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Production and Characterization of Human Monoclonal Antibody Fab Fragments to Vaccinia Virus from a Phage-Display Combinatorial Library

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A combinatorial, phage-display library of human Fab antibody fragments was generated from IgG heavy chain (HC) and light chain (LC) genes cloned from the lymphocytes of a vaccinia virus (VACV)-immune donor. To ascertain the complexity of the library, nucleotide sequences of the variable regions of the HC and LC genes were determined. Fourteen distinct HC and 18 distinct LC (7 κ and 11 λ) that formed a combinatorial library of 22 Fabs were identified. Immune-precipitation of radiolabeled VACV revealed that at least six different VACV proteins were recognized by the antibodies. Plaque-reduction neutralization demonstrated that six of the Fabs neutralized VACV in the presence of anti-human antibody. ELISA studies indicated that 15 of the Fabs were cross-reactive with monkeypox virus.

INTRODUCTION

Vaccinia virus (VACV) was used in the past as an effective vaccine against smallpox. Because smallpox was eradicated in natural settings as a result of a 10-year global vaccination campaign with live VACV (Fenner et al., 1988), the virus is now used primarily as a vector for expressing foreign genes and as a vaccine for laboratory workers. Concerns regarding the use of smallpox virus for biological warfare or terrorism (Breman and Henderson, 1998), the controversy over the proposed destruction of laboratory strains of smallpox virus (Roizman *et al.,* 1994; Donohoe, 1996), and the reemergence of monkeypox as a major human disease in Africa (Ivker, 1997; Mukinda *et al.,* 1997) have fueled renewed interest in VACV as a vaccine and as a diagnostic tool for other poxvirus infections.

Although VACV is a generally safe vaccine, disseminated, life-threatening infections occur infrequently, especially in individuals with impaired immunity (Keane *et al.,* 1983). Such infections can be treated by therapeutic administrations of human VACV immune globulin (VIG). VIG is clearly efficacious in treating disseminated VACV infections (Feery, 1976); however, the reason for its efficacy is not well defined. Neutralization assays are used to measure potency of VIG, but there are no convincing data to indicate that the neutralizing antibodies are the only, or even the primary, therapeutic components in VIG. VIG is in short supply, largely due to the small available immune-serum donor population and to the high cost of preparing and safety testing a product derived from human donors. Human monoclonal antibodies (MAb) offer an obvious alternative to VIG. If VACV-specific MAb mimic the therapeutic effects of VIG, they would serve as a stable source of well-characterized, high-quality immune globulin without the risks typically associated with human blood products.

VACV is structurally and antigenically complex, with a genome of ~192 kb encoding >100 polypeptides (Moss, 1990). Most of what is known about humoral immunity to VACV comes from studies with mouse MAb (Rodriguez *et al.,* 1985; Demkowitz *et al.,* 1992; Wolffe *et al.,* 1995). To date, human MAb to VACV have not been reported. Common techniques used for making human MAb, e.g., fusion of spleen cells with an immortal partner or transformation with Epstein–Barr virus (EBV), are labor intensive and often result in cells lines that are unstable or produce low levels of antibody (Glassy 1993). To circumvent these problems, molecular techniques have been developed to produce combinatorial libraries of human Fab fragments by bacterial expression of heavy chain (HC) and light chain (LC) antibody genes and display of the fragments on the surface of filamentous bacteriophage. The antibody genes can be recovered from immune or non-immune donors by RT–PCR using human antibody-specific primers. The amplified genes are cloned into phagemid vectors such as pComb3 (Barbas and Lerner, 1991). Within this vector, both the HC and LC genes follow a *lac Z* promoter and *pel B* leader sequence. The leader sequence dictates transport of the expression products to the bacterial periplasmic space. Because the HC gene is cloned in-frame with a portion of the M13 bacteriophage gene III, expression results in a

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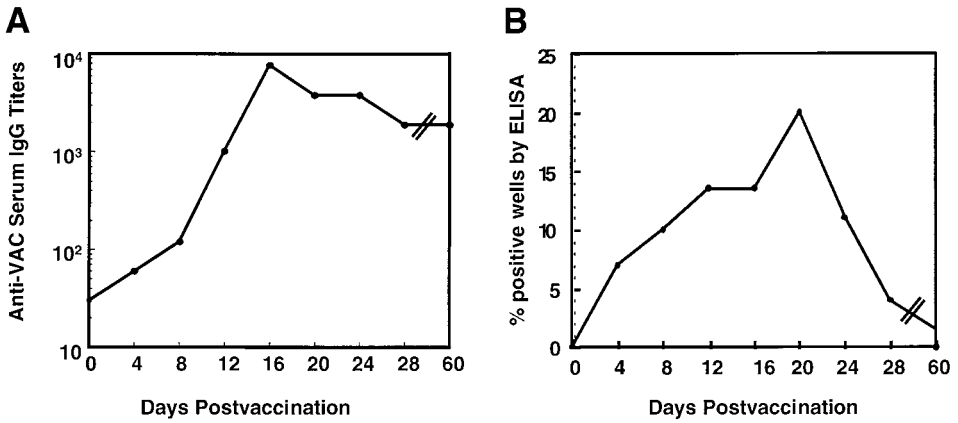

FIG. 1. Circulating VACV-specific antibodies and B cells. A VACV-immune volunteer was boosted with the licesnsed New York Board of Health VACV vaccine. Circulating anti-VACV IgG was measured by ELISA at various times after infection. B cells were transformed with Epstein–Barr virus and limiting dilutions were added to 96-well plates. The frequency of circulating B cells secreting antibodies to VACV was estimated by ELISA of wells containing cell clones.

fusion protein of the HC and the carboxyl-terminal portion of a minor coat protein. The gene III portion of the fusion protein tethers the HC to the inner membrane of the bacterial host. For phage-display, bacteria harboring the pComb3 constructs are infected with helper phage. The phage acquires the gene III product and consequently the Fabs when they pass through the inner membrane of the bacteria. After selection of phage that display appropriate Fabs, it is possible to remove the gene III region from the phagemid constructs; thus expressed HC and LC gene products are both secreted into the periplasmic space of the bacteria. Lysates of such bacteria will generate soluble Fabs (Barbas and Lerner, 1991).

Such phage-display methodology permits rapid construction of large combinatorial antibody libraries and has been used successfully to produce human MAb to a number of pathogens (e.g., Burton, 1991; Williamson *et al.,* 1993; Zebedee *et al.,* 1992; Hoogenboom *et al.,* 1998). The studies reported here are intended to lead toward a better understanding of the feasibility of developing human MAb that might be used to replace VIG, and secondarily, to generate defined human antibodies for use in poxvirus diagnosis.

RESULTS

Dynamics of VACV-specific B cells in peripheral blood after a secondary vaccination

To optimize our chances of obtaining a combinatorial library of VACV-specific Fabs, we first examined the kinetics of the humoral immune response to VACV. Sequential bleeds were obtained from a donor who had received a primary vaccination >10 years earlier, and a booster vaccination at the start of our study. An initial sample was taken just before vaccination, and seven additional samples were obtained at 3- to 4-day intervals thereafter. A final sample was obtained 60 days after the boost. Circulating VACV-specific IgG, as measured by ELISA, peaked 16 days after vaccination and remained high through Day 60 (Fig. 1A). To measure circulating B cells secreting antibodies to VACV, we transformed lymphocytes with Epstein–Barr virus (EBV) and performed ELISA to detect VACV-reactive clones. Approximately 5×10^7 peripheral blood lymphocytes (PBL) were recovered from each sample of heparinized blood. Of these, $\sim 10^7$ were transformed, and the remainder were frozen at $-70°C$ for subsequent library construction. The number of VACV-reactive cell clones increased during the 3 weeks after vaccination and peaked in samples obtained at Day 20 (Fig. 1B). After Day 24, the frequency of VACV-specific circulating B cells decreased rapidly. By Day 60, only one well had ELISA-positive cells. Based on these results, we used samples from Days 16 to 24 to construct the phage-display library. The neutralizing antibody titer for samples collected during this time frame was 1:80. Although we did not measure neutralizing antibody levels in other samples, results of a previous study indicated that development of neutralizing antibodies paralleled the antibody response detected by ELISA. In that study, sera from 21 volunteers, who were given booster vaccinations with the same vaccine that was used in our study, were analyzed by ELISA and by plaque-reduction neutralization tests (PRNT). In both assays, peak titers were observed 14 days after vaccination and remained nearly constant through Day 42 after vaccination (A. Schmaljohn, unpublished information).

Library construction and enrichment for phage-displaying VACV-specific Fabs

To construct the HC and LC cDNA libraries, we performed RT–PCR using total cytoplasmic RNA of VACV-infected cells (~49 μg) and primers specific for human

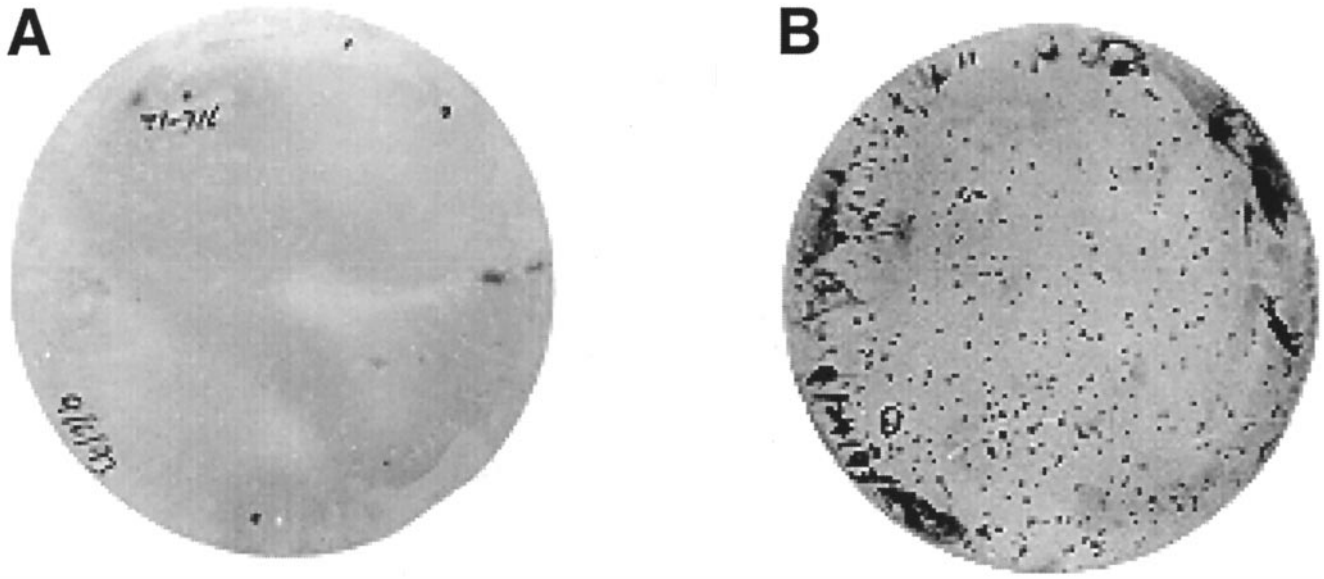

FIG. 2. Colony hybridization of bacteria infected with phage recovered after panning on VACV-coated microtiter wells. Bacteria infected with phage recovered after each of three rounds of panning were streaked on ampicillin-containing agar plates and colonies lifted onto nitrocellulose filters. The filters were incubated with partially purified VACV, fixed with formaldehyde, and incubated with VACV-specific MAb followed by alkaline phosphatase-labeled goat anti-human IgG (Fc-specific) and an enzymatic substrate. Few positive colonies were observed before panning (A), but after the third panning, >95% of the colonies were hybridization positive (B).

HC IgG1 (Fd region) or for κ and λ LC. The cDNA was cloned into the phagemid vector pComb3 (Barbas and Lerner, 1991).

To enrich for phage-displaying VACV-specific Fabs, bacterial supernatants were panned three times on VACV-coated microtiter plates. Because phagemids that have been packaged into virions are capable of infecting male *Escherichia coli* to form colonies on selective plates, we used a colony hybridization assay to estimate the proportion of VACV-specific phage (packaged phagemid) at each panning step. For this assay, ~300 colonies, grown in the presence of ampicillin, were lifted onto nitrocellulose filters, and the membrane-bound human Fab fragments were detected by sandwich hybridization. Few positive colonies were observed before panning, 32 positive colonies after the first panning, 156 positive colonies after the second panning, and 288 positive colonies after the third panning. Therefore after the third panning, >95% of the colonies were hybridization positive (Fig. 2).

Selection of clones

One hundred colonies were randomly selected from the hybridization-positive population for additional characterization. Periplasmic extracts of each clone were assayed by ELISA both for the presence of human-specific antibodies as well as VACV-specific antibodies. All 100 preparations contained human Fabs as indicated by ELISA results obtained with wells coated with goat anti-human IgG (Fab-specific). Control wells, coated with goat anti-mouse IgG (Fab-specific), were negative (data not shown). Of these, 98 periplasmic preparations were also positive when assayed by ELISA on plates coated with VACV-infected cell lysates (data not shown). Restriction enzyme analysis of phagemid DNA revealed that 11 of the clones had not retained complete HC and LC DNA. We were unable to maintain 21 other clones in culture; therefore a final combinatorial library of 66 clones were further characterized.

Complexity of the library

To identify unique Fabs, we determined the nucleotide sequences of the variable regions of the HC and LC and compared their deduced amino acid sequences. Fourteen HC variable regions were identified, each of which differed by at least one predicted amino acid from all of the others (Fig. 3A). HC5 and HC11 had differences only in framework region 4, which probably would not influence antigen specificity. HC85 had several framework differences, but only two CDR differences when compared to HC5. Similarly, HC7 and HC52 had only one conservative amino acid substitution (S *vs* T) in CDR2 but were otherwise identical (Fig. 3A). However, nine distinct lineages were apparent on a phylogenetic tree constructed from the HC variable region amino acid sequences (Fig. 4).

Eighteen unique LC, 7 κ, and 11 λ, were identified (Figs. 3B and 4). As with the HC, some of the LC had only minor differences in their CDR (Fig. 3B). Nine clearly unique lineages (4 κ and 5 λ) were apparent on a phylogenetic tree constructed from LC variable region

A

Clone	Fr1	CDR1	Fr2	CDR2	Fr3	CDR3	Fr4
27	-SGGGLVKPGGSLRLSCSASGFTFN-	-SYSMH	WVR--QAPGKGLEWVS	SISSSGGFIKDAESMRG	RFTISRDNAKNAVFLQMNSLRAEDTAVYYCAR	ESG----------SSRSGALDI	WGQGTLVTVSS
17	-SGGGVVQPGRSLRLSCAASGFTLS-	-LYPMH	WVRQGQAPGKGLEWVA	AISNDGNEKYYADSVKG	RFTISRDNSKRTMYLQMNSLRADDTAVYYCAR	DSTS-------ILWFGELSDGFDV	WGQGTMVTVSS
40	-SGGGLVQPGGSLRLSCAASGFTFR-	-SYAMN	WVR--QAPGKGLEWVS	SISGSGGNTYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTALYYCAK	G----------VEYLPRPKDAFDI	WGQGTMVTVSS
37	QSGGGLVKPGGSLRLSCAASGFTIS-	-DTHMS	WIR--QAPGKGLEWIS	QINSRGDTMYYADSVKG	RFTISRDNTKNSVYLQMNSLRVEDTADYYCAR	HG---------QLYDLWSRGYFDY	WGQGTLVTVSS
7	-SGPGLVKPSETLSLSCTVSGGSISS	SDYYWG	WIR--QPPGKGLEWIG	SIYYIG-STYYNPSLKT	RATISVDTSKNHFSLKLNSVTAADTAVYYCAR	LR---------LGVRRATGYFFDY	WGQGTPVTVSS
52	-SGPGLVKPSETLSLSCTVSGGSISS	SDYYWG	WIR--QPPGKGLEWIG	SIYYIG-STYYNPSLKS	RATISVDTSKNHFSLKLNSVTAADTAVYYCAR	LR---------LGVRRATGYFFDY	WGQGTPVTVSS
60	-SGPGLVKPSETLSLSCSVSGGSISS	SDYYWG	WIR--QPPGKGLEWIG	SIYYIG-STYYNPSLNS	RATISVDTSKNHFSLKLDSVTAADTAVYYCAR	LR---------LGVRRATGYYFDY	WGQGTPVTVSS
79	-SGPGLVKPSETLSLSCTVSGGSISS	SDYYWG	WIR--QPPGKGLEWIG	SIYYIG-STYYNSSLNS	RAILSVDTSKNHFSLKLNSVTAADTAVYYCAR	LR---------LGVRRASGYYFDY	WGQGTPVTVSS
1	-SGPGLLKPSETLSLTCAVSGDSISS	SDYYWG	WVR--QPPGKGLEWIG	AIYSSG-STYYKSSLKS	RATTSVDTSKNHFSLRLTSVSAADTAVYYCAR	LR---------LGVRRASGYYFDS	WGQGTLVTVSS
53	-SGPGLVKPSETLSLTCSVSGGSIKR	GPHYWA	WIR--RPPGEGLQGIG	SIYSSGNTYYYNPSLQS	RVTLSVDTSKNQFSLRLNSVTAADTAMYYCAR	G----------GDMAATGDYFDY	WGQGILVTVSS
5	QSGAEVKKPGASVKVSCKASGYTFT-	-TSDIS	WLR--QTTGQGPEWMG	WMNPQSGNSGLARRFRG	RVTLTRDNSISTAYMEISGLTFDDTAVYYCAA	HPG----------ECGGGSCYSAH	WGQGTLVTVSS
11	QSGAEVKKPGASVKVSCKASGYTFT-	-TSDIS	WLR--QTTGQGPEWMG	WMNPQSGNSGLARRFRG	RVTLTRDNSISTAYMEISGLTFDDTAVYYCAA	HPG----------ECGGGSCYSAH	WGQGTLVAVSA
85	QSGAEVKKPGASVKVSCKASGYTFT-	-TSDIS	WVR--QAPGQGPEWMG	WMNPQSGNSGLARRFRG	RVTLTRDTSISTAYMEISSLTFDDTAIYYCAA	HPG----------ECGGGSCYSAH	WGQGTLVTVSS
80	-SGAEVKKPGSSLKVSCEASGGTFD-	-NYAVS	WVR--QAPGQGLEWMG	RIIPLLGLTGYAHKFKG	RVTITADRSTNTVYMELTSLRSEDTAVYYCTR	DRVDIVTLNSPTLERNGYFFGMDV	WGQGTLVTVSS

B

Clone	Fr1	CDR1	Fr2	CDR2	Fr3	CDR3	Fr4
60	TQPP-SVSAAPGQKVTISC	SGRNSNIGNNF-----VS	WYQQLPGTAPKLLIY	DNNKRPS	GIPDRFSGS--KSGTSATLGIAELQTGDEADYYC	GTWDTGLS-AWVF	GGGTKLTVLG
77	TQPP-SVSAAPGQKVTISC	SGRNSNIGNNY-----VS	WYQQLPGTAPKLLIY	DNNKRPS	GIPDRFSGS--KSGTSATLGIAELQTGDEADYYC	GTWDTGLS-AWVF	GGGTKLTVLG
52	TQPP-SVSAAPGQKVTISC	SGRNSNIGNNY-----VS	WYQQVPGTAPKLLIY	DNNKRPS	GTPDRVSGS--KSGTSATLGIAGLQTGDEADYYC	GTWDSGLS-AWVF	GGGTKLTVLG
1	TQPP-SVSAAPGQKVTISC	SGSNSNIGNNY-----VS	WYQHLPGTAPKLLIY	DNNKRPS	GIPDRFSGS--KSGTSATLGIAGLQTGDAADYYC	GTWDTGLS-AWVF	GGGTKLTVLG
54	TQPP-SVSAAPGQKVTISC	SGSNSNIGNNF-----VS	WYQRLPGTAPKLLIY	DNNKRPS	GIPDRFSGS--KSGTSATLGITGLQTGDEADYYC	GTWDSGRS-VWVF	GGGTKLTVLG
17	TQPP-SVSAAPGQRVTISC	SGSSSNIGNHY-----VS	WYQQLPGTAPKLLIY	DNHKRPS	GIPDRFSGS--KSGTSATLGIAGLQTGDEADYYC	GTWDNNLS-AVVF	GGGTKLTVLG
53	TQPP-SVSGAPGQRVTISC	TGSSSNIGAGYD----VH	WYQQLPGTAPKLLIY	GNSNRPS	GVPDRFSGS--KSGTSASLAITGLQAEDEADYYC	QSYDSSLSGSVVF	GGGTKLTVLG
4	TQPP-SASGTPGQRVTISC	SGSSSNIGSNY-----VN	WYQQLPGTAPKLLIY	RNNQRPS	GVPDRFSGS--KSGTSASLAISGLRSEDEADYYC	APWDDSLSGSVVF	GGGTKLTVLG
21	TQPH-SVSESPGKTATISC	TRSGGDIANYY-----VQ	WYQQRPGSAPTTVIY	ETYLRPS	GIPDRFSGSIDRSSNSASLTISGLKTEDEADYYC	QSYNTSDQ---VF	GGRTKLTVLG
3	QDP--VVSVALGQTVRITC	QG--VSLRNYY-----AS	WYQQKPGQAPVLVIY	GKNNRPS	GIPDRFSGS--SSGNTASLTITGAQAEDEADYYC	NSRDSSGN-HVVF	GGGTKLTVLG
27	QDP--VVSVALGQTVRIKC	QG--DGLRSYY-----AS	WYQQKPGQAPELVIY	GKNNRPS	GIPDRFSGS--SSGTTASLTITGAQAEDEADYYC	NSRDSSGN-HLVF	GGGTKVTVLG
11	TQSPDSLAVSLGERATINC	KS-SQSVWYSSNNKNYLA	WYQQKPGQPPKLLIY	WASTRES	GVPDRFSGS--GSGTDFTLTISSLQAEDVAVYYC	QQYY---NT-QTF	GQGTKVEIKR
36	TQSPDSLAVSLGERATINC	KS-SQSVFYSSNNKNYLA	WYQQKPGQPPKLLIY	WASTRES	GVPDRFSGS--GSGTDFTLTISSLQAEDVAVYYC	QQYY---NT-QTF	GQGTKVEIKR
85	TQSPDSLAVSLGERATINC	KS-SQSVLYSSNNKNYLA	WYQQKPGQPPRLLIY	WASTRES	GVPDRFSGS--GSGTDFTLTISSLQAEDVAVYYC	QQYY---NT-QTF	GQGTKVEIKR
5	TQSPDSLAVSLGERATINC	KS-SQSVLYSSNNKNYLA	WYQQKPGQPPKLLIY	WASTRES	GVPDRFSGS--GSGTDFTLTISSLQAEDVAVYYC	QQYY---ST-QTF	GQGTKVEIKR
7	TQSPGTLSLSPGDGATLSC	RA-SQSVSSSY-----LA	WYQQKPGQAPRLLIY	GASNRAT	GIPDRFSGS--GSGTDFALTIDRLEPEDFALYYC	QLYGDSNRE-YTF	GQGTKLEIKR
40	TQSPGTLVSVSRERAALSC	RA-SQTVRSSY-----LA	WYQQKPGQPPRLLIS	GASTRAT	GIPDRFSGS--GSGTDFTLTISRLEPEDFAVYYC	QQYG-ASP---TF	GQGTKVEIKR
37	TQSPATLSVSPGERATLSC	RA-SQSVNNN-----LA	WYQQKPGQAPRLLIY	GASTRAT	GIPARFSGS--GSGTEFTLTISSLQSEDFAVYYC	QQYN-NWPP-WTF	GQGTKVEIKR

FIG. 3. Deduced amino acid sequences of the variable regions of unique HC (A) and LC (B). Nucleotide sequences were determined by automated sequence analysis using cycle sequencing and specific primers. Variable region framework (Fr) and complementarity determining regions (CDR) were assigned by comparison to previously published antibody sequences. Complete sequences available in GenBank under Accession Nos. AJ241344–AJ241375.

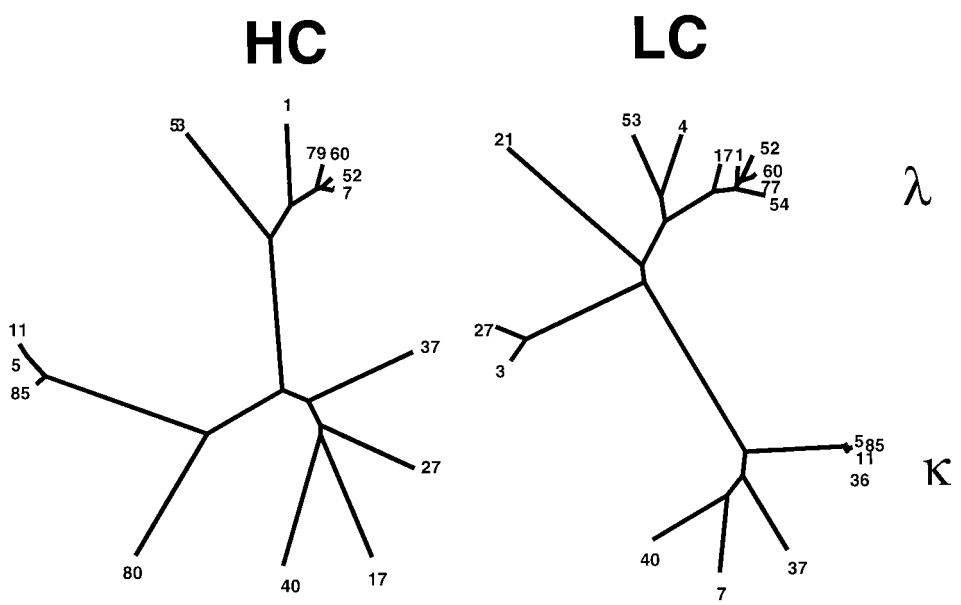

FIG. 4. Amino acid sequence relationships of HC and LC variable regions. Trees were derived by using Clustal W multiple sequence alignment program (http://www.clustalw.genome.ad.jp/). Trees were viewed by using TreeView program (http://taxonomy.zoology.gla.ac.uk/rod/rod.html).

amino acid sequences (Fig. 4). Combinations of these HC and LC produced 22 human Fabs (Table 1).

Immune precipitation of VACV proteins

Each of the unique Fabs was used to immune-precipitate radiolabeled, intracellular VACV polypeptides. Six distinct sizes of polypeptides were observed with apparent M_r of ~95-, 62-, 39-, 35-, 34-, and 14-kDa (Table 1, Fig. 5). We did not perform competition experiments with the Fabs, so it is possible that more than one VACV protein of the same apparent size was recognized by different Fabs.

TABLE 1

Characteristics of Human Fabs to VACV

						ELISA (OD405 1:2 diln)	
Fab	Genotype	No. of clones	IP[a] (kDa)	$PRNT_{50}$[b]	$PRNT_{50}$ + aFab	VACV[c]	MPXV[d]
1	5H/5L	3	95 k	−	−	1.006	1.041
2	11H/11L	1	95 k	−	−	1.138	1.037
3	85H/85L	13	95 k	−	−	0.922	0.924
4	60H/60L	9	95 k	−	+	0.327	0.516
5	85H/36L	1	95 k	−	−	0.947	0.703
6	52H/85L	1	95 k	−	−	0.265	0.587
7	7H/7L	1	95 k	−	−	0.244	0.526
8	60H/77L	1	None	−	−	0.204	0.656
9	7H/54L	1	None	−	−	0.213	0.414
10	52H/54L	2	35k	−	−	0.250	0.300
11	52H/52L	7	35 k	+	+	0.168	0.501
12	79H/52L	4	35 k	−	−	0.163	0.393
13	1H/1L	1	35 k	+	+	0.161	0.413
14	37H/37L	2	34 k	+	+	1.154	0.043
15	27H/27L	6	39 k	−	+	0.422	0.134
16	27H/3L	3	39 k	−	+	0.403	0.406
17	53H/53L	4	62 k	−	−	0.147	0.015
18	80H/7L	1	14 k	−	−	0.249	0.329
19	17H/21L	1	14 k	−	−	0.202	0.033
20	17H/4L	2	14 k	−	−	0.049	0.000
21	17H/17L	1	None	−	−	0.149	0.007
22	40H/40L	1	None	−	−	0.092	0.008

[a] IP, immune-precipitation; [b] PRNT, plaque-reduction neutralization test; [c] VACV, vaccina virus; [d] MPXV, monkeypox virus.

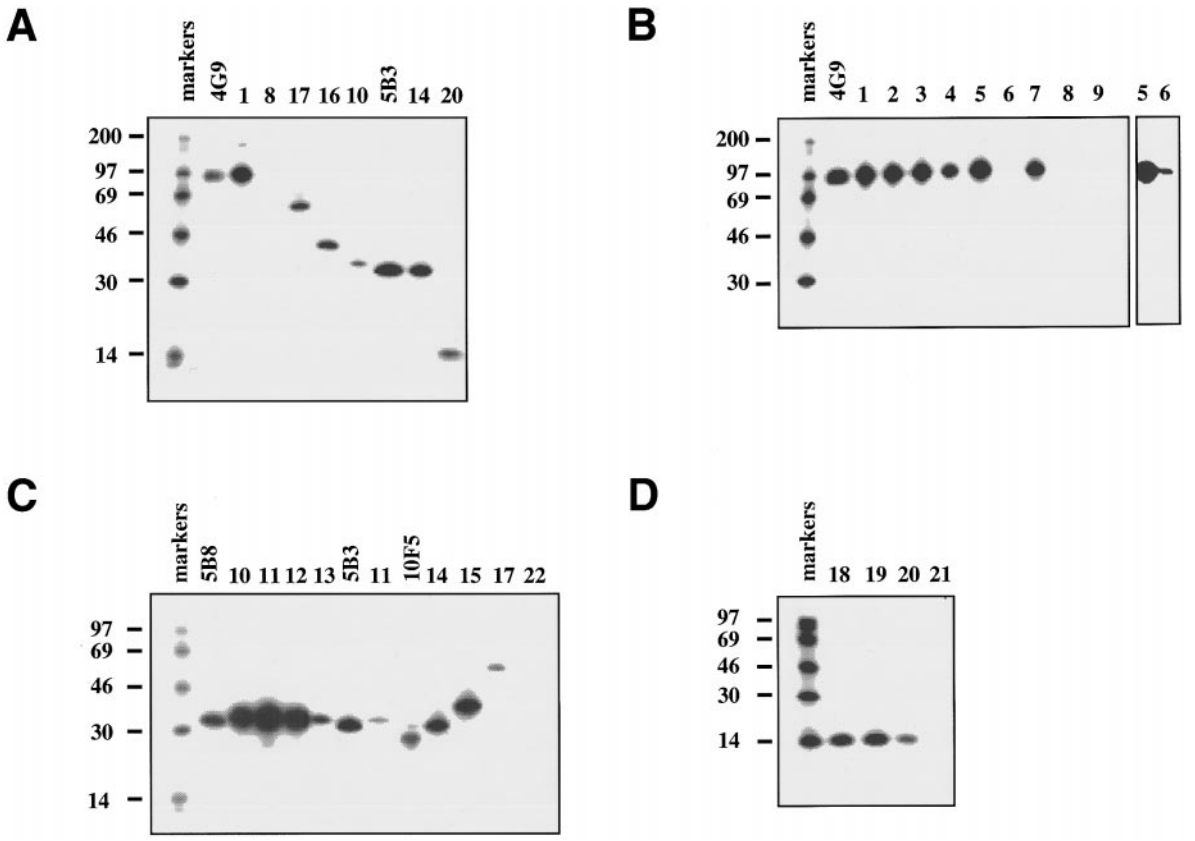

FIG. 5. Immune precipitation of radiolabeled VACV proteins. VACV proteins were radiolabeled with ^{35}[S]methionine and cysteine 1–12 h after infection of Vero E6 cells. Numbers above lanes correspond to those in Table 1. (A) Immune precipitation with Fabs representative of those that recognize each of six distinct VACV polypeptides are shown compared to immune precipitation with mouse MAb to the VACV hemagglutinin (MAb 4G9) and to the VACV D8L polypeptide (mouse MAb 5B3). (B) Immune precipitation with Fabs recognizing a 95-kDa polypeptide. The right panel shows a longer exposure of the film, which was required to visualize immune precipitation by Fab 6. (C) Immune precipitation by Fabs recognizing polypeptides of ~34-, 35-, 39-, and 62-kDa are shown compared to immune precipitation with mouse MAb recognizing the VACV D8L protein (MAb 5B8 and 5B3), or the VACV L1R protein (MAb 10F5). (D) Immune precipitation by Fabs recognizing 14-kDa polypeptide(s).

Within the library, Fabs reactive with the 95-kDa protein(s) were most common (29 Fabs, seven genotypes) (Table 1, Fig. 5). Of the remaining Fabs, the most commonly recognized proteins had apparent sizes of 35-kDa (14 Fabs, four genotypes), 39-kDa (nine Fabs, two genotypes), 14-kDa (four Fabs, three genotypes), 62-kDa (four Fabs, one genotype), and 34-kDa (two Fabs one genotype) (Table 1, Fig. 5). Four of the Fabs (8, 9, 21, and 22) did not immune precipitate any detectable VACV polypeptide.

Plaque-reduction neutralization tests (PRNT)

One goal of our study was to generate human neutralizing antibodies to VACV. To screen our library of Fabs for neutralizing activity, we performed PRNT with 1:10 dilutions of periplasmic bacterial extracts of each of the Fabs. Three of the Fabs, 11, 13, and 14, reduced the number of VACV plaques by >50% (Table 1). Because the Fabs were monovalent and neutralization might require divalent antibodies, and/or Fc regions, we repeated the PRNT with the addition of goat anti-human IgG, Fab-specific antibody. In this assay, three additional Fabs also neutralized VACV (Fab 4, 15, and 16, Table 1). Thus Fabs reactive with the 34-, 35-, 39-, and 95-kDa proteins were neutralizing.

Of the neutralizing Fabs, Fabs 4 and 14 (reactive with 95- and 34-kDa proteins, respectively), appeared to neutralize VACV most effectively. To more closely examine their neutralizing properties, we excised the gene III fusion protein portion of the plasmid constructs, tranformed *E. coli* with the modified plasmids, and affinity-purified the resultant soluble Fabs. We examined the purified Fabs by gel electrophoresis and Coomassie blue staining and estimated their quantities by comparing them to bovine serum albumin (BSA) standards (Fig. 6A). For a control, we prepared Fabs by papain digestion of a mouse MAb, 10F5, which is known to neutralize VACV and to recognize a 26-kDa protein encoded by the VACV L1R ORF (Fig. 5C and unpublished information). We also cloned and expressed the Fab regions of the HC and LC antibody genes of the 10F5 hybridoma. The expressed and papain-derived Fabs appeared as dou-

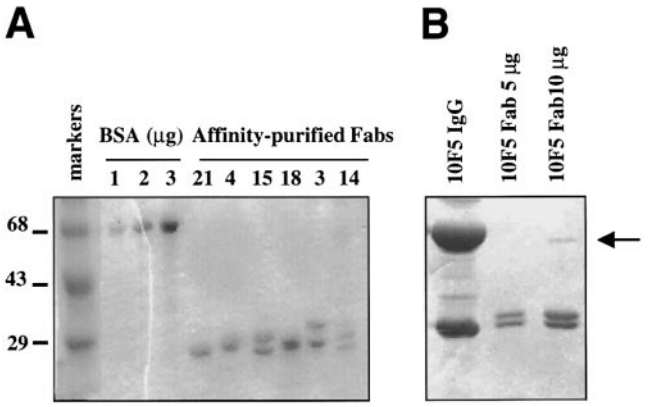

FIG. 6. SDS–polyacrylamide gel electrophoresis of affinity-purified Fabs or IgG. Numbers above lanes correspond to those in Table 1. Coomassie-blue-stained gels (A and B) showing affinity purified Fabs, prepared from periplasmic extracts of cells containing phagemids without the gene III region. Approximately 1 μg of recovered samples was compared to BSA standards. The mouse MAb 10F5, which recognizes the VACV L1 R protein, was purified by affinity chromatography (10F5 IgG) or was first digested with papain, and the Fab purified by affinity chromatography (10F5Fab). Arrow indicates the small amount of IgG that remained after papain digestion.

blets of ~23- to 30-kDa on Coomassie blue-stained gels (Figs. 6A and 6B). Not all of the Fabs had identical apparent M_r, most likely reflecting their different amino acid sequences. A small amount of the uncleaved IgG was seen in the papain-digested Fab preparation (arrow, Fig. 6B).

The complete IgG of the 10F5 mouse MAb neutralized VACV (>50%) at all 10-fold dilutions ≥0.075 μg/ml (Fig. 7A). In contrast, >50 μg/ml of the papain-digested IgG was required to neutralize VACV (Fig. 7A). A 100-fold decrease in concentration (0.5 μg/ml) was required for neutralization if the Fab was first incubated with anti-mouse IgG Fab-specific antibody (Fig. 7A). Similarly, expressed 10F5 Fab in crude periplasmic bacterial extracts did not neutralize VACV unless incubated in the presence of the anti-Fab antibody (Fig. 7A). Thus as we determined with the expressed human Fabs, a secondary antibody can increase the neutralizing activity of the papain-derived mouse Fab. For the human expressed Fabs 4 and 14, ~1 or 2 μg/ml, respectively, was required to achieve >50% neutralization (Fig. 7B).

Cross reactivity of the Fabs with monkeypox virus

To further characterize the library and to identify antibodies that might be useful for diagnosis of human disease, we evaluated the reactivity of the Fabs with another pathogenic orthopoxvirus, monkeypox virus (MPXV), by ELISA. Most of the Fabs that reacted well in ELISA with VACV also reacted well with MPXV. Fab 14, which had the best neutralizing activity for VACV, appeared to be specific for VACV (Table 1).

DISCUSSION

This study demonstrates the feasibility of using phage-display techniques to produce human antibodies to VACV. The 22 Fabs that we characterized reacted with at least six different VACV proteins. At least four of those proteins had neutralizing epitopes. Although it is likely that the Fabs recognized immunodominant epitopes on VACV, the nature of the VACV antigen used to pan the recombinant phage surely influenced which Fabs were obtained. Our panning antigen consisted of partially purified VACV recovered from infected cells; consequently, antibodies reactive with proteins present on the intracellular mature virus (IMV) rather than extracellular enveloped virus (EEV) were favored. Purification was not sufficiently extensive, however, to exclude VACV proteins not found in IMV. Therefore the library of Fabs that we generated represents an unknown subset of total antibodies to VACV produced by this donor. In addition, our Fab library may not represent the human immune response to VACV in general, because it was derived from a single donor. In future studies, we intend to select additional antibodies reactive with antigens known to elicit protective immunity to VACV (such as L1R and D8L gene products) by panning on lysates from cells transfected with genes expressing defined VACV genes. We also have obtained PBL samples from several additional vaccinated donors for use in expanding our Fab library.

Although we did not attempt to identify the VACV proteins recognized by the Fabs, we compared their sizes to those precipitated by mouse MAb of known specificity by gel electrophoresis. The largest polypeptide recognized by our library comigrated with VACV hemagglutinin (HA), which is recognized by mouse MAb, 4G9 (A. Schmaljohn, unpublished information) and is encoded in ORF A56R (Shida, 1986). Because HA is found in EEV and on membranes of infected cells, but not in IMV (Moss, 1990), it is possible that the protein recognized by the Fabs was not HA. Another VACV protein of this size is a major core protein, P4a, which has a predicted molecular mass of 102 kDa (Van Meir and Wittek, 1988) and an observed M_r of 95 kDa (Vanslyke *et al.,* 1991). However, this protein is cleaved to yield a 62-kDa mature core protein. (Demkowicz and Ennis, 1993), which was not apparent in our precipitation experiments. Conversely, one of our Fabs, Fab 17, did recognize a protein of ~62 kDa (but did not recognize a larger precursor).

Electrophoretic comparison of VACV proteins immune precipitated with our Fabs or with mouse MAb 5B3 and 5B8, which are specific for the D8L protein (A. Schmaljohn, unpublished information), demonstrated that Fab 14 recognized a protein of the same size (34 kDa) and that Fabs 10, 11, 12, and 13 recognized protein(s) that appeared slightly larger (~35 kDa). A similar size protein (36 kDa) was described previously to be immunodomi-

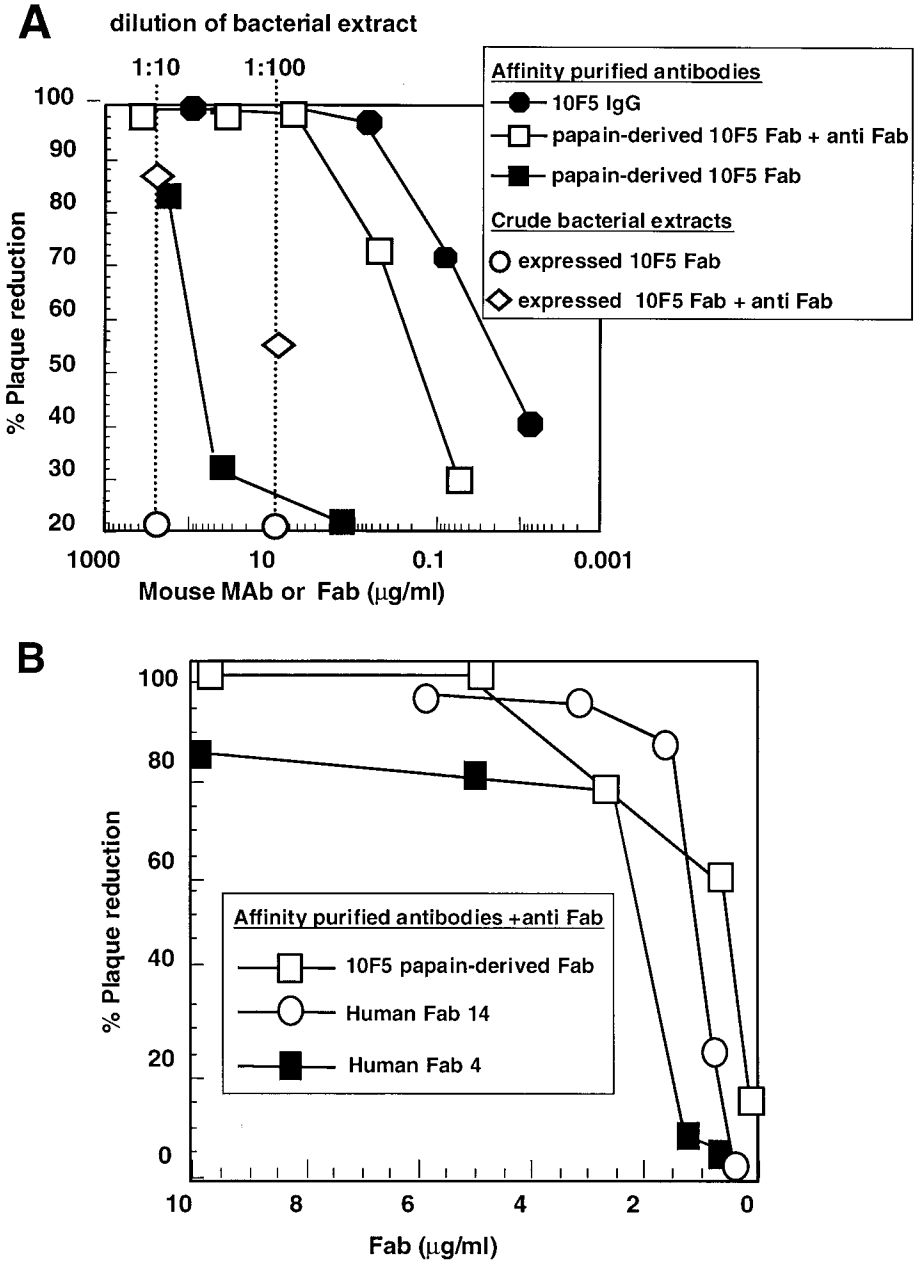

FIG. 7. Neutralization of VACV with affinity-purified antibodies or Fabs. (A) Neutralization by the mouse MAb 10F5 was compared to that of papain-derived Fab in the presence or absence of anti-Fab. Also compared was neutralization achieved with periplasmic extracts of *E. coli* containing phagemids expressing the 10F5 Fab. Dilutions of 1:10 and 1:100 of the periplasmic extracts, in the presence or absence of anti-Fab antibodies were assayed. (B) Comparison of VACV-neutralization by papain-derived Fab 10F5, Fab 14, and Fab 4 in the presence of anti-Fab antibodies.

nant in humans, but its specificity was not determined (Demkowicz *et al.,* 1992).

We did not have defined mouse MAb available to compare with our Fabs that recognized either the 39- or the 14-kDa polypeptide(s). One possible identity of the larger polypeptide is the VACV protein encoded in the A4L ORF and which was also reported to be immunodominant in humans (Maa and Esteban, 1987; Demkowicz *et al.,* 1992). Several VACV polypeptides of ~14 kDa have been described (Dallo *et al.,* 1987; Rodriguez and Esteban, 1987; Rodriguez *et al.,* 1987, 1997; Salmons *et al.,* 1997). One of these is an immunodominant polypeptide encoded in ORF A27L (Demkowicz *et al.,* 1992) reported to be involved in fusion of the virus and cell membrane (Rodriguez and Esteban, 1987).

Four of our Fabs did not immune precipitate any detectable VACV polypeptide. Of these, Fabs 8 and 9 had HC variable regions that were identical to those of Fab 4 and 7, respectively. Because both Fabs 4 and 7 reacted with the 95-kDa protein(s), Fabs 8 and 9 may also be specific for that protein. Likewise, because Fab 21 had the same HC as Fabs 19 and 20 (both of which react with

a 14-kDa protein), we suspect that Fab 21 is also specific for a 14-kDa protein. Clone 22 had unique HC and LC variable regions, thus we could not infer specificity for that antibody.

None of our Fabs was strongly neutralizing without added anti-Fab antibodies. Even then, only Fabs 4 and 14 neutralized VACV at dilutions >1:20. Whether or not the neutralizing activity of these antibodies will be improved once they are engineered as complete IgG, remains to be determined. If the re-engineered antibodies do display good neutralizing activity, then we will be interested in evaluating their protective and therapeutic properties in animal models.

In addition to possible use in therapy, human antibodies to VACV may have application in diagnosis of other pathogenic poxviruses. For example, although MPxV cases in humans were previously rare, an outbreak of >500 cases occurred recently in the Democratic Republic of Congo (CDC, 1998). We found that many of our Fabs reacted equally well with VACV and MPxV in ELISA. We intend to further study potential of these Fabs for orthopox virus diagnosis.

MATERIALS AND METHODS

Vaccination and lymphocyte preparation

A volunteer, who had been vaccinated with VACV >10 years previously, was boosted with the licensed New York Board of Health VACV vaccine (Connaught Laboratories). Fifty-milliliter blood samples were withdrawn at various intervals after vaccination and peripheral blood lymphocytes (PBL) were isolated by centrifugation, with Histopaque-1077 (Sigma).

EBV transformation of lymphocytes and assay for VACV-specific circulating B cells

Isolated PBL were infected with Epstein–Barr virus (EBV) and grown in 96-well U-bottom plates (Costar), at ~100,000 cells/well, in Cyclosporin A-containing medium (1 μg/ml in RPMI 1640/10% fetal bovine serum (FBS) (Melamed *et al.,* 1985). When vigorous growth of transformed cells was observed in all wells (12–15 days after infection), supernatants from each well were assayed for IgG-anti-VACV antibody by indirect ELISA. The proportion of IgG-anti-VACV-producing B cells in circulation was estimated by the number of ELISA-positive wells/the number of the wells with cell growth after EBV-transformation.

RNA preparation and library construction

Total cytoplasmic RNA was extracted from ~2×10^7 PBL. RNA was converted to first-strand cDNA by using a cDNA synthesis kit (Boehringer Mannheim) and Oligo (dT) primers as directed by the manufacturer. PCR amplification of the IgG1 (Fd region) genes or the κ and λ LC genes was performed by using combinations of 20 primers described in Table 2 of a previous study (Kang *et al.,* 1991). Approximately equal amounts of PCR-amplified κ and λ chain cDNAs were pooled and cut with the restriction enzymes *Xba*I and *Sac*I, then ligated to the *Xba*I/*Sac*I linearized phagemid vector, pComb3 (Barbas and Lerner, 1991; Barbas *et al.,* 1991). *E. coli* XL1-Blue cells were transformed with the DNA by electroporation. Phagemid DNA was prepared, cut with *Xho*I and *Spe*I, and ligated to *Xho*I/*Spe*I-digested, PCR-amplified IgG1(Fd) genes. To generate recombinant phage, *E. coli* transformed with the pComb3 construct was infected with helper phage (VCS-M13) and the culture was incubated at 37°C overnight. Phage was precipitated from the supernatant of the overnight culture, then resuspended in 2 ml of phosphate-buffered saline (PBS) and stored at −20°C as described previously (Barbas and Lerner, 1991; Barbas *et al.,* 1991).

Panning and colony hybridization assays

To enrich for phage-displaying VACV-specific Fabs on their surfaces, infected-cell supernatants were panned by methods similar to those reported earlier (Barbas and Lerner, 1991; Burton, 1991) but with VACV that was partially purified as follows. Infected Vero E6 cells were harvested by scraping with a rubber policeman and were centrifuged for 5 min at 500 *g.* The cells were resuspended in 1 mM Tris–HCl (pH 8.8) and disrupted with 20–25 strokes of a Dounce homogenizer. Nuclei were removed by centrifugation and the supernatant was layered onto a 35% sucrose cushion prepared in Tris–HCl, pH 8.8. After centrifugation in an SW27 rotor (Beckman) at 18,000 rpm for 80 min, the pellet was resuspended in 1 mM Tris–HCl and layered onto a 34-ml 25–40% sucrose gradient. The samples were centrifuged at 13,500 rpm in an SW27 rotor at 4°C for 40 min. The visible viral band was collected with a Pasteur pipet and diluted threefold in 1 mM Tris–HCl. The virus was concentrated by centrifuging at 13,000 rpm in an SW27 rotor for 40 min. The resultant pellets were resuspended in 1 mM Tris–HCl. For panning, the partially purified virus was measured by using a BCA assay kit (BioRad) and diluted to 50 μg/ml in carbonate-bicarbonate buffer, pH 9.6, and 100 μl was added to wells of 96-well microtiter plates.

Phage libraries (packaged phagemids) were assayed by colony hybridization after each of three successive pannings to estimate the proportion of specific clones. For the hybridization assays, bacterial clones from each round of panning were streaked on LB/ampicillin plates, and nitrocellulose filters were prepared and blocked as described elsewhere (Barbas and Lerner, 1991; Barbas *et al.,* 1991). The filters were incubated with partially purified VACV (20 μg/ml) in 1% BSA/PBS, fixed with 3.7% formaldehyde (w/v) in PBS (pH 7.2) for 30 min at room temperature, then washed and incubated with ~50

μg/ml of a VACV-specific MAb secreted from a mouse-human hybridoma (Cui, unpublished data). The filters were next incubated with alkaline phosphatase-labeled goat anti-human IgG (Fc-specific) (Sigma), which was extensively preadsorbed with *E. coli* XL1-Blue cell lysates and then diluted 1:1000 with blocking buffer (3% BSA and 3% powdered milk in PBS). After three washes in PBS/0.05% Tween 20, the filters were reacted with BCIP and NBT phosphatase substrates (Kirkegaard & Perry), which form colored precipitates (Harlow and Lane, 1988).

Preparation of Fab fragments

After three rounds of panning, the packaged phagemids were used to infect fresh *E. coli* XL1-Blue cells (OD_{600} = 1.0). The phage and cells were incubated for 15 min at room temperature and then plated on LB/ampicillin plates. One hundred colonies were randomly selected, and each clone was grown in 10 ml of super broth (30 g of tryptone, 20 g of yeast extract, and 10 g of MOPS per liter, pH 7.0) containing 50 μg/ml of ampicillin, 10 μμg/ml of tetracycline, and 20 mM $MgCl_2$. Fab synthesis was induced by adding isopropyl B-D-thiogalactopyranoside (IPTG) to 1 mM, and the soluble Fab fragments were prepared as bacterial crude extracts as described previously (Barbas and Lerner, 1991; Barbas *et al.,* 1991; Burton, 1991). These extracts were used for ELISA, immune precipitation, and PRNT or stored at −20°C.

For selected clones, the gene III portion of the plasmid was excised by digesting pComb3 contructs with *Spe*I and *Nhe*I, purifying the linearized plasmid by agarose gel electrophoresis, recovering the fragment by binding to glassmilk, and religating. The plasmids (without gene III) were then used to transform competent *E. coli* (dH5, Gibco-BRL) and 1-L cultures were prepared. The bacteria were lysed and Fabs purified from crude extracts by affinity-chromatography with goat anti-human IgG (Fab specific) (Sigma) immobilized on a Protein G matrix, prepared as described elsewhere (Harlow and Lane, 1988). Fab fragments were eluted in 0.2 M glycine: HCl (pH 2.5) and immediately brought to neutral pH with 1 M Tris–HCl (pH 9.0).

Preparation of papain-derived or expressed Fabs from a neutralizing, VACV-specific mouse MAb

Bacterially expressed recombinant Fab fragments were cloned from a mouse hybridoma, 10F5, which secretes a MAb that neutralizes VACV to high titer (A. Schmaljohn, unpublished information). Fabs were generated by the same methods as preparation of the human Fabs to VACV described above except that the primers for PCR-amplification of the antibody genes were specific for mouse IgG2a-κ (Kang *et al.,* 1991). 10F5 IgG molecules were purified by affinity chromatography with the MAb Trap G kit, (Pharmacia), and papain-digested Fab fragments of 10F5 IgG molecules were prepared with the ImmunoPure Fab Preparation Kit (Pierce), according to the manufacturer's directions.

Sequence analysis of HC and LC genes

The nucleotide sequences of the variable regions of the HC and LC antibody genes were determined by cycle sequencing and analysis on an Applied Biosystems automated sequencer. Sequence primers for HC (V_H) or LC (V_L) were: V_H 40–140 5′-GGG CGC CAG GGG GAA GAC-3′; V_H 40–159 5′-CGC TGG ATT GTT ATT ACT CG-3′; V_L 40–158 5′-CTA AAC TAG CTA GTC GCC AAG-3′; V_L 40–156 5′- TTG AAG CTC CTC AGA GGA G-3′; V_L 40–157 5′-CCA GAT TTC AAC TGC TCA TC-3′. An additional primer for κ LC sequencing was: Cκ 116–111 5′-GAA GAC AGA TGG TGC AGC-3′. Nucleotide sequences of both strands of every clone were determined. DNA and deduced amino acid sequences were analyzed by using DNA Strider and Clustal Software (http://www.clustalw.genome.ad.jp/). Trees were viewed by using TreeView Software (http://taxonomy.zoology.gla.ac.uk/rod/rod.html).

Enzyme-linked immunosorbent assay (ELISA)

Antigen preparation. The Connaught human vaccine strain of VACV, which originated from the New York City Board of Health strain, was previously plaque purified three times on monolayers of MRC-5 human diploid lung cells (ATCC CCL171) (Schmaljohn *et al.,* 1990). Vero cells were cultured in minimal essential medium (MEM) with Earle's salts, supplemented with 5% (cell propagation) or 2% (virus growth and assay) FBS and with gentamicin (40 μg/ml). VACV was grown in Vero cells and purified essentially as previously described (Schmaljohn *et al.,* 1990). In brief, cells in roller bottles were infected at a multiplicity of infection of <1. The cells were harvested 3–4 days after infection when cytopathic effects had decimated the monolayers. Cells were centrifuged and the pellet resuspended in 10 mM Tris–HCl (pH 8.0). Lysates were prepared by Dounce homogenization, and clarified by low-speed centrifugation.

For indirect ELISA, VACV-infected cell lysate, diluted in carbonate-bicarbonate buffer (pH 9.6), was added to 96-well polystrene plates (50 μl/well) (Costar), and plates were incubated at 4°C overnight. An antigen dilution of 1:100 was chosen empirically from prior titration. After 5% powdered milk (w/v in PBS) was added to block nonspecific binding, wells were rinsed and were ready to use. Uninfected Vero cell lysates at the same dilutions were used as antigen controls. The indirect ELISA was conducted as previously described with alkaline phosphatase-conjugated goat anti-human or anti-mouse IgG (Fab specific) (Sigma) as the secondary antibody (Harlow and Lane, 1988).

For sandwich ELISA, affinity-purified goat anti-human or anti-mouse IgG (Fab specific) (Sigma) was diluted to

10 μg/ml in carbonate-bicarbonate buffer (pH 9.6) and added to the wells of plates (50 μl/well), and incubated overnight at 4°C. The wells were washed three times with PBS/0.05% Tween 20 and then blocked by adding 100 μl of 5% powdered milk (w/v in PBS) and incubating overnight at 4°C. The plates were then incubated with 50 μl of human or mouse Fab-containing bacterial extracts at 4°C overnight, washed three times with PBS/0.05% Tween 20, and 50 μl of diluted alkaline phosphatase conjugated goat anti-human or anti-mouse antibody was added, and incubated for 1 h at 37°C. After five washes with PBS/0.05% Tween 20, 50 μl of *p*-nitrophenyl phosphatase substrate (Kirkegaard & Perry) was added and color development was monitored at 405 nm. Clones were scored as positive if OD_{405} readings were >0.3. Alkaline phosphatase-conjugated goat anti-human Fc or anti-mouse Fc was used as an antibody control.

Plaque-reduction neutralization test (PRNT)

To assess plaque formation, virus was sonicated, allowed to adsorb to subconfluent monolayers of Vero cells in six-well plates for 60 min at 37°C, and overlaid with MEM containing 2% FBS and 0.5% melted agarose. Three to 4 days after infection, viable cells were stained with Neutral red and clear plaques visualized. VACV was concentrated and partially purified by pelleting homogenized and sonicated VACV-infected cell lysates through 20% sucrose prepared in 10 mM Tris–HCl (pH 8.8) at 25,000 rpm for 2 h. VACV-containing pellets were resuspended in 10 mM Tris–HCl, sonicated, diluted to yield ~200 plaque-forming units (pfu) per 0.1 ml, and mixed with an equal volume of antibody solutions at selected dilutions. To assay neutralization by complete antibody molecules, the VACV-antibody mixtures were incubated at 4°C overnight before plating. For measuring neutralization by human or mouse Fab fragments, VACV was diluted to 250 pfu per 0.1 ml and mixed with 0.125 ml of the Fab solutions at selected dilutions. After the VACV-Fab mixtures were incubated for 60 min at 37°C, 25 μl of affinity-purified goat anti-human or anti-mouse IgG (Fab specific) (Sigma) was added to the VACV-Fab mixture to a final concentration of 20 μg/ml. An equal volume (25 μl) of PBS, pH 7.2 was used as a control. The VACV-Fab/anti-Fab mixtures were incubated at 4°C overnight then assayed as above.

Immune precipitation

VACV-infected Vero E6 cells, in 75 cm^2 flasks, were radiolabeled from 1 to 6 h or from 6 to 12 h p.i. with 1 mCi ^{35}S-Promix (Amersham) in 5 ml of methionine- and cysteine-deficient medium per flask. Cells were lysed with a nonionic detergent, Zwittergent 3–14 (Calbiochem), the lysates pooled, and proteins were precipitated with immune sera essentially as previously described (Schmaljohn *et al.,* 1987), except that 50% protein G-agarose beads coupled with the affinity-purified goat anti-human IgG-Fab (Sigma) were used to precipitate the VACV antigen-human Fab complexes. The Protein G/anti-human IgG-Fab beads were prepared as described previously (Harlow and Lane, 1988).

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REFERENCES

Barbas, C. F., Kang, A. S., Lerner, R. A., and Benkovic, S. J. (1991). Assembly of combinatorial antibody libraries on phage surfaces: The gene III site. *Proc. Natl. Acad. Sci. USA* **88,** 7978–7982.

Barbas, C. F., and Lerner, R. A. (1991). Combinatorial immunoglobulin libraries on the surface of phage (Phabs): Rapid selection of antigen-specific Fabs. *Methods: A Companion to Methods in Enzymology* **2,** 119–124.

Breman, J. G., and Henderson, D. A. (1998). Poxvirus dilemmas–monkeypox, smallpox, and biologic terrorism. *N. Engl. J. Med.* **339,** 556–559.

Burton, D. R. (1991). Human and mouse monoclonal antibodies by repertoire cloning. *Trends Biotechnol.* **9,** 169–175.

CDC. (1996). Human monkeypox—Kasai Oriental, Democratic Republic of Congo, February-October 1997. *JAMA* **279,** 189–190.

Dallo, S., Rodriguez, J. F., and Esteban, M. (1987). A 14K envelope protein of vaccinia virus with an important role in virus-host cell interactions is altered during virus persistence and determines the plaque size phenotype of the virus. *Virology* **159,** 423–432.

Demkowicz, W. E., Jr., and Ennis, F. A. (1993). Vaccinia virus-specific CD8+ cytotoxic T lymphocytes in humans. *J. Virol.* **67,** 1538–1544.

Demkowicz, W. E., Maa, J. S., and Esteban, M. (1992). Identification and characterization of vaccinia virus genes encoding proteins that are highly antigenic in animals and are immunodominant in vaccinated humans. *J. Virol.* **66,** 386–398.

Donohoe, M. T. (1996). A call for the destruction of smallpox virus stores [letter]. *Am. J. Public Health* **86,** 268.

Feery, B. J. (1976). The efficacy of vaccinia immune globulin. A 15-year study. *Vox Sang.* **31,** 68–76.

Fenner, F., Henderson, D., Arita, I., Jezek, Z., and Ladnyi, I. (1988). *Smallpox and Its Eradication.* World Health Organization, Geneva.

Glassy, M. C. (1993). Production methods for generating human monoclonal antibodies. *Hum. Antibodies Hybridomas* **4,** 154–165.

Harlow, E., and Lane, D. (1988). *Antibodies A Laboratory Manual.* Cold Spring Harbor Laboratory, New York.

Hoogenboom, H. R., de Bruine, A. P., Hufton, S. E., Hoet, R. M., Arends, J. W., and Roovers, R. C. (1998). Antibody phage display technology and its applications. *Immunotechnology* **4,** 1–20.

Ivker, R. (1997). Human monkeypox hits beleaguered Zaire [news]. *Lancet* **349,** 709.

Kang, A. S., Burton, D. R., and Lerner, R. A. (1991). Combinational immunoglobulin libraries in phage l. *Methods: A Companion to Methods in Enzymology* **2,** 111–118.

Keane, J. T., James, K., Blankenship, M. L., and Pearson, R. W. (1983). Progressive vaccinia associated with combined variable immunodeficiency. *Arch. Dermatol.* **119,** 404–408.

Maa, J. S., and Esteban, M. (1987). Structural and functional studies of a 39000-M_r immunodominant protein of vaccinia virus. *J. Virol.* **61,** 3910–3919.

Melamed, M. D., Gordon, J., Ley, S. J., Edgar, D., and Hughes-Jones,

N. C. (1985). Senescence of a human lympho-blastoid clone producing anti-rhesus (D). *Eur. J. Immunol.* **15,** 742–746.

MMWR (1984). Smallpox: Post-eradication policy–destruction of variola virus stocks. *Morb. Mortal. Wkly. Rep.* **33,** 24.

Moss, B. (1990). Poxviridae and their replication. *In* "Virology" (B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. Melnick, T. P. Monath, and B. Roizman, Eds.), 2nd ed., Vol. 2. pp. 2079–2111. Raven Press, New York.

Mukinda, V. B., Mwema, G., Kilundu, M., Heymann, D. L., Khan, A. S., and Esposito, J. J. (1997). Re-emergence of human monkeypox in Zaire in 1996. Monkeypox Epidemiologic Working Group [letter]. *Lancet* **349,** 1449–1450.

Rodriguez, J. F., and Esteban, M. (1987). Mapping and nucleotide sequence of the vaccinia virus gene that encodes a 14-kilodalton fusion protein. *J. Virol.* **61,** 3550–3554.

Rodriguez, J. F., Janeczko, R., and Esteban, M. (1985). Isolation and characterization of neutralizing monoclonal antibodies to vaccinia virus. *J. Virol.* **56,** 482–488.

Rodriguez, J. F., Paez, E., and Esteban, M. (1987). A 14000-M_r envelope protein of vaccinia virus is involved in cell fusion and forms covalently linked trimers. *J. Virol.* **61,** 395–404.

Rodriguez, J. R., Risco, C., Carrascosa, J. L., Esteban, M., and Rodriguez, D. (1997). Characterization of early stages in vaccinia virus membrane biogenesis: Implications of the 21-kilodalton protein and a newly identified 15- kilodalton envelope protein. *J. Virol.* **71,** 1821–1833.

Roizman, B., Joklik, W., Fields, B., and Moss, B. (1994). The destruction of smallpox virus stocks in national repositories: A grave mistake and a bad precedent [editorial]. *Infect. Agents Dis.* **3,** 215–217.

Roper, R. L., Payne, L. G., and Moss, B. (1996). Extracellular vaccinia virus envelope glycoprotein encoded by the A33R gene. *J. Virol.* **70,** 3753–3762.

Salmons, T., Kuhn, A., Wylie, F., Schleich, S., Rodriguez, J. R., Rodriguez, D., Esteban, M., Griffiths, G., and Locker, J. K. (1997). Vaccinia virus membrane proteins p8 and p16 are cotranslationally inserted into the rough endoplasmic reticulum and retained in the intermediate compartment. *J. Virol.* **71,** 7404–7420.

Schmaljohn, C., Chu, Y., Schmaljohn, A., and Dalrymple, J. (1990). Antigenic subunits of Hantaan virus expressed by baculovirus and vaccinia virus recombinant. *J. Virol.* **64,** 3162–3170.

Schmaljohn, C. S., Schmaljohn, A. L., and Dalrymple, J. M. (1987). Hantaan virus M RNA: Coding strategy, nucleotide sequence, and gene order. *Virology* **157,** 31–39.

Shida, H. (1986). Nucleotide sequence of the vaccinia virus hemagglutinin gene. *Virology* **150,** 451–462.

Van Meir, E., and Wittek, R. (1988). Fine structure of the vaccinia virus gene encoding the precursor of the major core protein 4 a. *Arch. Virol.* **102** 19–27.

Vanslyke, J. K., Whitehead, S. S., Wilson, E. M., and Hruby, D. E. (1991). The multistep proteolytic maturation pathway utilized by vaccinia virus P4a protein: A degenerate conserved cleavage motif within core proteins. *Virology* **183,** 467–478.

Williamson, R. A., Burioni, R., Sanna, P. P., Partridge, L. J., Barbas, C. F., III, and Burton, D. R. (1993). Human monoclonal antibodies against a plethora of viral pathogens from single combinatorial libraries. *Proc. Natl. Acad. Sci. USA* **90,** 4141–4145.

Wolffe, E. J., Vijaya, S., and Moss, B. (1995). A myristylated membrane protein encoded by the vaccinia virus L1R open reading frame is the target of potent neutralizing monoclonal antibodies. *Virology* **211,** 53–63.

Zebedee, S. L., Barbas, C. F., III, Hom, Y. L., Caothien, R. H., Graff, R., DeGraw, J., Pyati, J., LaPolla, R., Burton, D. R., Lerner, R. A., and Thornton, G. B. (1992). Human combinatorial antibody libraries to hepatitis B surface antigen. *Proc. Natl. Acad. Sci. USA* **89,** 3175–3179.