Elsevier has created a Monkeypox Information Center in response to the declared public health emergency of international concern, with free information in English on the monkeypox virus. The Monkeypox Information Center is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its monkeypox related research that is available on the Monkeypox Information Center - including this research content - immediately available in publicly funded repositories, with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the Monkeypox Information Center remains active.
Production and Characterization of Human Monoclonal Antibody Fab Fragments to Vaccinia Virus from a Phage-Display Combinatorial Library

Connie Schmaljohn,* Yunchang Cui,* Stephen Kerby,† David Pennock,* and Kristin Spik*

*Virology Division, United States Army Medical Research Institute of Infectious Diseases, and †Geo-Centers, Inc., Fort Detrick, Frederick, Maryland 21702-5011

Received January 26, 1999; returned to author for revision February 18, 1999; accepted March 10, 1999

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; Wolfe 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
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 $5 \times 10^7$ peripheral blood lymphocytes (PBL) were recovered from each sample of heparinized clones. Of these, $10^7$ were transformed, and the remainder were frozen at $-70^\circ C$ 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 $\mu g$) and primers specific for human
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 Fab 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
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.
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.

![Diagram](image)

**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).

**TABLE 1**

Characteristics of Human Fabs to VACV

| Fab | Genotype | No. of clones | IP*(kDa) | PRNT<sub>50</sub> | PRNT<sub>50</sub> + aFab | ELISA (OD405 1:2 diln) |
|-----|----------|---------------|---------|----------------|------------------------|------------------------|
| 1   | 5H/5L    | 3             | 95 k    | -              | -                      | 1.006                  |
| 2   | 11H/11L  | 1             | 95 k    | -              | -                      | 1.138                  |
| 3   | 85H/85L  | 13            | 95 k    | -              | -                      | 0.922                  |
| 4   | 60H/60L  | 9             | 95 k    | +              | -                      | 0.327                  |
| 5   | 85H/36L  | 1             | 95 k    | -              | -                      | 0.947                  |
| 6   | 52H/85L  | 1             | 95 k    | -              | -                      | 0.265                  |
| 7   | 7H/7L    | 1             | 95 k    | -              | -                      | 0.244                  |
| 8   | 60H/77L  | 1             | None    | -              | -                      | 0.204                  |
| 9   | 7H/54L   | 1             | None    | -              | -                      | 0.213                  |
| 10  | 52H/54L  | 2             | 35 k    | -              | -                      | 0.250                  |
| 11  | 52H/52L  | 7             | 35 k    | +              | +                      | 0.168                  |
| 12  | 79H/52L  | 4             | 35 k    | -              | -                      | 0.163                  |
| 13  | 1H/1L    | 1             | 35 k    | +              | +                      | 0.161                  |
| 14  | 37H/37L  | 2             | 34 k    | +              | +                      | 1.154                  |
| 15  | 27H/27L  | 6             | 39 k    | -              | +                      | 0.422                  |
| 16  | 27H/38L  | 3             | 39 k    | -              | +                      | 0.403                  |
| 17  | 53H/53L  | 4             | 62 k    | -              | -                      | 0.147                  |
| 18  | 80H/7L   | 1             | 14 k    | -              | -                      | 0.249                  |
| 19  | 17H/21L  | 1             | 14 k    | -              | -                      | 0.202                  |
| 20  | 17H/14L  | 2             | 14 k    | -              | -                      | 0.049                  |
| 21  | 17H/17L  | 1             | None    | -              | -                      | 0.149                  |
| 22  | 40H/40L  | 1             | None    | -              | -                      | 0.092                  |

*IP, immune-precipitation; **PRNT, plaque-reduction neutralization test; VACV, vaccina virus; MPXV, monkeypox virus.
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, transformed *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. 5.** Immune precipitation of radiolabeled VACV proteins. VACV proteins were radiolabeled with 35S-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).
In contrast, at all 10-fold dilutions 50% neutralization was achieved with VACV L1 R protein, was purified by affinity chromatography (10F5 Fab). Arrow indicates the small amount of IgG that remained after papain digestion.

**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.

bles of ~23- to 30-kDa on Coomassie blue-stained gels (Figs. 6A and 6B). Not all of the Fabs had identical apparent Mₚ, 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 M₀ of 95 kDa (Vanslyke et al., 1991). However, this protein is cleaved to yield a 62-kDa mature core protein. (Demko-wicz 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-
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 (Dalio 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

![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.]

A

Dilution of bacterial extract

Affinity purified antibodies

- 10F5 IgG
- papain-derived 10F5 Fab + anti Fab
- papain-derived 10F5 Fab

Crude bacterial extracts

- expressed 10F5 Fab
- expressed 10F5 Fab + anti Fab

B

Mouse MAb or Fab (µg/ml)

% Plaque reduction

1000 10 1.0 0.1 0.01

1:10 1:100

100 80 60 40 20 0

10 8 6 4 2 0

% Plaque reduction

Affinity purified antibodies + anti Fab

- 10F5 papain-derived Fab
- Human Fab 14
- Human Fab 4

Fab (µg/ml)
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 × 10⁷ 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 XbaI/SacI 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 XhoI/SpeI-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 (OD600 = 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 µg/ml of tetracycline, and 20 mM MgCl₂. 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 constructs with SpeI and NheI, 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).

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 polystyrene 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 OD405 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 4°C overnight before plating. For measuring 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, the VACV solutions at selected dilutions. After the VACV-antibody mixtures were incubated at 4°C overnight then diluted to 250 pfu per 0.1 ml and mixed with 0.125 ml of 5% powdered milk (w/v in PBS) and then blocked by adding 100 μl of 5% powdered milk (w/v in PBS) and incubating overnight at 4°C. 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 4°C overnight before plating. For measuring neutralization by complete antibody molecules, the VACV-antibody mixtures were incubated at 4°C overnight before plating. For measuring neutralization by complete antibody molecules, the VACV-antibody mixtures were incubated at 4°C overnight before plating. For measuring neutralization by complete antibody molecules, the VACV-antibody mixtures were incubated at 4°C overnight before plating.

Immune precipitation

VACV-infected Vero E6 cells, in 75 cm² flasks, were radiolabeled from 1 to 6 h or from 6 to 12 h p.i. with 1 mCi 35S-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).

ACKNOWLEDGMENTS

We thank Dr. Alan Schmaljohn for the gifts of his monoclonal antibodies and for his advice and criticisms of this study. We thank Dr. Erik Henchal, Chief of the Diagnostic Systems Division, USAMRIID, for contributing time and effort of his staff for nucleotide sequencing studies. Dr. Y. C. Cui was supported by a Research Associateship Award from the National Research Council.

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, 1538–1644.
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. Immunootechnology 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). Combinatorial immunoglobulin libraries in phage I. 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, 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. *MMWR* **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., Janeckzko, 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-Mr 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**, 461–462.

Van Meir, E., and Wittek, R. (1988). Fine structure of the vaccinia virus envelope glycoprotein encoded by the precursor of the major core protein 4a. *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. I., 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., II, 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.