally considered to be the main target of neutralizing antibodies (1). Therefore, we tested by ELISA each of the individual serum samples at high serum dilutions to detect antibodies against H5 HA proteins [see supporting information (SI) Fig. S1]

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§To whom correspondence may be addressed. E-mail: ramesh.bhatt@sealanebio.com or rlerner@scripps.edu.

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Table 1. Example sequences displaying the immunochemical basis of neutralization found from survivor 5 libraries after H5N1 Vietnam panning

Group 1 heavy chains	FR1 ‡ † ¶ § 1-29	CDR1 30-35	FR2 36-46	CDR2 47-56	FR3 59-92	CDR3 93-101	FR4 102-113
Vh1e	QVQLVQSGAEVKKPGSSVKVSCKASGGTF	SSYAIS	WVRQAPGQGLE	WMGGIIPIFGTAN	YAQKFQGRVTITADKSTSTAYMELSSLRSEDTAVYYC	ARGSYYYESSLD	YWGQGTLVTVSS
1	-----------------------------	------	-----------	------GM---T-	--------------EL---------------------	------------	---K--T-----
2	-----------------------------	------	-----------	---A--GM---T-	----------L---EL---------------------	------------	---K--M-----
3	-----------------------------	------	-----------	---A--GM---T-	--------------EL-------------D-------	------------	---K--M-----
4*	-----------------------------	----VT	-----------	---A--GM---T-	----------L---EL---------------------	------------	---K--M-----
5	-----------------------------	----VT	-----------	---A--GM---T-	--------------EL-------------D-------	------------	------------
6	-----------------------------	----VT	-----------	---A--GM---T-	----------L---EL---------------------	------------	---K--M-----
7†	-----------------------T-----	----VT	-----------	------GM---T-	--------------ELR--------------------	------------	------------
8*	-----------------------T-----	----VT	-----------	------GM---T-	--------------EL---------------------	------------	---R--------
9‡	-----------------------T-----	----VT	-----------	---A--GM---T-	--------------EL-------------D-------	------------	------------
10	-----------------------T-----	----VT	-----------	---A--GM---T-	--------------EL-------------D-------	------------	---R--M-----
11*	-----------------------T-----	----VT	-----------	---A--GM---T-	--------------EL-------------D-------	------------	------M-----
12§	-----------------------T-----	----VT	-----------	---A--GM---T-	----------L---EL---------------------	------------	------------
13*	-----------------------T-----	----VT	-----------	---A--GM---T-	----------L---EL---------------------	------------	------M-----
14	-----------------------T-----	----VT	-----------	---A--GM---T-	----------L---EL---------------------	------------	---K--M-----
15	-----------------------T-----	----VT	-----------	---A--GM---T-	----------L---EL---------------------	------------	------------
16*	-----------------------T-----	----VT	-----------	---A--GM---T-	----------L---EL---------------------	------------	------------
17	------------------R----T-----	----VT	-----------	------GM---T-	--------L-----EM---------------------	--------TT--	---R--------
18	-----------------------T---S-	----VT	-----------	------GM---T-	--------------EL---------------------	------------	------M-----
19	-----------------------TT----	----VT	-----------	------GM---T-	--------------EM---------------------	------------	---K--M-----
20¶	-----------------------TT----	----VT	-----------	------GM---T-	--------------EM---------------------	------------	------------
21	-----------------------TT----	----VT	-----------	------GM---T-	--------------EM---------------------	------------	---K--------
22	-----------------------TT----	----VT	-----------	------GM---T-	--------------EM---------------------	------------	------M-----
23	-----------------------------	----VT	-----------	---A--GM---T-	--------------EL-------------D-------	------------	------------
24	E----------------------------	------	-----------	---A--GM---T-	--------------EL-------------D-------	------------	------------
25	E-------T----------L---------	----VT	-----------	------GM---T-	--------L-----EM---------------------	--------TT--	---R--M-----
26	E-------T--------------V-----	----VT	-----------	---A--GM---T-	--------------EL-------------D-------	------------	---G--T-----
27	E----------Q-----------T-----	----VT	-----------	---A--GM---T-	----------L---EL---------------------	------------	------------
28	E--------------A-----------A-	----VT	-----------	------GM---T-	--------------EL---------------------	------------	------M-----
29‡	E-----------------R----T-----	----VT	-----------	------GM---T-	--------L-----EM---------------------	--------TT--	------------
30‡	E-----------------R----T-----	----VT	-----------	------GM---T-	--------L-----EM---------------------	--------TT--	---K--------
31	E-----------------R----T-----	----VT	-----------	------GM---T-	--------L-----EM---------------------	--------TT--	---R--M-----
32	E----------------------T-----	----VT	-----------	---A--GM---T-	----------L---EL---------------------	------------	---R--------
33‡	E----------------------T-----	----VT	-----------	---A--GM---T-	--------------EL-------------D-------	------------	---K--------
34†	E----------------------T-----	----VT	-----------	---A--GM---T-	--------------EL-------------D-------	------------	------------
35	E----------------------T-----	----VT	-----------	---A--GM---T-	--------------EL-------------D-------	------------	------M-----
36	E----------------------T-----	----VT	-----------	---A--GM---T-	--------------EL-------------D-------	------------	---K--------
37	E----------------------T-----	----VT	-----------	---A--GM---T-	--------------EL-------------D-------	------------	---K--T-----
38	E----------------------T-----	----VT	-----------	---A--GM---T-	--------------EL-------------D-------	------------	---K--M-----
39¶	E----------------------T-----	----VT	-----------	---A--GM---T-	----------L---EL---------------------	------------	------------
40¶	E----------------------T-----	----VT	-----------	---A--GM---T-	----------L---EL---------------------	------------	---K--------
41*	E----------------------T-----	----VT	-----------	---A--GM---T-	----------L---EL---------------------	------------	---K--M-----
42‡	E----------------------T-----	----VT	-----------	---A--GM---T-	----------L---EL---------------------	------------	---R--------
43	E----------------------T-----	----VT	-----------	---A--GM---T-	----------L---EL---------------------	------------	---R--------
44	E----------------------T-----	----VT	-----------	---A--GM---T-	----------L---EL---------------------	------------	---R--M-----
45	E----------------------T-----	----VT	-----------	------GM---T-	----------M---EM---------------------	------------	---K--------
46	E----------------------T-----	----VT	-----------	------GM---T-	--------------EL---------------------	------------	------------
47	E----------------------T-----	----VT	-----------	------GM---T-	--------------EL---------------------	------------	---K--------
48‡	E----------------------T-----	----VT	-----------	------GM---T-	--------------EM---------------------	------------	---R--------
49	E----------------------T-----	----VT	-----------	------GM---T-	--------------EM---------------------	------------	---R--M-----
50‡	E----------------------TT----	----VT	-----------	------GM---T-	--------------EM---------------------	------------	------------
51	E----------------------TT----	----VT	-----------	------GM---T-	--------------EM---------------------	------------	------M-----
52	G----------------------T-----	----VT	-----------	---A--GM---T-	----------L---EL---------------------	------------	---K--M-----
53	G----------------------T-----	----VT	-----------	---A--GM---T-	--------------EL-------------D-------	------------	---R--------
54	-M---------------------T-----	----VT	-----------	---A--GM---T-	--------------EL-------------D-------	------------	---K--M-----
55‡	-M---------------------T-----	----VT	-----------	---A--GM---T-	----------L---EL---------------------	------------	------------
56	----L------------------TT----	----VT	-----------	------GM---T-	--------------EM---------------------	------------	---K--------
57*	----Q------------------T-----	----VT	-----------	---A--GM---T-	----------L---EL---------------------	------------	------------
58	----Q------------------T-----	----VT	-----------	---A--GM---T-	--------------EL-------------D-------	------------	---K--M-----
59	----Q------------------T-----	----VT	-----------	------GM---T-	--------------EL---------------------	--------N---	---K--------
60*	----Q-------------R----T-----	----VT	-----------	------GM---T-	--------L-----EM---------------------	--------TT--	------M-----
61*	----Q-------------R----T-----	----VT	-----------	------GM---T-	--------L-----EM---------------------	--------TT--	---K--------

The 61 unique heavy chain sequences aligned with their germ-line variable regions from the 115 unique heavy and light chain combinations. Required mutations are highlighted in blue, and predominant mutations are highlighted in red. Heavy chain sequences also discovered in H1N1 New Caledonia panning are highlighted in gray. Antibody regions and Kabat numbering ranges are listed at the top of each sequence column.

*Paired with 2 unique light chains.

†Paired with 4 unique light chains.

‡Paired with 3 unique light chains.

§Paired with 12 unique light chains.

¶Paired with 5 unique light chains.

and intact viruses (data not shown). This analysis showed that the patients had readily detectable serum antibodies, even when the serum was diluted 10,000-fold. We selected the Vietnam/1203/04 HA as a target because it was readily available and is thought to be related to the influenza virus strain that caused the disease outbreak in Turkey.

Library Construction. Our primary objectives were to understand the nature of the immunological response to infection and to identify specific antibodies that might be used passively for the prevention of spread and/or for treatment of H5N1 influenza virus infections. We wished to recover every possible solution to H5N1 infections, with minimal or no bias. Because gene expression for the individual Ig families is not equal—making them prone to bias and overrepresentation—we decided against using the standard pooled approach to Ig recovery. Instead, we individually rescued 20 of the 23 distinctly amplifiable gene families during construction of the libraries. The remaining three gene families (V_H 2, 5, and 6) were recovered as a pool because they are infrequently used. We further normalized gene content by creating equimolar pools of each Ig family DNA for cloning into a phagemid display vector.

A unique DNA barcode was embedded into a nondisruptive portion of the phagemid vector to allow each clone to be tracked back to the original patient source (Fig. S2). This barcoding enables assignment of clones to individual patients, even when phage libraries from multiple survivors are screened simultaneously. As a

result of this tagging, every clone isolated from any library can be confidently attributed to the cognate survivor.

Using this vector with its coding system, we successfully cloned repertoires from the bone marrow of five of the six survivors in both single-chain (scFv) and Fab phagemid formats. Each collection from an individual survivor has a diversity $>1.0 \times 10^8$ members. Furthermore, we created additional barcoded libraries comprising mixed survivor light and heavy chains with a final diversity of 1.1×10^9. Collectively, the five donor-specific collections and the pooled libraries from all donors have a total diversity of 1.0×10^9 as an scFv collection and 4.2×10^9 as a Fab-displayed collection (Table S1).

Selecting Binding Antibodies. As indicated above, one interesting feature of these studies was that we initially selected antibodies for binding against a virus strain and antigen that were related to, and different from, the one that caused the infection. This was done because viral isolates from the patients were not available. The necessity of using a related strain to select antibodies could have proven fortuitous because it may have led to the isolation of more broadly neutralizing antibodies (see below).

The libraries were panned against inactivated virus containing the Vietnam/1203/04 virus HA and neuraminidase proteins and recombinant purified HA (10). Typically, after three to four rounds of phage panning, individual clones from enriched phage pools were analyzed by ELISA against H5N1 virus or purified HA, and the positive clones were sequenced to determine their heavy and light chain sequences and to read their survivor barcode (11). From these studies, we isolated specific H5 HA binding clones from all five of the individual libraries from survivors. We have thus far recovered >300 hundred different antiviral antibodies, of which 146 specifically bind the H5 HA protein.

General Features of the Selected Clones. Overall, the individual patients use different germ lines for both heavy and light chains, demonstrating that these individuals have found different solutions to the same potentially lethal immunological challenge. The major features of combinatorial antibody libraries that can be used both to give confidence as to the quality of the obtained repertoire and to provide information about the chemistry of antibody binding and/or neutralization are seen in these clones. The clones contain all of the hallmarks of the previously described repeated clones ("jackpot solution") to antigen binding that is found in the natural progression of affinity maturation, as well as in selected synthetic antibody libraries (8, 12). The presence of jackpots in these large collections validates the screening procedure because, unless the phage was selected on the basis of activity, the chance of obtaining the same clone multiple times is highly improbable. Moreover, when one analyzes the heavy chain differences within groups, it is observed that many of the amino acid substitutions were chemically and structurally conservative (Table 1). As with repeated clones, the appearance of multiple amino acid substitutions that are chemically reasonable is unlikely to be a random event.

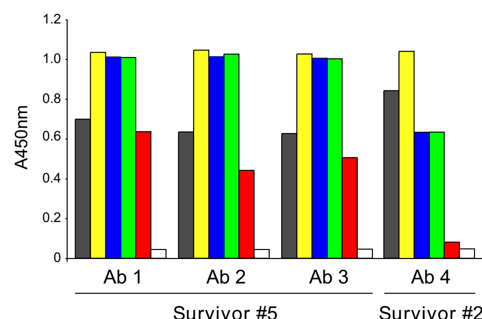

Fig. 1. H5N1 antibodies from two survivors cross-react with HAs from H1N1 viruses. Bars are H5N1 Vietnam/1203/04 (dark gray), H5N1 Turkey/65596/06 (yellow), H5N1 Indonesia/5/05 (blue), H1N1 New Caledonia/20/99 (green), H1N1 South Carolina/1/18 (red), and H3N2 Wisconsin/67/05 (white).

Binding Specificity of Recovered Antibodies. Initial testing of a set of Fabs by using biolayer interferometry binding to the H5 Vietnam HA protein indicated that we had identified at least four distinct epitopes (data not shown). We selected six clones, from three survivors, that recognized two different epitopes for conversion into full IgG1 proteins. The binding of three of these antibodies was mapped to the HA1 subunit of the HA protein by Western blot analysis (data not shown).

One goal of these studies was to recover those rare antibodies that broadly neutralize divergent viral strains. It was suggested that some of our antibodies might be broadly reactive because the serum from the donors had high-titer antibodies against a divergent subfamily of H5N1 viruses that extended beyond the virus with which they were infected. To determine the degree of cross-reactivity at the level of individual antibodies, we analyzed binding of our clones to various influenza HA antigens (Fig. 1 and Table 2). Not surprisingly, these antibodies recognize HA from the corresponding infecting Turkey/65596/06 strain and, in addition, recognize the heterologous HA from the Vietnam/1203/04 strain used for selection. Furthermore, they recognize the antigenically divergent Indonesian/5/05 H5 HA. We performed kinetic binding analyses on the four prototype antibodies and found that the antibodies from survivor 5 bound Vietnam/1203/04 HA with single-digit nanomolar affinities, whereas the survivor 2 antibody bound more strongly, with a measured affinity of 13 pM (data not shown).

To determine whether our antibodies are even more broadly reactive, we studied their binding to a larger collection of HAs from different influenza A subtypes (Fig. 1 and Table 2). We found that the four prototype antibodies bound HA from the closely related subtype H1N1 contemporary reference strain New Caledonia/20/99. Notably, the three neutralizing antibodies belonging to survivor 5 also bound HA from the H1N1 South Carolina/1/18 isolate that emerged during the 1918 Spanish flu pandemic. Conversely, none of these four antibodies bound HA from the contemporary H3N2 Wisconsin/67/05 reference strain, indicating that even though the antibodies display broad-spectrum binding among and between

Table 2. Relative ranking of antibodies by their ELISA signal over background on the various purified proteins

	Protein					
	H5			H1		H3
Antibody	Vietnam 1203/04	Turkey 65596/06	Indonesia 5/05	New Caledonia 20/99	South Carolina 1/18	Wisconsin 67/05
Ab 1	+++	++++	++++	++++	++	–
Ab 2	+++	++++	++++	++++	+++	–
Ab 3	+++	++++	++++	++++	++	–
Ab 4	+++	++++	++	++	–	–

+, above background and <2-fold; ++, between 2- and 9-fold; +++, between 9- and 15-fold; ++++, >15-fold above background; –, not measurably above background.

Table 3. MDCK cells were inoculated with 100 $TCID_{50}$ of virus in the presence of 2-fold serial dilutions of monoclonal antibodies

Antibody	Virus: H5 A/Vietnam/ 1203/04*	A/Vietnam/ 1203/04*	A/Indonesia/ 5/05	A/Turkey/ 65596/06	A/Egypt/ 14725/06	H1 A/New Caledonia/ 20/99	H3 A/Hong Kong/68
Ab 1†	11–21	2.3–9.3	9.3	9.3	1.2–2.3	9	>333
Ab 2†	63	54–217	27	108	7–13	54–108	>333
Ab 3†	58	18	16	31	4–8	8–16	>333
Ab 4†	1.7–6.3	0.5–2.2	>333	Not done	Not done	>333	>333
Mab #8‡	2.7	Not done	Not done	Not done	Not done	Not done	Not done

*Viral neutralization results for two independent experiments are shown.
†Minimum inhibitory concentrations required to neutralize virus in duplicate samples, in micrograms per milliliter.
‡A mouse monoclonal H5N1 neutralizing antibody raised against A/Vietnam/1203/04 (P.P., unpublished data).

influenza subtypes, the reactivity did not extend to all influenza subtypes.

To further explore the immunochemical basis of the H1/H5 cross-reactivity, we rescreened the libraries against the H1N1 New Caledonia/20/99 HA protein. From this selection, we found clones (Table S2) that bore significant similarity to the sequences obtained from survivor 5 when the H5 HA protein was used in the panning (Table 1).

Neutralization Studies. Initially, the antibodies were assayed for their ability to neutralize an H5 HA (Vietnam/1203/04) containing influenza virus. One antibody derived from survivor 2 and three from survivor 5 that recognized a common epitope (epitope "A") were all neutralizing, whereas the two antibodies derived from survivor 1 that recognized a second epitope (epitope "B") were not.

Based on the striking sequence similarity of clones separately isolated from survivor 5 against either H5N1 or H1N1 HA, we predicted that their cross-reactivity would extend beyond simple binding and they would also have the highly unusual property of neutralizing both H5N1 and H1N1 virus. To examine the cross-neutralizing activity of the IgGs, we tested representative antibodies from the H5N1 screen in a neutralization assay to see whether they would also neutralize H1N1 or H3N2 virus (Table 3). We studied the H1-bearing virus A/New Caledonia/20/99 and the H3-bearing virus A/Hong Kong/68. A collection of viruses bearing H5 subtype HA (A/Vietnam/1203/04; A/Indonesia/5/05; A/Turkey/65596/06; A/Egypt/06) was also tested. The antibodies showed no activity against H3 subtype influenza; however, three of the monoclonal antibodies that neutralized H5-containing viruses also strongly neutralized A/New Caledonia/20/99, which bears an H1 subtype HA (Table 3).

Immunochemical Basis of Neutralization. One advantage of antibody libraries is that when large numbers of antibodies are obtained, they can be grouped as to their relatedness. Thus, when a function for a given antibody in the collection is observed, one can predict that other members of the group to which the antibody belongs will have similar activity.

All members of the group that contained the neutralizing antibody collection against epitope A from survivor 5 analyzed to date are shown in Table 1. The group consists of 61 unique members that most closely resemble the V_H1e germ-line heavy chain. Some heavy chains are paired with more than one light chain. In total, these heavy chains have 115 unique pairings to both κ and λ light chains. In comparing these heavy chains to the highly related V_H1e germ line, we observe three types of point substitutions. Some changes appear to be required, others are dominant, and some residues have only been changed sporadically. The required changes occur in every clone in the group within CDR2 at positions 52A (Pro → Gly), 53 (Ile → Met), and 57 (Ala → Thr), as well as in the framework 3 region at positions 73 (Lys → Glu) and 74 (Ser → Leu or Met), all of which vary from the germ-line side chain chemistries, suggesting that these mutations are critical to antigen binding and neutralization. The second set of mutations are dominant and are found in most clones. The first mutation, in framework 1 at position 24 (Ala → Thr), represents a significant chemical change. The next three are conservative changes in CDR1 at positions 34 (Ile → Val) and 35 (Ser → Thr) and also in CDR2 at position 50 (Gly → Ala). All four of these dominant substitutions, however, are dispensable, suggesting that, although beneficial, they are not essential. The sporadic changes found throughout framework regions 1, 3, and 4, as well as CDR3, are all conservative and likely represent minor optimization events. The positions of the required mutations in the structure of the antibody are shown in Fig. 2 superimposed on the crystal structure of a highly related anti-HIV Fab called 47e (Protein Data Bank ID code 1RZI) (13). The required mutations 52A (Pro → Gly), 53 (Ile → Met), 73 (Lys → Glu), and 74 (Ser → Leu or Met) create a remarkably tight cluster on the exposed

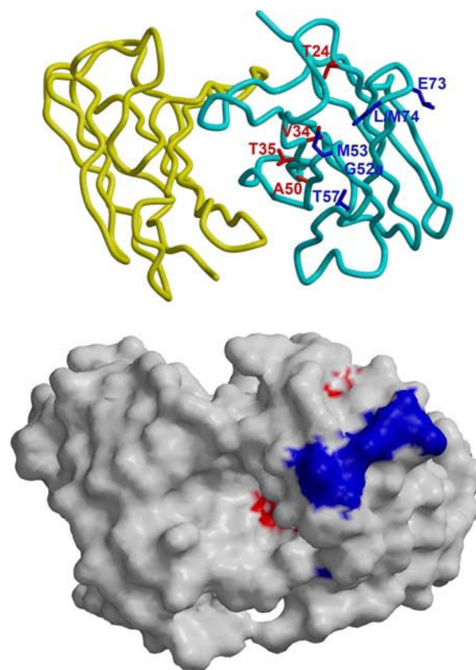

Fig. 2. The positions of H5 HA binding group 1 required and dominant mutations on the crystal structure of Fab 47e. Required (blue) and dominant (red) mutations from group 1 heavy chain sequences identified in H5 Vietnam/1203/2004 HA biopanning are superimposed on the crystal structure of the highly related anti-HIV Fab 47e. Mutations are shown in both backbone (*Upper*) and space-filling (*Lower*) models. A tight cluster is formed by four of the required mutations in, and adjacent to, CDR2.

BIOCHEMISTRY

Table 4. Example sequences displaying donor 2 neutralizing antibody and related clones

Group 2	FR1 1-29	CDR1 30-35	FR2 36-46	CDR2 47-58	FR3 59-92	CDR3 93-101	FR4 102-113
Heavy chain	QVQLQESGPGLVKPSETLSLTCTVSGYSF	DSGYYWG	WLRQPPGKGLE	WIGSIYHSRNTY	YNPSLKSRVTISVDTSKNQFSLQLSSVTAADTAVYYC	ARGTWYSSNLRYWFD	PWGKGTLVRVSS
	FR1 1-29	CDR1 30-35	FR2 36-46	CDR2 47-58	FR3 59-92	CDR3 93-101	FR4 102-113
Lambda light chains	FMLTQPHSVSESPGKTVTISCTGSGGN	IARNYVQWY	QQRPGSAPV	TVILEDDKRP	SGIPDRFSGSIDRSSNSASLTISGLRTEDEALYYC	QSYDDSDLV	VFGGGTKLT
	FMLTQPHSVSESPGKTVTISCTGSSGS	IASNYVQWY	QQRPGSAPT	TVIYEDYQRP	SGVPDRFSGSIDSSSNSASLTISGLKTEDEADYYC	QSYDDSDHL	IFGGGTKLTVL
	SVLTQPPSASGTPGQRVTISCSGSSSN	IGSNTVNWY	KQLPGTAPR	LLIYSNDQRP	SGVPDRFSGSKSGTSASLAISGLQSEDEANYYC	AAWDDSLSGW	VFGGGTKLTVL
	SVLTQPPSASGTPGQRVTLSCSGSSSN	IGGNSVNWY	QHVPGTAPK	LLMHSDDQRP	SGVPDRFSGSKSGTSXSLAISGLQSEDEADYYC	AXWDDSLNAW	VFGGXTKVTVL
	PELTQPHSVSESPGKTVTISCTGSGGR	IATNHVQWY	QQRPGSAPT	IVIYENNQRP	SGVPNRFSGSIDDSSNSASLTISALRTEDEADYYC	QSADATNV	FFGGGTKVTVL
	PELTQPPSASGTPGQRVTISCSGSSSN	IGSNTVNWY	QQLPGTAPK	LLIYSNNQRP	SGVPDRFSGSKSGTSASLAISGLQSEDEADYYC	AAWDDSLNGW	VFGGXTKLTVL
	FR1 1-29	CDR1 30-35	FR2 36-46	CDR2 47-58	FR3 59-92	CDR3 93-101	FR4 102-113
Kappa light chain	DIQMTQSPSSLSAFVGDRVTITCQASQDI	SNYLNWY	QQKPGKAPK	LLIYDATNLE	TGVPSRFSGSGSGTDFTFTISSLQPEDIATYYC	QQYDNLPL	TFGGGTKVDIKR

Antibody regions and Kabat numbering ranges are given at the top of each sequence column.

surface of the heavy chain variable domain, where they form a ridge that protrudes prominently from the protein surface (Fig. 2). The remaining required mutation, 57 (Ala → Thr), is partially buried at the base of the CDR2 loop. The surface-exposed changes in CDR 2 and framework 3 are likely to have a direct role in antigen binding, whereas the less exposed required mutation and the nonessential dominant mutations may have indirect effects through stabilizing and/or positioning of the CDR2 loop.

The probability that a given mutation is important to the activity of an antibody increases as a function of the number of times the mutation was independently selected. To determine whether the required mutations were selected during somatic mutation from independent clones or were from the progeny of a single clone that mutated further during subsequent replications, the codon usage of the dominant mutations was analyzed (Dataset S1). The data reveal that, although different codons were used, they resulted in the same amino acid changes, demonstrating that these mutations arose independently in different clones and thus were selected multiple times. This convergent outcome for independently selected events is strong evidence that these dominant mutations play a critical role in the binding to the virus and/or in its neutralization.

The antibodies from survivor 2 consist of two unique heavy chains that most closely resemble the V_H4–4b germ-line heavy chain (Table 4). The first heavy chain has been found paired with five unique λ light chains, four of which are from the infrequently used λ 6 light chain family, and the other is paired with a single κ light chain. Antibody 4, whose neutralization profile was more restricted, came from this group.

Conclusions

This article raises two central issues regarding the prevention and treatment of infections caused by the avian influenza neutralized virus. The first issue concerns the importance of antibodies relative to other components of the immune system. Although it has been known for more than 80 years that passive administration of immune sera can prevent infection (3), more recent studies with monoclonal antibodies (6, 13, 14) also offer encouragement. For example, Hanson *et al.* (6) showed that a monoclonal antibody to H5N1 virus was completely protective against lethal infection, even when administered 3 days after viral inoculation in mice (6). Given the possibility of a catastrophic epidemic, the way forward seems clear to many in the field. It has been suggested that governments should maintain stocks of neutralizing antibodies such as those reported here. The fact that our antibodies are fully human and have been isolated from individuals who successfully combated viral infection may offer advantages. However, even if such antibodies are stockpiled, hurdles remain. For instance, if the gene encoding the epitope to which the antibody binds were to mutate, the antibody might be less effective. Also, there is some evidence that cellular immunity enhances clearance of the virus. Nevertheless, if the only effect of passive immunization was to diminish the severity of infection—thereby giving the necessary time for other immune effectors to operate—it could be of critical importance for lessening mortality in patients with weakened immune, cardiovascular, and respiratory systems and in the elderly. Passive immunization might prevent the cytokine storm against rapidly proliferating virus, as occurred even in healthy young adults during the 1918 influenza outbreak.

The second important issue relates to the special advantages that antibodies from combinatorial libraries bring to the problem (8). The most general aspect is that, because such libraries are nucleic acid-based, they are not dependent on whether an important antibody is currently being produced. This obviates any concern about when in the course of the disease the sample was obtained. Indeed, as is the case here, when the source of antibody genes is the bone marrow, the entire immunologic history of an individual's antibody response may be obtained, irrespective of whether an antibody is actively expressed or is stored in the memory compartment. Thus, in the analysis of antibody ontogeny in the individuals studied here, the time factor is eliminated and one can obtain a clearer view of the precursor–product relationships between related antibodies. In this respect, one of the most remarkable features of some of our antibody collections (i.e., group 1) is that the required somatic mutations are confined to framework 3 or CDRH2 rather than CDRH3, where they would be expected to occur. This may be because the extreme virulence of the virus imposes time pressure on the evolution of the immune response. To survive an H5N1 avian influenza virus infection, one must mount an effective immune response rapidly. Because the framework regions and CDR2 of the protein are structurally rather constrained, the evolutionary search of sequence space for increased binding energy through somatic mutation may be more efficient for these regions than for a similar search through the more flexible and diverse CDR3 region. Indeed, it is well known—mostly from attempts to humanize antibodies—that framework mutations can directly or indirectly affect binding energy and/or specificity (15, 16). Alternatively, the immune system may use frameworks and/or CDRs that have been previously optimized, perhaps in response to an earlier exposure to a similar virus. Regardless of the exact mechanism, our results are in broad agreement with those of Zinkernagel and colleagues (17, 18), who studied the immune response against lethal vesicular stomatitis virus infections in mice. In their studies, only one V_H germ-line gene was used, and the primary neutralizing immune response was devoid of somatic mutations. Only later did somatic mutations appear in the CDRs. It should be emphasized that, although our analysis to date has revealed many interesting antibodies, so far only a small fraction of the library has been analyzed. As further analyses are carried out, we expect to see many other immunochemical solutions to the problem of virus infection.

From an antibody-engineering viewpoint, the large database unique to antibody libraries creates a roadmap for improving the binding energy and/or specificity of the antibodies, if necessary. For

example, one understands immediately that heavy chains (Tables 1 and 4 and Table S2) exist that are highly promiscuous with respect to their light chain partners. These heavy chains are ideal for light chain shuffling experiments in which very large numbers of new light chains are paired with a single promiscuous heavy chain (8, 19). Ultimately, the best features of different antibodies can be amalgamated into a single antibody that can be highly effective and even overcome viral escape by mutation. This is especially likely when consensus sequences important to neutralization occur in the different antibody chains, in different CDRs or frameworks within a chain, or both. Thus, many combinations can be tested, and an amalgamated antibody could contain the best elements of these various loops and frameworks. Critically, when some of the features incorporated into the amalgamated antibodies represent alternative binding modes to a neutralization target on the virus, one would expect viral escape to be more difficult.

Another feature that derives from the large numbers of antibodies obtained from libraries may be of particular importance to the influenza problem. Many, if not most, of the antibodies that result from an infection have little to do with prevention of further infectivity and are simply a response to the foreign nature of the virus. Thus, if one has only a few antibodies to choose from, one might miss the most important rare antibodies because they are underrepresented in the bulk immune response. Indeed, this may be a feature of the most potent antibodies because they need only be present in small concentrations and/or may occur late in an infection, only after many other "attempts" were made during the evolution of an immune response. We have seen this phenomenon in human libraries from cancer patients, where antibodies that prevent metastasis are present at the very rare frequency of ≈ 1 in 1.0×10^8 library members (20). The features that one might screen for that would be expected to be rare are, for example, antibodies that exhibit broad neutralization or have unusual access to important tissue compartments. Toward this end, it will be interesting to see whether any neutralizing antibodies in our collection of clones bind virus but are not directed to the HA.

Analysis of the immune response from actual cases can give guidance for both new passive antibody therapy and vaccine design. For example, we already know that patients make antibodies against the HAs that are broadly reactive between H5 and H1 strains and skip H3. We could not learn this from simple serology because serum contains a collection of activities, as seen here for our patients, and thus it is impossible to determine the clonal basis of any reactivity from an analysis of sera. Localization of the cross-reactive epitopes already found here, as well as others, is now relatively straightforward using antibodies from the library as a guide. Access to multiple antibodies from several survivors of the viral infection also enables the mapping of common epitopes, other than HA, to which all survivors have developed high-affinity antibodies. The identification of several previously unknown epitopes could provide the foundation for the design of novel vaccines.

Characterized neutralizing antibodies can also provide information regarding the potential efficacy of candidate vaccines. For instance, one can determine whether particular traditional or recombinant vaccine preparations generate antibody classes that have proven to be neutralizing on the basis of analysis of survivors of actual infections. Furthermore, these antibodies can be used as test reagents to ensure that epitopes that are important to neutralization are properly presented in the vaccine constructs. Although this latter point might seem trivial, there has heretofore been no simple way to learn whether critical epitopes are destroyed during construction of subunit vaccines or even during formulation of intact virus preparations.

Finally, we come to the often-asked and interesting question of whether it matters that the libraries were prepared from patients who successfully combated an infection, as opposed to animals or people that simply have been immunized with viral antigens. Because a substantial fraction of patients in our cohort died, it is tempting to speculate that the survivors made antibodies that were related to the patients' favorable clinical outcome. This is a difficult argument to address because so many factors contribute to patient survival, several of which have little to do with the robustness of the immune response. Natural antibodies obtained from survivors can reasonably be expected to be at least as good as, and perhaps better than, those obtained after simple immunization with inert antigens. At the very least, one can be certain that the virus has been presented in a manner that allows an immune response appropriate to survival of the individual. From this analysis we have gained insight into how the immunological repertoire searches sequence space when, because of the virulence of the infectious agent, time is short.

Materials and Methods

The recovery of bone marrow and the preparation of the combinatorial antibody libraries are detailed in *SI Materials and Methods*. Experimental procedures for serological analysis and virus neutralization studies are also provided.

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