Superantigen Properties of a Human Sialoprotein Involved in Gut-associated Immunity

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Abstract

Protein Fv (pFv) is a recently described 175-kD gut-associated sialoprotein with a potent capacity for augmentation of antibody-dependent immune functions. To investigate the molecular basis for Fab-mediated binding of pFv, we evaluated a panel of 52 monoclonal IgM and found that ~ 40% bound pFv. Whereas the majority ($\geq$ 75%) of V_H3 and V_H6 IgM strongly bound pFv, only a small minority (< 20%) of IgM from other V_H families bound pFv, and these antibodies had weaker binding interactions. Inhibition studies suggested that all binding occurred at the same (or overlapping) site(s) on pFv. Surface plasmon resonance studies demonstrated binding affinity constants up to $6.7 \times 10^8 \, M^{-1}$ for pFv. Biopanning of IgM and IgG Fab phage-display libraries with pFv preferentially selected for V_H3 and V_H6 antibodies, but also obtained certain V_H4 IgM. V_H sequence analyses of 36 pFv-binding antibodies revealed that binding did not correlate with CDR sequence, J_H, or L chain usage. However, there was preferential selection of pFv binders with V_H CDR3 of small size. These studies demonstrate that a protein which enhances immune defense in the gut has structural and functional properties similar to known superantigens. (*J. Clin. Invest.* 1995. 96:417–426.) Key words: repertoire • antibody • B cell • variable region • immunity

Introduction

Protein Fv (pFv)[1] is a 175-kD dimeric sialoprotein, produced in the human liver, which has unique properties that may aid in immune defense in the gut. In vitro studies have demonstrated that binding of pFv by antibodies enhances complement activation (1), and by interacting with the Fab domain of the surface membrane–associated IgE, pFv can also aid in the release of histamine and sulfidopeptide leukotriene C4 by human basophils and mast cells (2). In addition, in vitro studies indicate that the addition of pFv augments agglutination of pathogens

by antivirus and by anti–*Salmonella typhi* antibodies, which may improve conveyance in the mucus stream and excretion of pathogens from the gut (3).

pFv derives these diverse functional properties from its unique Fab binding activity. A single molecule of pFv can bind six F(ab')₂ fragments (2). The pFv binding capacities of different antibodies vary greatly, but IgM, IgA, IgG, and IgE can display pFv binding activity (4, 5). pFv binding capacity has also been detected in the Ig from a variety of mammalian, avian, reptilian, amphibian, and fish species (4). Studies using isolated Ig fragments have clearly demonstrated that binding to pFv is mediated by the V_H region (5). As this interaction does not block binding of either IgG or IgM antibodies to their conventional ligands, pFv presumably interacts with Fab outside of the conventional antigen-binding pocket (3, 5).

To investigate the molecular basis for antibody binding of pFv, we applied approaches and insights originally developed for studies of Fab-mediated binding of staphylococcal protein A (SpA) (6–10). We sought to evaluate the spectrum of V_H regions used in pFv binding and determine whether binding of pFv could preferentially select for antibodies encoded by certain V_H gene segments. The pFv binding capacity of a panel of human monoclonal IgM was first evaluated, and we investigated binding within large antibody repertoires of a healthy adult obtained from combinatorial Fab phage-display libraries. These studies revealed that the affinity of pFv binding is comparable to that of a conventional antigen binding interaction, but the frequency of binders and the structural correlates for the interaction are akin to those of a superantigen.

Methods

Immunoblot analysis of IgM binding to pFv. The isolation of pFv has previously been described (11), and the activity and purity of the pFv preparations from donors TER and FAR have been extensively documented (2–5, 11). To identify the IgM proteins that bind pFv, 10×-concentrated pFv samples or control proteins were dissolved in reducing (8 M urea and 2-mercaptoethanol) buffer and electrophoretically separated on 10% polyacrylamide gels, as previously described (10, 12). Replicate gels were electrotransferred to Immobilon P membrane (Millipore Corp., Bedford, MA), air dried, rewetted, and blocked with 1% nonfat dried milk, 0.1% gelatin in borate-buffered saline, pH 8.2 (BBS) for 1 h. Immunoblots were incubated with IgM preparations at 1 µg/ml in blocking solution for 1 h at room temperature with rocking, followed by extensive washing with 0.05% Tween 20–BBS and incubation with horseradish peroxidase–conjugated goat anti–human IgM (Novabiochem, La Jolla, CA) diluted in rabbit IgG at 10 µg/ml for 1 h at room temperature with rocking. Blots were washed and developed with a chemiluminescent detection reagent (Amersham Corp., Arlington Heights, IL), followed by exposure of autoradiograms.

ELISA studies. The current pFv binding assay was used because it enables the discrimination of strong from weak pFv binders and can be used for biopanning of phage-display antibody libraries. To evaluate for pFv binding, microtiter wells (Costar Corp., Cambridge, MA) were precoated with polylysine at 50 µg/ml in water, sealed, and incubated

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1. *Abbreviations used in this paper:* BBS, borate-buffered saline; FR, framework region; H and L, heavy and light chains; K_a, association constant; pFv, protein Fv; SpA, staphylococcal protein A.

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overnight at 4°C. Plates were emptied and rinsed once with distilled water and then incubated with pFv from donor TER (2–5, 11), or a control antigen, orosomucoid (Novabiochem) at 1 μg/ml in water with 0.25% glutaraldehyde for 1 h at 37°C. Wells were emptied and blocked by completely filling with 100 mM glycine, 0.1% BSA in PBS, pH 7.2, for 30 min at room temperature. Plates were further blocked with 3% BSA in 0.1 M NaHCO$_3$, pH 8.6, for 30 min at 37°C. Other control antigens, transferrin, actin, BSA, or tetanus toxoid was precoated on wells at 5 μg/ml in PBS overnight at 4°C and then blocked with 1% BSA in PBS for 1 h at room temperature. IgM proteins, Fab, or a polyclonal IgM standard at 1 μg/ml in 1% BSA in 0.1 M NaHCO$_3$, pH 8.6, was then added in replicate, and the plates were sealed and incubated for 90 min at 37°C. Plates were washed five times with 0.05% Tween 20–BBS and incubated with horseradish peroxidase–labeled anti–human IgG Fab (Pierce, Rockford, IL) diluted 1:3,000 in PBS for 1 h at room temperature. Plates were washed five times and developed with substrate. The human monoclonal IgM include antibodies with rheumatoid factor, anti–I/i cold agglutinin, anti–pneumococcal polysaccharide type 3, anti–DNA, and anti–Sm specificity (sources reported in references 6, 8, 13, and 14). Fab-producing bacterial clones were tested for pFv binding activity only if the plasmids contained the appropriately sized V gene inserts and produced detectable light (L) chains. Designation as an pFv binder required an absorbance greater than twice the mean of negative controls (or greater than five times SD above negative controls) and > 30% inhibition of pFv binding activity by soluble pFv in competition ELISA.

The evaluation of the antibody binding capacity of Fab from the phagemid libraries used previously described methods (9, 15–17). The bacterial supernatants were used directly or stored at −20°C. By comparison with human IgG Fab (Novabiochem) standards, the concentration of the Fab in each extract was measured as previously described except plates were precoated with affinity-purified goat anti–human IgG Fab (Sigma Chemical Co., St. Louis, MO) at 5 μg/ml in PBS, pH 7.2, overnight at 4°C and then blocked with 1% BSA in PBS for 1 h at room temperature before addition of the samples in replicate.

To compare binding avidities and determine whether antibodies recognized a common site on pFv, competition ELISA was performed using purified adult polyclonal IgM (Novabiochem) that was labeled with N-hydroxyl-succimide-biotin (Novabiochem). ELISA wells were precoated with pFv, as previously described, and replicate wells were incubated with either polyclonal IgM–biotin at 500 ng/ml alone or polyclonal IgM–biotin at 500 ng/ml with different concentrations of unlabeled antibodies or IgG Fc for 1 h at 37°C. By comparison with a series of dilutions of polyclonal IgM–biotin in the absence of inhibitor, the relative inhibition was determined (e.g., values equivalent to that for polyclonal IgM–biotin at 250 ng/ml represented 50% inhibition).

Surface plasmon resonance determinations of pFv binding affinity. Binding kinetics were measured using the surface plasmon resonance technique on the BIAcore instrument (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) (18, 19). This enables real-time kinetics analysis of antibody–antigen interactions in a label-free mode. Immobilization of the pFv and the binding of all subsequent molecules were measured using a direct optical sensing technique based on total internal reflectance. Adapting the ELISA precoating procedure, activation solution (Pharmacia LKB Biotechnology Inc.) was used to immobilize 0.1% poly-L-lysine (Sigma Chemical Co.) on the 100-nm-thick layer of dextran coating the gold film of the biosensor chip (18), resulting in a signal increase of 3,400 RU. Next, a 10×-concentrated pFv (donor TER) solution with 0.25% glutaraldehyde was added, resulting in a signal increase of 4,100 RU. The surface was then blocked by two consecutive injections of 3% BSA plus 0.1 M NaHCO$_3$, pH 8.6, and 1% BSA plus 0.1 M NaHCO$_3$, pH 8.6. To assess binding, each antibody was added at final concentrations of 5–500 μg/ml. PBS was allowed to flow over the surface after each injection for 10 min so that at least 30% of the bound antibody dissociated. The binding was displayed in real time as a sensogram, which was analyzed using kinetics software to give final affinity values (19).

Creation of combinatorial Ig libraries. Using previously described methods (9, 15–17), we recently constructed an IgG library (9), now

designated JG. An IgM library, designated JM, was made from the same peripheral blood sample that was the source of the JG library, and this also used the same L chain phagemid library used for the JG library. To amplify the heavy (H) chain genes, in separate reactions an antisense (downstream) primer (termed CM1) (5′-GCTCACACTAGTAGG-CAGCTCAGCAATCAC-3′) derived from the μ constant region was paired individually with sense (upstream) V$_H$ oligonucleotide primers from the previously described panel, with an additional V$_H$ primer, (5′-CAGGTGCAGCTACTCGAGTGGGGC-3′), designated V$_H$4gs, which ensured representation of rearrangements of the V$_H$4-21 gene segment. The H chain genes were cloned after the L chain genes to avoid destroying V$_H$4-21 rearrangements, which contain internal SacI sites. In these libraries, there were > 10^7 CFU for both H and L chain transformants. The PCR conditions used are associated with < 1 mutation in 1,500 bases due to Taq polymerase infidelity. Each pComb3 clone was designated according to the library from which it was obtained (JM or JG). Clones from the original library include the "O" prefix, and selected clones include the prefixes pFv1-, pFv2-, and pFv3-, based on the round of selection.

Antibody selection. As previously described (9, 15–17), antigen-specific clones were selected using a modified panning procedure (20). For selection of pFv-specific clones, microtiter wells were precoated and blocked as previously described. For each round of selection, two of these wells were filled with 50 μl of phage library containing 10^{11} CFU in PBS and incubated for 2 h at 37°C. After extensive washing, binding phage were eluted with glycine–HCl, pH 2.5, which was rapidly neutralized with 2 M Tris-base. Phage were then amplified.

Colony hybridization to survey V$_H$ family diversity. To survey V$_H$ chain diversity within the pComb3 clones, an oligonucleotide-based hybridization method was adapted (9, 10, 21) using 150–250 colonies per plate and Hybond-N discs (Amersham Corp.) Oligonucleotide hybridization studies used antisense sequences from diagnostic framework regions (designed by Dr. C. Rundle, University of Alabama, Birmingham, AL) that include the following: μ (H-13), 5′-CTCACA-GGAGACGAGGGGGAAAAGGGTTGG-3′; V$_H$1 (CR-8), 5′-AGC-TCCATGTAGGCTGTGCTCGTGGACGTGTCCCTGGTCA-3′; V$_H$2 (CR-2), 5′-GGTCCATGTTGGTCATTTTAAGGACCACCTGGTTT-3′; V$_H$3 (CR-3), 5′-GGCTCTCAGGCTGTTCATTTGCAGATACAG-CGTGTTCTTG-3′; V$_H$4 (CR-4), 5′-CGCGGCGGTCACAGAGCT-CAGCTTCAGGGAGAACT-3′; V$_H$5 (CR-5), 5′-ACTCCAGGCCTT-TCCCGGGCATCTGGCGCA-3′; and V$_H$6 (CR-6), 5′-AGCCAC-TCAAGGCCTCTCGATGGGGACTGCCTG-3′.

All hybridization studies were performed at least twice to ensure accuracy. On all blots, control V$_H$-containing plasmids were included to document specificity and signal intensity: 51P1 (V$_H$1), M60, (V$_H$2), 30P1 (V$_H$3), 58P2 (V$_H$4), 83P2 (V$_H$5), M71 (V$_H$6), and 10P1 (V$_H$7).

DNA sequence determination. The nucleotide sequences encoding V$_H$ and V$_L$ regions were determined from double-stranded DNA using automated sequencing (Applied Biosystems, Inc., Foster City, CA). The oligonucleotide 5′-ATGGAGTCGGGAAGGAAGTC-3′, termed SEQMB, from the μ constant region immediately adjacent to the V$_H$ region and other previously described oligonucleotides (9) were used to prime sequencing reactions. Sequences were analyzed with Macvector 4.0 (IBI, New Haven, CT) and Genetics Computer Group (Madison, WI) software packages and compared with comparison to the GenBank data base. To ensure accuracy, sequences were determined at least three times using two different primers.

Results

IgM that bind pFv. To evaluate the binding capacity of IgM for pFv, immunoblots of pFv and control proteins were incubated with different IgM preparations. Species within polyclonal adult IgM identified the same bands at 85 and 175 kD in pFv preparations from two different individuals (FAR and TER). The monoclonal V$_H$3V$_κ$3 IgM (GLO), V$_H$1V$_κ$3 IgM (KAS), and V$_H$6 λ IgM (L16) were also found to be reactive with the pFv by immunoblotting (data not shown). By compari-
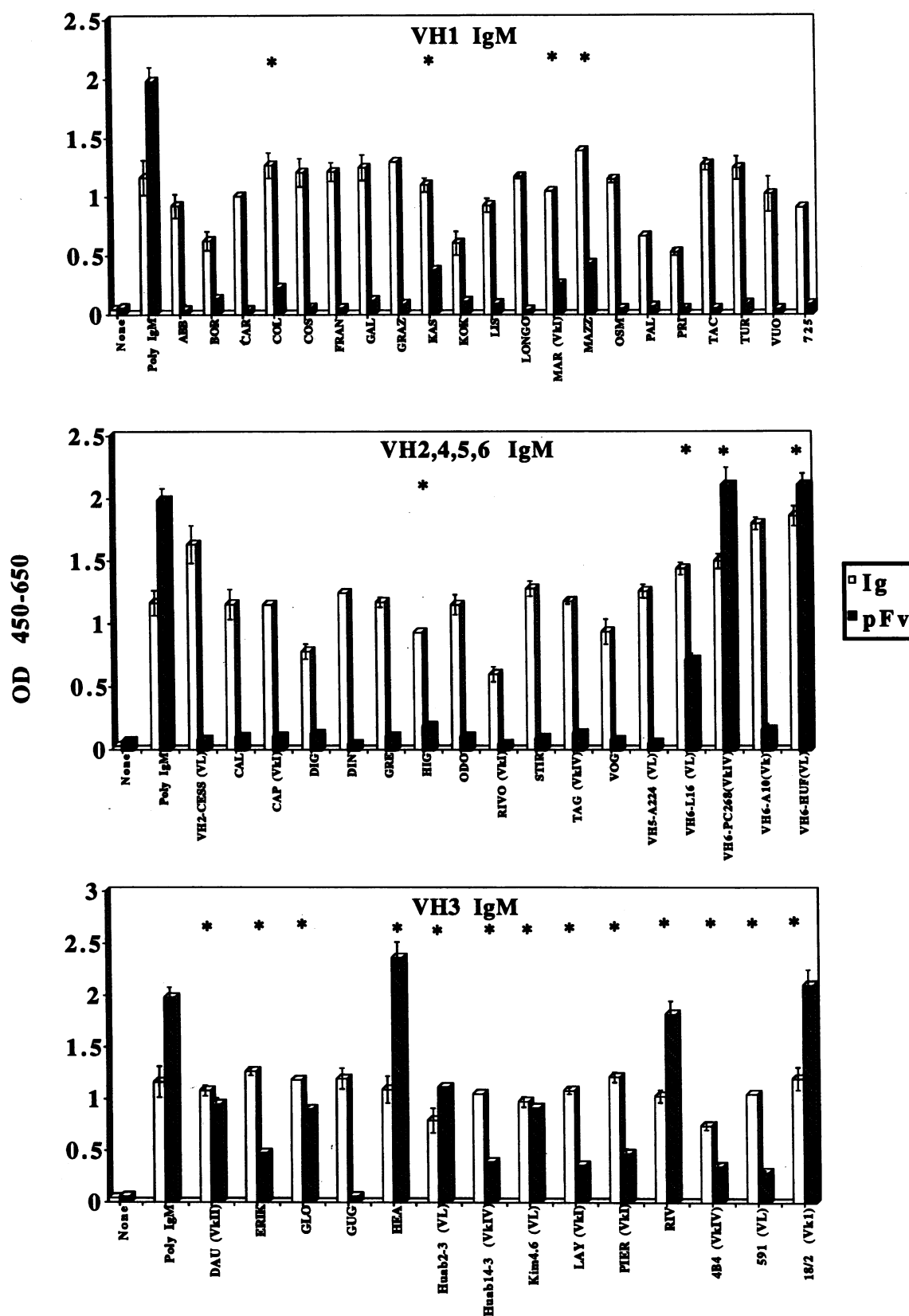
Figure 1. ELISA evaluation of human IgM for pFv binding activity. Direct binding evaluations were performed on a panel of 52 monoclonal human IgM. The proteins are organized according to V_H family, and if known, V_H region sequences of IgM with strong pFv binding activity are included in Fig. 3. Except where indicated, these IgM use $V_\kappa III$ L chains, and usage was presented in earlier publications (6, 8, 13, 14). For comparison, values for Ig content were also obtained. Values were determined in triplicate and are represented ±SD. An asterisk indicates IgM with significant pFv binding capacity (> 5 SD above negative controls).
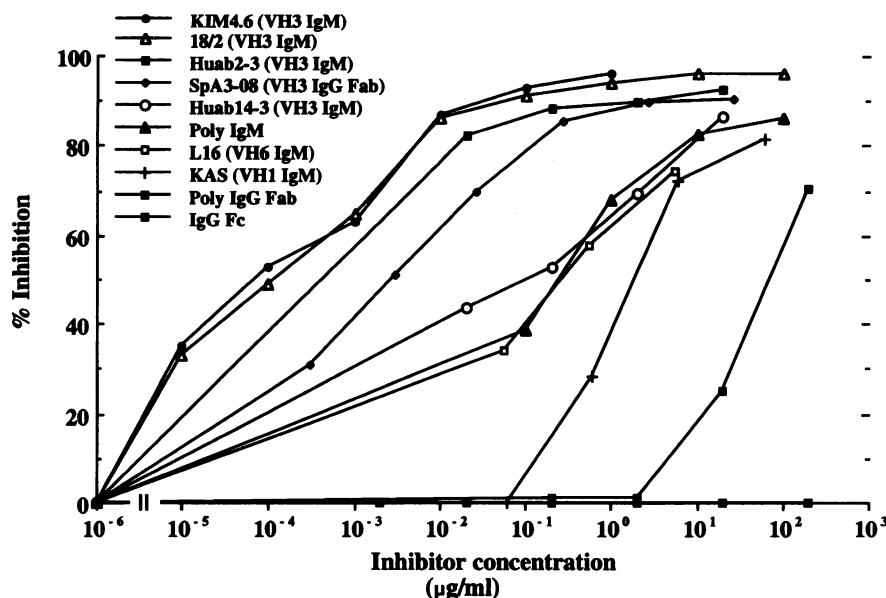
Figure 2. Competitive inhibition of polyclonal IgM binding of pFv by monoclonal IgM. Polyclonal IgM from the peripheral blood of healthy adults was biotinylated and added at a final concentration of 500 ng/ml to wells coated with pFv. To evaluate the ability of different purified Ig to inhibit the binding of polyclonal IgM to pFv, inhibitors were added at final concentrations between 0.01 and 200 μg/ml to aliquots of polyclonal IgM–biotin. All values were determined in duplicate. Without inhibitor, an OD > 1.5 was obtained. Percent inhibition was determined by comparison with a standard curve of polyclonal IgM–biotin without inhibitor.

son, SpA, which has been shown to bind most V_H3-derived Ig (6–9, 22), was recognized only by the polyclonal IgM and the V_H3 IgM (data not shown).

The binding capacity of a large panel of monoclonal IgM for pFv was evaluated by direct binding ELISA (Fig. 1). ELISA studies demonstrated that different IgM have a wide range of reactivities with pFv, and 42.3% (21/52) of this panel of monoclonal IgM, including 4/21 V_H1, 0/1 V_H2, 13/14 V_H3, 1/11 V_H4, 0/1 V_H5, and 3/4 V_H6 IgM, displayed significant ELISA reactivity with pFv. Binding specificity was confirmed by demonstration that soluble pFv inhibited the interaction with solid phase pFv (data not shown). IgM from the V_H1 and V_H4 families with pFv binding activity generally had weaker pFv binding activity than displayed by the V_H3 and V_H6 IgM binders. Binding to a panel of control antigens was also tested, but the IgM were uniformly nonreactive except for the V_H6 IgM, L16, which had significant binding activity with orosomucoid, a control sialoprotein preparation.

Inhibition of polyclonal IgM pFv binding by monoclonal IgM. In competitive binding studies, three V_H3 IgM, 18/2, Kim4.6, and Huab2-3, were potent inhibitors. The V_H3 IgM Huab14-3, the V_H6 IgM L16, and polyclonal IgM had intermediate inhibition capacity. The V_H1 IgM KAS was the weakest inhibitor (Fig. 2). At higher concentrations, the five different monoclonal IgM were each able to significantly inhibit (75–97%) pFv binding by polyclonal IgM (Fig. 2). Of those tested, the best inhibitors were the IgM-κ 18/2 and the IgM-λ KIM4.6, which use V_H3 regions encoded by the germline configuration of the $V_H26c/V3$-23 and 1.9III/V3-30 gene segments, respectively (23–25). Presumably because it is capable of only monovalent binding interactions, the monoclonal IgG-λ Fab JGSpA3-08 (9) encoded by a different rearrangement of the $V_H26c/V3$-23 gene was a ~ 30-fold weaker inhibitor. Polyclonal IgG Fab was by far the weakest of the inhibitors tested, and IgG Fc had no detectable inhibition capacity. The demonstration that monoclonal antibodies from different V_H families can each nearly completely inhibit binding of polyclonal IgM, which represents a mixture of IgM, suggests that Fab-mediated binding is not due to recognition of a multitude of conventional epitopes on pFv, but derives from interactions with a common or closely

adjacent Fab-binding site. Polyclonal IgM contains subpopulations of strong and weak (and non-) pFv binders (illustrated in Fig. 1), which is presumably the basis by which low concentrations (i.e., 0.01–0.1 ng/ml) of certain monoclonal V_H3 IgM with strong pFv binding activity caused significant inhibition of the binding of a portion of polyclonal IgM to pFv. In other studies, IgM and Fab from different V_H families, and with dissimilar L chain isotypes, could also inhibit the binding of each other to pFv (data not shown).

Binding affinities measured by surface plasmon resonance. Based on direct binding and competition ELISA, five IgM with stronger pFv binding interactions were selected for further evaluation. Based on the structures of pFv and intact IgM, these appear to represent multivalent binding interactions of strong avidity. Despite the use of diverse V_H regions (Fig. 3), surface plasmon resonance studies indicated that the V_H6 IgM L16 interacts with pFv with an apparent association constant (K_a) of 8.8×10^7 M^{-1}, and the four V_H3 IgM, Hum2-3, Hum 14-3, 18/2, and KIM4.6, bind with a K_a of 0.11–6.7×10^8 M^{-1} (Table I). Consistent with the results of the competitive binding assays, the IgM 18/2 and KIM4.6 displayed the strongest binding interactions with pFv. The V_H region of the IgM-κ Fab JMSpA3-15 expresses the germline configuration of $V_H26c/V3$-23, which differs by only 4 amino acids in this portion from the IgG-λ Fab JGSpA3-08 (Fig. 3). These Fab, which are capable of only monovalent interactions, bind pFv with K_a values of 1.9 and 3.5×10^6 M^{-1}, respectively.

Analysis of library composition. Combinatorial Ig phage-display libraries were used to investigate the pFv binding capacity of different IgM Fab and IgG Fab. Colony hybridization studies documented that the unselected JM library contained 19% V_H1, 0% V_H2, 22% V_H3, 39% V_H4, 0% V_H5, and 7% V_H6 (with ~ 13% unidentified) μ rearrangements (Table II). Although appropriate V_H primers were used, V_H2 family Fab clones were not detected in the library. By ELISA, of 45 IgM clones evaluated from the unselected library, 10 (22%) had a significant binding interaction with pFv (data not shown).

In an earlier report (9), the unselected JG library was shown to contain 26% V_H1, 26% V_H3, and 26% V_H4 γ rearrangements (other V_H families were not assessed). A total of 21 IgG Fab

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        VH Group  VL              FR1                        CDR1      FR2            CDR2           FR3                                 CDR3              FR4

VH1 sequences
VH1 Fab
JGpFv3-11    κ    QVKLLE SGAEVKKPGASVKVSCKASGYTST S  YDIN WVRQATGQGLEWMG RMNP  NSGHTGYAQKFQG  RVTMTTDSSLHTVYMELSSLKSEDTAVYYCAR    GGGPLYSWSWVDY     WGQGTLVTVSS
JM 0-131     λ    ------ ------------R--------SF- -  ---- ----T-------- WI--  D--I-----N---  ----SR-T-IS-A-------R--------SF      LVMRGY           -------I---

VH1 IgM
KAS          Vκ3  -Q-VQ ---------S----T-----G-FS -   -A-S -----P-------- GII-  IF-QAN-------  ---I-A-E-TN-A----R--R-D---M----K     BGYGDYGRPFDF     ----------

VH3 sequences
VH3 Fab
VH26c/V3-23 (germline)  EVQLLE SGGGLVQPGGSLRLSCAASGFTFS S  YAMS WVRQAPGKGLEWVS AISG  SGGSTYYADSVKG  RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR                     WGQGTLVTVSS
JMSpA3-15    Vκ3  Q-K--E------------------------- -  ---- -------------- ----  -------------  -----------------------------K       LGGQGSYYHFDY     ----------
JGSpA3-08    Vλ1  Q-K--- ------------------------- -   H--- -------------- D--A  -------------  -------------------------L----S     SGAGWGLPSLDY     ----------
JGpFv3-07    Vκ3  Q-K---E----V----R------------- -   HG-H -------------T S-WY  D-SKK-------   ------------------------------V-     DGSYHGLGTHRWFDP  ----------
JGpFv3-06    λ    Q-K---Q-----------K----V---S-- G   S-LY -----S-------G R-RSKTNYAN-E--E--R-  -------D----A-------KT--------M     WAGNGDHDYAVLDY   --------I---
JM 0-42      κ    Q-K--- ------T-------------N T     -S-N -------------- S--R  -SDYI-------   --------A--SV--------D----F----      DPGGMPTYGITWVY   ----------
JMpFv2-38    λ    Q-K--- --------------------- N       ---N -------------- G--D  --DR-HF-----   ------------V-----------P-----K      DNARTVAGTGGYFDY  ----------
JMpFv2-51         Q-K--E------------------------- -  ---- -------------- ----  -------------  ------------------------------K      LGGQGSYYHFDY     ----------
JMpFv2-36    A Vκ3  Q-K---E------------------AS--- R  --VD -------------- SFE   -A-G-H------   ---V------I-QH---------------T        RGEYYYYGMDV      -----T-I---
JMpFv2-40    A Vκ3  Q-K---E------------------AS--- R  --VD -------------- SFE   -A-G-H------   ---V------I-QH---------------T        RGEYYYYGMDV      -----T-I---
JMpFv2-43    A Vκ3  Q-K---E------------------AS--- R  --VD -------------- SFE   -A-G-H------   ---V------I-QH---------------T        RGEYYYYGMDV      -----T-I---
JMpFv2-62    A κ    Q-K---E------------------AS--- R  --VD -------------- SFE   -A-G-H------   ---V------I-QH---------------T        RGEYYYCGMDV      -----T-I---
JMpFv2-63    A Vκ3  Q-K---E------------------AS--- R  --VD -------------- SFE   -A-G-H------   ---V------I-QH---------------T        RGEYYYYGMDV      -----T-I---
JMpFv2-68    A κ    Q-K---E------------------AS--- R  --VD -------------- SFE   -A-G-H------   ---V------I-QH---------------T        RGEYYYYGMDV      -----T-I---

VH3 IgM
18/2         Vκ1  ------ ------------------------- -  ---- -------------- ----  -------------  --------------------------TK         GQVLYYGSGSYHWFDP ----------
RIV          Vκ3  ----V- ----V-----S----------- -   F--H -------------A VM-Y  --DNK--V-----  ------------------------L----K       LSTAASYPTFDTYGMD -----T-----
Huab14-3     Vκ4  ----V- ------------------------- T   -R-H ----------V--- R-NS  D-S--D-----E-  --------A--------------------        SNYYFDS          ----------
LAY          Vκ3  A----- ------------------------- A   S--- -------------A WKYE  N-NDKH-----N-  -----ND-----------Q---S-I----K       DAGPLVSPTFFAH    ----------
Kim4-6       Vλ1  Q---V- ----V----R------------- -    -G-H -------------A V--Y  D-SNK-------   ------------------------------K      VREYYYGSGYYYYYGMDV -----T-----
4B4          Vκ4  ----V- ------K---------------- N     AW-- -------------G R-KSKTD--T-D--AP---  -------D------------KT--------TT     DSLPPHRV         ----------
Huab2-3      Vλ7  ----V- ------------K---------- A     S-VH -----S-------G R-RSKANSYA-A--A----  -------D----A-------KT--------TG     HQPLTTVTTPHWFDP  ----------

VH4 sequences
VH4 Fab
VH4-21/V4-34 (germline)  QVQLQQ WGAGLLKPSETLSLTCAVYGGSFS G  YYWS WIRQPPGKGLEWIG EIN  HSGSTNYNPSLKS  RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR                     WGQGTLVTVSS
JM 0-133     κ    --K-LE ------------------------- -  ---- -------------- ---  -------------  --------------------------------     GQPFTAL          ----------
JM 0-26X     λ    --K-LE ------------------------- -  ---- -------------- ---  -------------  --------------------------------     GRGGYNSGHGGYFDY  ----------
JM 0-28X     λ    --K-LE S-P--V----------T-SR--I- S   ---- -------------- Y-Y  Y--T--------   -----L------L--R-I----E-----S---     HSPGDYGYYYGMDV   -----T-----
JMpFv2-07    B κ    --K-LE S-P--V----------W--S---MD SDN--YD -------------- NVH  YR-IIY-TA----  ---M-----KK----MT----I---E-----      HKGGDDLFDS       ----S------
JMpFv2-46    B κ    --K-LE S-P--V----------S---MD SDN--YD -------------- NVH  Y--IIY-TA----  ---M-----KK----MT----T---------      HKGGDDRFDS       ----S------
JMpFv2-26    C κ    --K-LE ------------------------- -  ---- -------------- ---  -------------  -------------------------------I     WFGELYPH         ----------
JMpFv2-50    C Vλ2  --K-LE ------------------------- -  ---- -------------- ---  -------------  -------------------------------I     WFGELYPH         ----------

VH6 sequences
VH6 Fab
6-1G1/V6-01 (germline)  QVQLQQ SGPGLVKPSQTLSLTCAISGDSVS SNSAAWN WIRQSPSRGLEWLG RTYYR  SKWYNDYAVSVKS  RITINPDTSKNQFSLQLNSVTPEDTAVYYCAR                    WGQGTLVTVSS
JGpFv3-13    κ    --K-LE ------E---------D--FY---- --T---- -------------- --F--  -----G--E----  ----S---------------IV----------     DRVYTSTMAGGIDV   -----T-----
JGpFv3-01    E Vκ3  --K-LEQ------E---------D------- --T---- -------G------ --F--  -----G--E----  ----S---------------------V-         GGGNFDH          ----------
JGpFv3-02    E Vκ3  --K-LEQ------E---------D------- --T---- -------------- --F--  -A-R-E--A----  ----------R--------------------V-     GGGNFDY          -A---------
JM 0-8X      E κ    --K-LE ------E----------------- ------- -------------- --F--  -A-R-E--A----  --------------V--------------V-       GGGNFDY          -A---------
JMpFv2-52         --K-LEQ---------------D------- R---G-- -------------- -----  -M--TN------   -------------------M------------     DVDFTSTMADVGMDV  -VR--T-----
JMpFv2-58    D Vκ3  --K-LEQ------------------------ N------- -------------- -----  -------------  --V-T----------------D---------      DISARHTD         ----------
JMpFv2-56    D κ    --K-LEQ---------------D------- R---G-- -------------- -----  -------------  ----T----------------D---------      DISARHTD         ----------

VH6 IgM
L16          Vλ   ------ ------------------------- ------- -------------- -----  -------------  --------------------------------     ELGDAFDI         -----M-----
                  FR1                      CDR1     FR2            CDR2           FR3                              CDR3             FR4
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Figure 3. V_H regions of antibodies that bind pFv. V_H region sequences from intact IgM antibodies with strong reactivity for pFv are included. All antibodies contain H and L chains and bind pFv, with the exception of JMpFv2-51, which was defective owing to an absent L chain. The germline gene segments V_H26/V3-23 (29, 30), V_H4-21/V4-34 (31), and 6-1G1/V6-01 (33) are included for comparison. Residues identical to those used in the master sequence are indicated by a dash. The first framework sequences encoded by the oligonucleotide primers are underlined. Residues in the CDR3 that are contributed by the J_H gene segment are underlined. The portion of the rearranged genes upstream of these primers is part of the vector. Subdomains were assigned as described by Kabat et al. (36) and modified by Chothia and Lesk (37). These sequence data are available from EMBL/GenBank/DDBJ under accession numbers U21248–U21272 and L06102 for Huab2-3 and L06101 for Huab14-3.

clones from the unselected JG library were evaluated, but only 2 (9.5%) IgG Fab were found to have significant binding capacity for pFv (data not shown). Fab with pFv binding capacities were less frequent in the unselected IgG library than in the unselected IgM library, but this difference was not statistically significant (Fisher exact test, $P < 0.21$), likely owing to the limited number of clones examined.

Biopanning of the combinatorial libraries for pFv binders. The JM library was subjected to two rounds of panning with pFv, and 21 representative clones from the JMpFv2 library were evaluated. With the exception of JMpFv2-51, which was defective owing to an absent L chain gene, all bound pFv (data not shown), indicating that panning resulted in significant selection of pFv binders (Fisher exact test $P < 0.0001$). Colony hybridization studies indicated a sequential selection for V_H3 and V_H6 clones (Table II). The JG library was subjected to three rounds of panning with pFv. 12 representative clones from the resultant library were evaluated, and 7 IgG Fab clones had significant pFv binding activity (data not shown).

V region sequence analyses of Fab with pFv binding capacity. The complete DNA sequences were determined for the V_H regions of six IgM Fab clones with pFv binding activity obtained from the unselected JM library, which included JM 0-131 (V_H1), JM 0-42 (V_H3) JM 0-28X (V_H4), JM 0-26X (V_H4), JM 0-133 (V_H4), and JM 0-8X (V_H6). From sequence analysis, all were derived from independent V_H rearrangements (Fig. 3; Table III). Included in Fig. 5 are the V_H sequences available for nine intact IgM with pFv binding capacity.

The V_H sequences of 15 representatives of the JMpFv2 library obtained after two rounds of selection with pFv were also determined (Fig. 3). These V_H genes appear to derive from only seven different rearrangements. JMpFv2-51 (V_H3), JMpFv2-38 (V_H3), and JMpFv2-52 (V_H6) are the only single representatives of their respective V_H gene rearrangements, and the other 12 V_H regions can be grouped based on the usage of identical or nearly identical gene rearrangements: V_H group A, JMpFv2-36/40/43/62/63/68 (V_H3); V_H group B, JMpFv2-07/46 (V_H4); V_H group C, JMpFv2-26/50 (V_H4); and V_H group D, JMpFv2-58/56 (V_H6).

A total of six V_H region sequences were determined for representative clones from the JG library obtained after three rounds of biopanning with pFv (Fig. 3). Of these, four, JGpFv3-

Table I. Binding Kinetics of Antibodies for Protein Fv

Ig	V_H usage	V_L usage	Ag*	Isotype[‡]	Reference		Binding constants	
Fab fragments (monomeric)								
JMSpA3-15	V_H3 V_H26c/V3-23 (100%)	$V_\kappa3$	SpA	IgM Fab		k_{on}	5.96×10^4 M^{-1} s^{-1}	
						k_{off}	3.1×10^{-3} s^{-1}	
						K_a	1.92×10^6 M^{-1}	
						K_d	5.2×10^{-7} M	
JGSpA3-08	V_H3 V_H26c/V3-23 (94.8%)	$V_\lambda1$	SpA	IgG Fab	(9)	k_{on}	9.8×10^3 M^{-1} s^{-1}	
						k_{off}	2.7×10^{-4} s^{-1}	
						K_a	3.5×10^6 M^{-1}	
						K_d	2.9×10^{-7} M	
IgM (polymeric)								
18/2	V_H3 V_H26c/V3-23 (100%)	$V_\kappa1$	DNA	IgM	(23)	k_{on}	3.75×10^4 M^{-1} s^{-1}	
						k_{off}	2.5×10^{-4} s^{-1}	
						K_a	1.5×10^8 M^{-1}	
						K_d	6.7×10^{-9} M	
KIM4.6	V_H3 1.9III/V3-30 (100%)	$V_\lambda1$	DNA	IgM	(25)	k_{on}	4.3×10^4 M^{-1} s^{-1}	
						k_{off}	6.4×10^{-5} s^{-1}	
						K_a	6.7×10^8 M^{-1}	
						K_d	1.5×10^{-9} M	
Hum2-3	V_H3 MTGL/V3-73 (90%)	$V_\lambda7$	PPS-3	IgM	(26)	k_{on}	3.4×10^3 M^{-1} s^{-1}	
						k_{off}	2.4×10^{-4} s^{-1}	
						K_a	1.4×10^7 M^{-1}	
						K_d	1.1×10^{-8} M	
Hum14-3	V_H3 H11 (97%)	$V_\kappa4$	PPS-3	IgM	(26)	k_{on}	5.0×10^3 M^{-1} s^{-1}	
						k_{off}	4.5×10^{-4} s^{-1}	
						K_a	1.1×10^7 M^{-1}	
						K_d	9.1×10^{-8} M	
L16	V_H6 6-1R1/V6-01 (100%)	V_λ	Polyspecific	IgM	(27)	k_{on}	1.76×10^4 M^{-1} s^{-1}	
						k_{off}	2.0×10^{-4} s^{-1}	
						K_a	8.8×10^7 M^{-1}	
						K_d	1.1×10^{-8} M	

The kinetics of binding interactions with pFv were measured by surface plasmon resonance determinations using the BIAcore apparatus (see Methods). The coefficients of correlation (r) were all >0.96. Values were reproducible to within 20%. Upon testing, binding of pFv by the V_H1 IgM KAS and the IgM Fab JM 0-8X (V_H6), JM 0-42 (V_H3), JM 0-133 (V_H4), and JMpFv2-43 (V_H3) was also detected by surface plasmon resonance, but binding coefficients could not be accurately determined owing to relative affinity and limitations of antibody concentrations. * The antigen binding specificity for which the antibody was originally selected. *PPS-3*, pneumococcal polysaccharide type 3. [‡] Isotype of the antibody.

11 (V_H1), JGpFv3-07 (V_H3), JGpFv3-06 (V_H3), and JGpFv3-13 (V_H6), represent single and unrelated V_H rearrangements. The other two, JGpFv3-01 and JGpFv3-02, were amplified by different upstream primers but appear to be intraclonal variants

Table II. V_H Family Distribution in the Combinatorial Libraries

Library	V_H1	V_H2	V_H3	V_H4	V_H5	V_H6	Total
JM 0	19*	0	22	39	0	7	87[§]
JMpFv1	13	0	19	22	2	15	71
JMpFv2	0.5	0	47	9	0	28	84.5

These data represent results of hybridization of membrane lifts of bacterial colonies with 150–250 colonies per plate. JM 0 represents the unselected IgM Fab library, and JMpFv1 and JMpFv2 are the libraries obtained after one or two sequential rounds of selection by the pFv protein. Data are presented as the percentage of colonies that hybridized with a μ-specific probe. * Controls included in each study were plasmids with V_H genes of all known families. In each survey certain colonies were unidentified, either because they did not contain V_H genes or presumably because V_H sequence variations abolished hybridization.

of the same V_H6 rearrangement and are therefore designated group E. JM 0-8X, a μ V_H6 rearrangement from the unselected JM library, was likely created by amplification with different upstream and downstream primers but appears to be clonally related to the γ chains in JGpFv3-01 and JGpFv3-02, so it has also been included in group E. The V_H gene sequence of JM 0-8X also shares most mutations present in the JGpFv3-02. This pattern, in which a μ V_H6 sequence (JM 0-8X) has accumulated more somatic mutations than a clonally related γ V_H6 rearrangement (JGpFv3-01), has previously been described (28). By comparisons of V_H rearrangement sequences with the GenBank data base, the closest known V_H germline genes were identified (Table III).

Among the V_H sequences of selected Fab, each of the members of V_H groups B, D, and E contains a level of sequence variation that greatly exceeded our experimental Taq polymerase infidelity error (< 1/1,500 nucleotides), which indicates that they were independently derived from in vivo clonally related B cells and therefore must have been independently selected by pFv. Whereas the V_H regions of group C are identical, the associated L chains are encoded by a κ gene and a λ

Table III. V_H Gene Usage

	Primer	V_H family	Nearest V_H*	Reference	V_H group	DNA homology	Protein homology	FR1‡		CDR1		FR2		CDR2		FR3		J_H	V_L usage
								R	S	R	S	R	S	R	S	R	S		
Unselected																			
JM IgM library																			
JM 0-131	1f/3f	1	DP-15/V1-08	(29)		93.5%	89.9%	2	2	0	2	1	0	4	3	2	1	4b	λ
JM 0-42	1f/3f	3	V_H26c/V3-23	(24, 30)		96.3%	91.3%	2	0	1	0	0	0	2	1	3	1	4	κ
JM 0-26X	4gs	4	4-21/V4-34	(31)		100%	100%	0	0	0	0	0	0	0	0	0	0	4b	λ
JM 0-28X	6f	4	4-11/V4-59	(31)		94.3%	91.3%	1	2	0	0	0	1	1	.1	6	3	6	λ
			T23-9*	(32)		95%	91.3%	1	2	0	0	0	1	1	1	6	1	6	λ
JM 0-133	4gs	4	4-21/V4-34	(31)		100%	100%	0	0	0	0	0	0	0	0	0	0	4	κ
JM 0-8X	6f	6	6-1G1/V6-01	(33)	D	95.6%	92.4%	1	0	0	0	0	0	5	4	1‡	1	4	κ
Selected																			
JM IgM library																			
JMpFv2-38	1f/3f	3	V_H26c/V3-23	(24, 30)		95.2%	88.8%	0	0	2	0	0	1	6	1	2	1	4b	λ
JMpFv2-36	3a	3	V_H26c/V3-23	(24, 30)	A	87.1%	82.2%	2	5	3	2	0	2	7	6	4	3	6	$V_\kappa 3$§
JMpFv2-40	3a	3	V_H26c/V3-23	(24, 30)	A	86.8%	82.2%	2	5	3	2	0	2	7	6	4	4	6	$V_\kappa 3$§
JMpFv2-43	3a	3	V_H26c/V3-23	(24, 30)	A	86.8%	82.2%	2	5	3	2	0	2	7	6	4	4	6	$V_\kappa 3$§
JMpFv2-62	3a	3	V_H26c/V3-23	(24, 30)	A	87.1%	82.2%	2	5	3	2	0	2	7	6	4	3	6	κ
JMpFv2-63	3a	3	V_H26c/V3-23	(24, 30)	A	86.8%	82.2%	2	5	3	2	0	2	7	6	4	4	6	$V_\kappa 3$§
JMpFv2-68	3a	3	V_H26c/V3-23	(24, 30)	A	86.8%	82.2%	2	5	3	2	0	2	7	6	4	4	6	κ
JMpFv2-51	3a	3	V_H26c/V3-23	(24, 30)		100%	100%	0	0	0	0	0	0	0	0	0	0	4b	
JMpFv2-07	2f/4f	4	4-18/V4-39	(31)	B	85.3%	75.0%	4	3	4	3	0	3	8	3	7	5	4	λ
JMpFv2-46	2f/4f	4	4-18/V4-39	(31)	B	86.8%	78.3%	3	3	4	3	0	3	7	4	6	4	4	κ
JMpFv2-50	4gs	4	4-21/V4-34	(31)	C	100%	100%	0	0	0	0	0	0	0	0	0	0	4	$V_\lambda 2$
JMpFv2-26	4gs	4	4-21/V4-34	(31)	C	100%	100%	0	0	0	0	0	0	0	0	0	0	4	κ
JMpFv2-52	6a	6	6-1G1/V6-01·	(33)		96.8%	92.6%	1	0	2	0	0	0	3	1	1	1	6	κ
JMpFv2-56	6a	6	6-1G1/V6-01	(33)	D	97.5%	94.7%	1	0	2	0	0	0	0	1	2	1	6	κ
JMpFv2-58	6a	6	6-1G1/V6-01	(33)	D	97.8%	95.6%	0	0	1	0	0	0	0	2	3	2	6	$V_\kappa 3$
Selected																			
JG IgG library																			
JGpFv3-11	1f/3f	1	DP-15/V1-08	(29)		94.8%	89.1%	1	0	0	0	0	0	2	1	7	3	4	κ
JGpFv3-06	1a	3	MTGL/V3-73	(34)		95.5%	90.2%	2	3	2	0	0	0	5	0	0	1	4b	λ
JGpFv3-07	3a	3	hv3019/V3-33	(35)		97.3%	95.6%	0	0	1	1	1	0	2	1	0	2	1	$V_\kappa 3$
JGpFv3-13	6a/6f	6	6-1G1/V6-01	(33)		91.8%	88.0%	4	0	1	0	0	0	3	2	3	5	6	κ
JGpFv3-01	6a/6f	6	6-1G1/V6-01	(33)	E	95.2%	91.3%	2	0	1	0	1	1	3	2	1	1	1	$V_\kappa 3$
JGpFv3-02	6a/6f	6	6-1G1/V6-01	(33)	E	93.7%	92.3%	1	0	0	0	0	0	5	4	1‡	2	4	$V_\kappa 3$

* Comparison with the closest known germline V_H gene segment, except in the case of T23-9, which represents a rearranged gene. Germline genes are designated as originally named and by the nomenclature of Matsuda et al. (64). ‡ Subdomains are assigned as described by Kabat et al. (36) and modified by Chothia and Lesk (37); these assignments have been modified based on crystallographic data, and calculations of homology were performed omitting the contribution of the upstream primer and ending with the codon for the terminal cysteine. R represents replacement mutations. S represents silent mutations. § The $V_\kappa 3$ genes encoding the L chains of group A are identical except for a single mutation in JMpFv2-40. Based on V_H and V_L homology, these likely represent duplications of the same phagemid clone. By similar criteria, the individual members of groups B, C, and E were probably different clones that were independently selected.

gene (Table III). Taken together, these data indicate that in many cases independent antibody clones with identical or highly similar V_H regions were recurrently selected, presumably based on preferential pFv binding activity. These data further support the overall impression that pFv binding is V_H dependent, with limited, if any, influence from the V_L region.

Analyses of the CDR3 of the Fab selected from the phage-display libraries revealed a remarkable consistency. While there were no recurrently used sequence motifs or charge–hydrophilicity distributions, there was evidence of selection based on the size of the CDR3. This trend was even more pronounced among the V_H genes that were repeatedly represented (and therefore assigned to V_H groups A–E). The CDR3 sizes of these 15 sequences exhibit a restricted range of 7–11 (9.1±1.8,

mean±SD) and are all smaller than the mean size of V_H CDR3 expressed in the adult repertoire (38, 39).

Discussion

The current report investigates the structural basis for antibody binding of protein Fv, a human sialoprotein with unique immunologic properties. These studies confirm that among antibody populations, the frequency of pFv binders is many orders of magnitude greater than occurs for the expression of a conventional antigen binding specificity. Within the panel of monoclonal IgM, pFv binding capacity was expressed by only a small proportion of V_H1 and V_H4 antibodies (< 20% and < 10%, respectively), whereas almost all V_H3 and V_H6 IgM bound pFv.

Significantly, among the five V_H3 IgM antibodies studied in detail, antibodies directly encoded by the germline $V_H26c/V3-23$, $1.9III/V3-30$, and V_H6 gene segments had the strongest binding interactions.

To extend these studies, phage-display library technology was used to investigate pFv binding within the repertoire from a healthy adult. As documented by sequence analysis and colony hybridization, panning of the IgM and IgG libraries with pFv selected predominantly for V_H3- and V_H6-encoded Fab, even though it is certain that the specific V_H gene compositions within the two Fab libraries were very different. Antibodies encoded by V_H4-21 were obtained only from the μ library, but this may have been due to differential in vivo representation of V_H4-21 rearrangements and/or primer usage in construction of the libraries. However, for both libraries, pFv binding repeatedly selected for antibodies with identical or clonally related V_H genes that are paired with different L chains. These data and the very similar binding affinities of the IgM-κ Fab and IgG-λ Fab represent independent evidence that L chains are relatively passive and that pFv binding is likely mediated by contact areas in the portion of the Fab that comes from the V_H gene segment. It is also clear that pFv interacts with a protein and not a carbohydrate site on Fab, because many of these studies used antibodies produced by bacteria.

In certain cases, panning with pFv selected for Fab with germline-encoded V_H regions (Table III), but all selected V_H6 Fab were found to be hypermutated, demonstrating that these mutations do not interfere with pFv binding. A likely explanation comes from previous reports that have detected only hypermutated V_H6 regions in the adult repertoire (40–43). Thus, even if nonmutated V_H gene products are more commonly associated with optimal pFv binding, they could not be selected if they were not represented in the libraries.

Crystallographic studies have demonstrated that binding pockets for conventional antigens generally occur at the central confluence of the H and L chain CDR loops, and in most cases CDR3 loops, the subdomains of greatest diversity, are highly involved (36, 44, 45). In contrast, the current variable region analyses did not detect conserved amino acid sequences in any of the CDR of pFv binders, and diverse V_H CDR3 were demonstrated to be permissive of pFv binding. However, the library studies did reveal preferential selection for V_H CDR3 of a small size. Although the CDR3 could still directly contribute to the pFv-binding site, another plausible explanation is that CDR3 of a particular size are more favorable for the expression of desired conformations of contact sites in another subdomain(s) of the Fab. Alternatively, significantly larger V_H CDR3 loops may be selected against because they often protrude from the surface of the classic antigen-binding site (46) and may interfere with access of pFv to another surface of the Fab.

Conserved sequences in the V_H framework regions (FRs) correlated best with pFv binding capacity. The FR1 and FR3 subdomains create a large solvent-exposed surface separate from the classic antigen-binding pocket, and this surface is highly superimposable on antibodies of the same V_H family (44, 45, 47). Significantly, antibodies encoded by $V_H26c/V3-23$, which were found to have among the strongest pFv binding interactions, contain one of the most highly conserved V_H FR1/FR3 surfaces within the human genome (reference 47; G. J. Silverman, manuscript in preparation). This gene also shares high homology with antibody genes from other species with prevalent Fab-mediated binding activity for pFv (4, 47).

The current study was not designed to discriminate amino acids that correlate with pFv binding from those retained because they help to maintain the overall "immunoglobulin fold" (45), but comparison of V_H sequences indicate that the recurrently selected pFv binders from the libraries all share cationic residues at the tip of the FR3 loop that are absent in V_H1 chains which bind pFv weakly or not at all. The 3 sequential lysines at this site in the V_H regions of the group B antibodies have not been previously reported in germline or somatic V_H variants, but the selection of this unusual variant further supports a role for cationic residues in V_H FR3 loop in binding of this endogenous sialoprotein. The unexpected finding of pFv binding among bacterially expressed V_H4 Fab, but the uncommon binding among intact V_H4 IgM, also raises the question of whether N-linked glycosylation at potential sites in V_H4 antibodies (that are generally absent in other families) can adversely influence binding. N-linked glycosylation of equivalent sites on a T cell receptor has been demonstrated to abolish binding of a T cell superantigen (48). Mutagenesis studies will be required to address these topics further.

The implications for the production of pFv in the body are little understood, and all of the immune properties of pFv described to date derive from in vitro studies. Though it has not yet been examined, we speculate that after pFv associates with immune complexes within the gut, it may accompany the immune complexes during transepithelial cell transport and influence antigen processing (49, 50). B lymphocytes that express surface Ig with rheumatoid factor (anti-IgG Fc) activity have been shown to be highly efficient at the capture of small amounts of antigen-associated immune complexes, which results in efficient presentation of a conventional antigen at 1,000-fold lower concentrations than monocytes or macrophages (51). By analogy, nearly all B lymphocytes should be able to interact via their surface Ig with pFv-associated immune complexes, which should greatly amplify the capacity to internalize and process conventional antigens in an immune complex. This proposed mechanism, in which pFv assists the immune response to ingested antigens, may contribute to the process in which oral immunization of humans results in B cell sensitization and not tolerance (52).

An influence on repertoire selection should also be considered. In the mouse, there is evidence that during lymphogenesis, certain V_H gene segments are used based on their relative position in the Ig locus, with more J_H proximal genes being preferentially rearranged (53). Although this may explain the preferential use of the human V_H6 gene (54–58), which is most J_H proximal (33, 59–61), it certainly does not explain the overexpression of other genes, such as $V_H26c/V3-23$, $1.9III/V3-30$, and $4-21/V4-34$ (40, 41, 54, 55, 57, 62–64), that are scattered throughout the V_H locus (61). Auto-antibody–mediated interactions, which include anti-idiotypes, rheumatoid factors, anti–red cell membrane, anti-DNA, and other specificities, have all been discussed as surrogates for the foreign antigens that later direct B cell repertoire selection (23, 25, 65–67). However, the earliest B lymphogenesis occurs in the liver, which suggests that if it is produced during in utero development, pFv could potentially bias clonal selection of the early B cell repertoire. The evidence that V gene segments which are overexpressed in the early repertoire may have preferential binding interactions with pFv makes this hypothesis even more appealing. Preferential binding interactions with B cell receptors with small V_H CDR3, a pattern more common during early ontogeny (38, 39), are also consistent with this speculation. Taken together, the demonstrated high affinity of antibody interactions, potential

for multivalent interactions, known localization to the liver, and the broad range of Fab that can bind all favor a role for pFv in receptor–mediated selection of the human B lymphocyte repertoire.

In summary, recent studies have demonstrated that there is a class of proteins for which there are naturally occurring B cell receptor–binding sites that are created predominantly by the contribution of the V_H gene segment. The minimal structural requirements for the creation of these sites explain why they are highly prevalent among human lymphocyte populations. Within the current studies, we have investigated the molecular basis for Fab-mediated binding of pFv. This human liver protein has the broadest of known variable region mediated–Fab binding capacities and thus the highest frequency of binding interactions with the antibody repertoire of any protein characterized to date. Within the gut, the compartmentalized and regulated expression of pFv may greatly contribute to B cell/antibody–mediated immune defenses. Recent reports suggest that SpA and the gp120 envelope proteins of certain HIV-1 strains also interact with similar V_H sites (6–9, 22, 68–70). Although yet to be proven, these infectious pathogens may have co-opted a physiologic Fab binding functional capacity, to interfere with opsonization of pathogens and normal B cell regulation within extravascular sites. Further studies will be required to investigate the physiologic expression of pFv and consider the role of pFv in the acquisition of immune competence.

Acknowledgments

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