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# Site-Directed C3a Receptor Antibodies from Phage Display Libraries[1]

**Heiko Hawlisch,* Ronald Frank,‡ Meike Hennecke,* Melanie Baensch,* Bettina Sohns,* Lubomir Arseniev,† Wilfried Bautsch,* Axel Kola,* Andreas Klos,* and Jörg Köhl[2]***

**Recent cloning of the human C3a receptor (C3aR) revealed that this receptor belongs to the large family of rhodopsin-type receptors. A unique feature of the C3aR is the large second extracellular loop comprising about 175 amino acid residues. We constructed combinatorial phage Ab libraries expressing single chain Fv Abs from BALB/c mice immunized with the affinity-purified second extracellular loop of the C3aR, fused to glutathione-*S*-transferase. A panel of anti-C3aR single chain Fv fragments (scFvs) was selected after four rounds of panning using the second extracellular loop of the C3aR, fused to the maltose binding protein as Ag. Sequencing of the clones obtained revealed three different groups of scFvs, the epitopes of which were mapped to two distinct regions within the loop, i.e., positions 185 to 193 and 218 to 226, representing the immunodominant domains of the loop. By flow cyotmetric analyses, the scFvs bound to RBL-2H3 cells transfected with the C3aR, but not to cells transfected with the C5aR or to nontransfected RBL-2H3 cells. In addition, the scFvs bound to the human mast cell line HMC-1. Immunofluorescence studies showed C3aR expression on polymorphonuclear granulocytes and monocytes, but not on lymphocytes. In addition, no C3aR expression was observed on human erythrocytes or platelets. Surprisingly, none of the scFvs alone or in combination inhibited C3a-induced $Ca^{2+}$ mobilization from RBL-2H3 cells transfected with the C3aR. In addition, C3a did not displace binding of the scFvs to the receptor, strongly suggesting that the N-terminal part of the second extracellular loop is not involved in ligand binding.** ***The Journal of Immunology,* 1998, 160: 2947–2958.**

Activation of the complement system results in the release of a variety of potent proinflammatory mediators, such as the anaphylatoxins C3a, C4a, C5a, and C5adesArg. In the guinea pig system C3a exerts numerous biologic effects, such as smooth muscle contraction probably induced by mast cell degranulation, enhanced vascular permeability, and aggregation of platelets (reviewed in Ref. 1). However, the physiologic relevance of the 77-amino acid C3a in humans has been an enigma for a long time. Only in the last few years have biologic effects, such as histamine release from IL-3-stimulated basophils (2), chemotaxis of eosinophils (3) and mast cells (4), or release of reactive oxygen metabolites from polymorphonuclear leukocytes (5) and eosinophils (6), been determined.

The pleiotropic effects of the anaphylatoxins are mediated through specific receptor interactions. C3a receptors (C3aR)[3] have been convincingly demonstrated on human leukemia-derived basophils (7), eosinophils (3, 6, 8), neutrophils, monocytes (9), and mast cells (4, 10, 11). Furthermore, C3a has been recently shown to modulate LPS-induced mRNA and protein synthesis for TNF-$\alpha$ and IL-1$\beta$ in PBMC (12) and to suppress IgG, TNF-$\alpha$, and IL-6 production in activated tonsil-derived B cells (13).

The human C5a anaphylatoxin receptor (C5aR) and the C3aR belong to the family of G protein-coupled receptors (14–18). The most conspicuous feature of the C3aR is its large second extracellular loop, which is unique among the family of seven-transmembrane receptors and which implies a functional role, e.g., in ligand binding.

Northern blot hybridization revealed a broad expression of the C3aR in different tissues, such as lung, brain, spleen, thymus, lymph nodes, bone marrow, and peripheral blood leukocytes (16–18). This wide distribution of the C3aR suggests a role for C3a even beyond its function as a pure proinflammatory mediator.

C3aR-specific mAbs would be useful to assess the expression of the C3aR on cells and tissues and might provide information about the functional role of extracellular domains. In addition, they could prove helpful in receptor mutagenesis studies. However, in general, it has been difficult to isolate mAbs to G protein-coupled receptors. In fact, only two groups have reported isolation of mAbs to the C5aR, and one of these mAbs was an IgM (19, 20).

In the last few years methods have been developed to clone the entire repertoire of Ab genes from mice or human donors and to express the encoded Abs on filamentous bacteriophage (reviewed in Ref. 21). Phage displaying specific Ab fragments on their surface can be selected for binding on Ag-coated surfaces. Ab fragments specific for haptens or proteins, e.g., C5a (22), and several human viruses have been recovered from such phage display combinatorial libraries (21, 23).

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[3] Abbreviations used in this paper: C3aR, complement 3a receptor; C5adesArg, complement 5a without C-terminal arginine residue; scFv, single chain Fv fragment; GST, glutathione-*S*-transferase; hC3aR, human complement 3a receptor; MBP, maltose binding protein; IPTG, isopropyl $\beta$-D-thiogalactoside; TYE/ap/glu, TYE plates containing ampicillin (100 $\mu$g/ml) supplemented with 1% glucose; MPBS, phosphate-buffered saline containing 2% nonfat dry milk; SN, supernatant; PBS-BSA, phosphate-buffered saline supplemented with 1% bovine serum albumin; $[Ca^{2+}]_i$, intracellular $Ca^{2+}$ concentration; CDR, complementarity-determining region.

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Here, we report the selection of site-directed C3aR Abs from scFv phage libraries derived from mice immunized with the second extracellular loop of the C3aR fused to glutathione-*S*-transferase (GST). We obtained a panel of scFv Ab that could be divided into three groups according to their nucleotide sequences. The scFvs from the three different groups reacted with two distinct regions within the second extracellular loop of the C3aR. These scFvs were used 1) to analyze the role of the second extracellular loop in terms of C3a binding, and 2) to assess C3aR expression on human polymorphonuclear leukocytes, monocytes, lymphocytes, erythrocytes, and platelets.

## Materials and Methods

### *Ag preparation*

The second extracellular loop of the hC3aR from amino acid positions 173 to 332 (C3aR-(173–332)) was amplified from plasmid pTC12 (which is pcDNAI/Amp + cDNA hC3aR) (16) using the following primers: TC1, 5′-GGCATA*G*↓*AATTC* GGCCACAAATTTGGTCTC-3′; and TC2, 5′-GGCATA*G*↓*TCGAC***TCATTA**GGGTGTTGGCACTTGATC-3′ (stop codons are in boldface; restriction sites are in italics).

The DNA fragment was digested with *Eco*RI and *Sac*I and ligated into vector pMAL-c (New England Biolabs, Beverly, MA), resulting in a fusion protein composed of maltose binding protein (MBP) and the second extracellular loop of the C3aR, MBP-C3aR-(173–332). In addition, the DNA was ligated into vector pGEX-4T-1 (Pharmacia, Freiburg, Germany), resulting in a fusion protein composed of GST and the second extracellular loop of the C3aR (GST-C3aR-(173–332), using standard protocols (24). *Escherichia coli* BL21 cells (Pharmacia, Freiburg, Germany) were transfected with plasmids pMAL-c and C3aR-(173–332) or pGEX-4T-1 and C3aR-(173–332) by electroporation (25). Protein expression was induced by adding IPTG (0.3 mM) to the bacterial culture at an $OD_{600}$ of 0.4 to 0.6. To purify the fusion proteins, bacteria were lysed by sonification, and the cytoplasmic extract containing MBP-C3aR-(173–332) was purified by chromatography using an amylose resin column (New England Biolabs). GST-C3aR-(173–332) was purified using a glutathione-Sepharose 4B column (Pharmacia). The purified proteins were concentrated by filtration through a Centriprep-10 membrane (Amicon, Danvers, MA). In silver-stained SDS gels, a band with the expected molecular mass of MBP-C3aR-(173–332) was observed at approximately 60 kDa, with an additional band at 45 kDa. In the case of GST-C3aR-(173–332), a prominent band with the expected size of 45 kDa was found, with a second band at 38 kDa. The low molecular mass bands probably represent degradation products yielded during the purification procedure (data not shown).

### *Immunization*

Five BALB/c mice were immunized i.p. with 100 μg of GST-C3aR-(172–332), with two booster injections 3 and 6 wk later. Four weeks after the second boost, a final boost (7.4 μg of Ag) was applied directly into the spleen of three mice, which exhibited the highest Ab titer as determined by ELISA using MBP-C3aR-(173–332). Total RNA was prepared from both spleen and blood cells as previously described (26).

### *Construction of the scFv library*

First-strand cDNA was generated from $poly(A)^+$ RNA (obtained from the Oligotex mRNA kit, Quiagen, Hilden, Germany) using Superscript II reverse transcriptase (Life Technologies, Eggenstein, Germany) and an $oligo(dT)_{12-18}$ primer (Boehringer Mannheim, Ingelheim, Germany). From each mouse Igγ1 and Igγ2a heavy chain repertoires as well as the κ light chain repertoire were amplified separately (Fig. 1).

In PCR I, γ1 and γ2a heavy chains and κ light chains were amplified using the following primer combinations: γ1, primers MH1 to MH7/MH9; γ2a, MH1 to MH7/JK10; and κ, MH14 to MH20/MH21 (Table I).

As template, 1.8 μl of cDNA was used. Ten microliters of Pfu buffer (10-fold concentrated), 0.8 μl of dNTP mix (25 mM of each), and 2 μl each of the 3′ and 5′ primers were added (10 pmol/μl), and the volume was adjusted to 100 μl with distilled water. The PCR cycles were as follows. Cycle I was 94°C for 300 s and XX°C for 60 s (XX = 64°C heavy chain; 58°C light chain). After the first cycle, 2.5 U of Pfu polymerase were added. Cycle II was 72°C for 120 s, 94°C for 90 s, and XX°C for 120 s. The second cycle was repeated 30 times. Cycle III was 72°C for 660 s. The correct length of the PCR products was assessed (γ1 heavy chain = 378 bp; γ2a heavy chain = 705 bp; κ light chain = 348 bp) by agarose gel electrophoresis. Bands with the correct size were purified using glass beads (Jetsorb gel extraction kit, Genomed, Bad Oeynhausen, Germany).

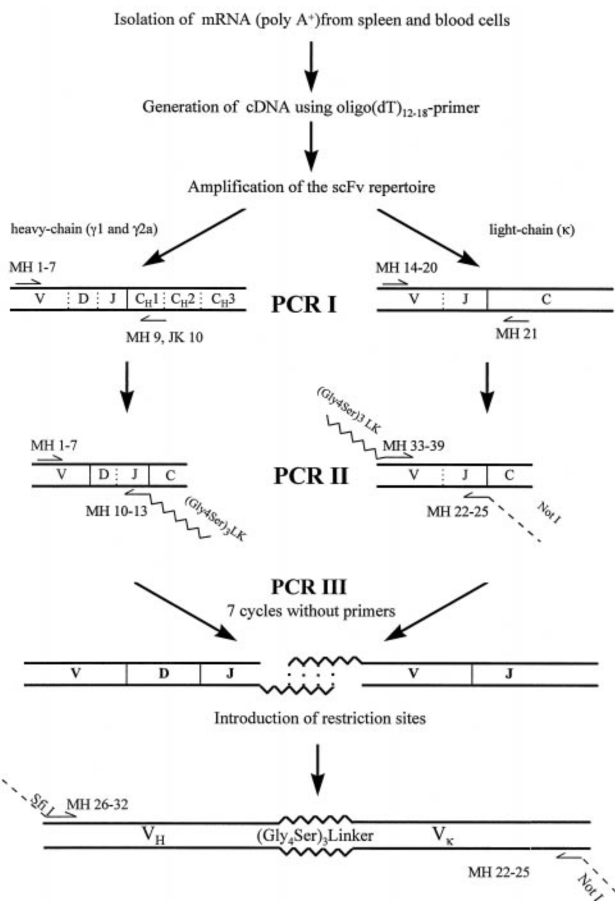


**FIGURE 1.** Flow chart of the cloning scheme used to construct the scFv repertoire.

In PCR II, the seven purified γ1 heavy chain, γ2a heavy chain, and κ light chain fragments were used as templates. Heavy chains were amplified with the same 5′ primers as those described for PCR I, however, they were now combined with four different 3′ primers, i.e., primers MH10 to MH13 (Table I and Fig. 2), specific for the four different joining region gene segments. They have a long 5′ overhang encoding for a part of the $(Gly^4Ser)_3$-linker at the 3′ end of the heavy chain. The κ light chain was amplified analogous to the heavy chains using primer combinations MH33 to MH39/MH22 to MH25 (Table I, and Figs. 1 and 2).

Two nanograms of purified PCR I product (12.8 μl PCR I product, diluted 1/100) was mixed with 10 μl of Taq buffer (10-fold concentrated; Life Technologies), 3 μl of $MgCl_2$ (50 mM), 0.8 μl of dNTP mix (25 mM each), and 2 μl each of the 3′ and 5′ primers (10 pmol/μl) and adjusted to a final volume of 100 μl with distilled water. PCR cycling was performed as described for PCR I, except that Taq polymerase (Life Technologies) was used. The annealing temperature was 55°C.

PCR II resulted in 28 γ1 heavy chain, 28 γ2a heavy chain, and 28 κ light chain fragments, with lengths of 377 bp (heavy chain) and 381 bp (light chain) (Fig. 2). Fragments of the correct size were purified as described above.

For the combination of heavy and light chains in PCR III, all PCR II products amplified by the same 5′ primer were pooled, resulting in seven γ1 and seven γ2a heavy chain pools, i.e., pools 1 to 7, each of which contained four different PCR II heavy chain products (Figs. 1 and 2). In the case of the light chain, all PCR II products amplified with the same 3′ primer, e.g., all MH22 templates, were combined, leading to four κ light chain pools, i.e., pool $J_\kappa1$ to pool $J_\kappa4$, each pool of which contained seven different PCR II light chain products (Figs. 1 and 2).

As template for PCR III we combined 10 ng of each heavy chain pool (pools 1–7) with 10 ng of every light chain pool (pools $J_\kappa1$-$J_\kappa4$), resulting in 28 different γ1 and 28 different γ2a mixtures (Fig. 5). These mixtures were cycled seven times with an annealing temperature of 60°C. Then, 2 μl of both $V_H$-specific 5′ primers (MH26–31) and $V_L$-specific 3′ primers (MH22–25; each 10 pmol/μl) were added, and the mixture was cycled

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Table I. *PCR primers used for PCR amplification of mouse Igγ1, Igγ2a, and κ repertoire*[a]

| Primer | Sequence |
|---|---|
| **PCR I** | Backward primers |
| JK10 ($C_{H1}$ γ2a) | 5′-GTT CTG ACT AGT GGG CAC TCT GGG CTC-3′ |
| MH9 ($C_{H1}$ γ1) | 5′-AGG GGC CAG TGG ATA GAC AGA TGG-3′ |
| MH21 ($C_κ$) | 5′-GGA TAC AGT TGG TGC AGC ATC AGC-3′ |
| | Foreward primers heavy-chain |
| MH1 (I A+B) | 5′-CAG GTG CAG CTK MAG GAG TCA GG-3′ |
| MH2 (II B) | 5′-CAG GTC CAR CTG CAG CAG YCT GG-3′ |
| MH3 (II A+C) | 5′-GAG GTY CAG CTG CAR CAG TCT GG-3′ |
| MH4 (III A+D) | 5′-GAR GTG AAG CTG GTG GAR TCT GG-3′ |
| MH5 (III B) | 5′-GAG GTG AAG CTT CTC GAG TCT GG-3′ |
| MH6 (III C) | 5′-GAA GTG AAG CTK GAK GAG TCT GG-3′ |
| MH7 V | 5′-GAG GTY CAG CTT CAG CAG TCT-3′ |
| | Forward primers κ-light chain |
| MH14 (I+III) | 5′-GAC ATT GTG MTG ACM CAR TCT-3′ |
| MH15 (IV+VI) | 5′-CAA AWT GTK CTC ACC CAG TCT-3′ |
| MH16 (II) | 5′-GAT GTT KTG ATG ACC CAR RCT CCA C-3′ |
| MH17 (II) | 5′-GAT GTT KTG ATG ACC CAR ACT CCA C-3′ |
| MH18 (II) | 5′-GAT RTT GTG ATG ACC CAA RCT CCA C-3′ |
| MH19 (V) | 5′-GAC ATC MAG ATG ACM CAG TCT-3′ |
| MH20 (V) | 5′-GAY ATT GTG ATG ACM CAG WCT-3′ |
| **PCR II** | $J_H$ backward primers with linker (underlined) |
| MH10 ($J_H$1) | 5′-CC TCC AGA ACC TCC GCC TCC TGA TCC GCC ACC TCC ACC TGA GGA GAC GGT GAC CGT GGT C-3′ |
| MH11 ($J_H$2) | 5′-CC TCC AGA ACC TCC GCC TCC TGA TCC GCC ACC TCC ACC TGA GGA GAC TGT GAG AGT GGT-3′ |
| MH12 ($J_H$3) | 5′-CC TCC AGA ACC TCC GCC TCC TGA TCC GCC ACC TCC ACC TGC AGA GAC AGT GAC CAG AGT-3′ |
| MH13 ($J_H$4) | 5′-CC TCC AGA ACC TCC GCC TCC TGA TCC GCC ACC TCC ACC TGA GGA GAC GGT GAC TGA GGT T-3′ |
| | Foreward primers $V_κ$ with linker (underlined) |
| MH33 (I+III) | 5′-GGC GGA TCA GGA GGC GGA GGT TCT GGA GGA GGT GGG AGT GAC ATT GTG MTG ACM CAR TCT-3′ |
| MH34 (IV+VI) | 5′-GGC GGA TCA GGA GGC GGA GGT TCT GGA GGA GGT GGG AGT CAA AWT GTK CTC ACC CAG TCT-3′ |
| MH35 (II) | 5′-GGC GGA TCA GGA GGC GGA GGT TCT GGA GGA GGT GGG AGT GAT GTT KTG ATG ACC CAR RCT CCA C-3′ |
| MH36 (II) | 5′-GGC GGA TCA GGA GGC GGA GGT TCT GGA GGA GGT GGG AGT GAT GTT KTG ATG ACC CAR ACT CCA C-3′ |
| MH37 (II) | 5′-GGC GGA TCA GGA GGC GGA GGT TCT GGA GGA GGT GGG AGT GAT RTT GTG ATG ACC CAA RCT CCA C-3′ |
| MH38 (V) | 5′-GGC GGA TCA GGA GGC GGA GGT TCT GGA GGA GGT GGG AGT GAC ATC MAG ATG ACM CAG TCT-3′ |
| MH39 (V) | 5′-GGC GGA TCA GGA GGC GGA GGT TCT GGA GGA GGT GGG AGT GAY ATT GTG ATG ACM CAG WCT-3′ |
| | Backward primers $J_κ$ with *Not*I restriction site (underlined) |
| MH22 ($J_κ$1) | 5′$(GA)_{10}$ GC GGC CGC ACG TTT GAT TTC CAG CTT GGT GCC-3′ |
| MH23 ($J_κ$2) | 5′$(GA)_{10}$ GC GGC CGC ACG TTT TAT TTC CAG CTT GGT CCC-3′ |
| MH24 ($J_κ$3) | 5′$(GA)_{10}$ GC GGC CGC ACG TTT TAT TTC CAA CTT TGT CCC-3′ |
| MH25 ($J_κ$4) | 5′$(GA)_{10}$ GC GGC CGC ACG TTT CAG CTC CAG CTT GGT CCC-3′ |
| | Foreward primers heavy-chain with *Sfi*I restriction site (underlined) |
| MH26 (I A+B) | 5′$(GA)_{10}$ G GCC CAG CCG GCC ATG GCC CAG GTG CAG CTK MAG GAG TCA GG-3′ |
| MH27 (II B) | 5′$(GA)_{10}$ G GCC CAG CCG GCC ATG GCC CAG GTC CAR CTG CAG CAG YCT GG-3′ |
| MH28 (II A+C) | 5′$(GA)_{10}$ G GCC CAG CCG GCC ATG GCC GAG GTY CAG CTG CAR CAG TCT GG-3′ |
| MH29 (III A+D) | 5′$(GA)_{10}$ G GCC CAG CCG GCC ATG GCC GAR GTG AAG CTG GTG GAR TCT GG-3′ |
| MH30 (III B) | 5′$(GA)_{10}$ G GCC CAG CCG GCC ATG GCC GAG GTG AAG CTT CTC GAG TCT GG-3′ |
| MH31 (III C) | 5′$(GA)_{10}$ G GCC CAG CCG GCC ATG GCC GAA GTG AAG CTK GAK GAG TCT GG-3′ |
| MH32 (V) | 5′$(GA)_{10}$ G GCC CAG CCG GCC ATG GCC GAG GTY CAG CTT CAG CAG TCT-3′ |
| **Sequencing** primers | |
| JK61 | 5′-ATG AAA TAC CTA TTG CCT ACG GC-3′ |
| JK75 | 5′-CC TCC AGA ACC TCC GCC TCC-3′ |
| JK76 | 5′-GGC GGA TCA GGA GGC GGA GG-3′ |
| JK77 | 5′-CTA TGC GGC CCC ATT CA-3′ |

[a] Primers used for sequencing are depicted. Primer degenerations are shown in bold face. The ambiguity codes used were the following: M = A or C; R = A or G; W = A or T; Y = C or T; K = G or T.

another 25 times. After purification, 56 scFv gene fragments (28 γ1+κ and 28γ2a+κ) with a length of 711 bp were obtained.

The 28 scFv gene fragments of each library and the vector DNA (pHEN-1) were digested with both *Sfi*I and *Not*I as previously described (21). The digested scFv DNA fragments were purified by phenol/chloroform extraction and subsequently ethanol precipitated. Vector DNA was purified from an agarose gel using glass beads (Jetsorb, Genomed). Five hundred nanograms of vector DNA was ligated with 500 ng of scFv DNA in a total volume of 100 μl with 8 μl of T4 ligase (400 U/μl; New England Biolabs) for 72 h at 4°C. The mixture was heated to 70°C for 30 min to stop the reaction. After phenol/chloroform extraction and ethanol precipitation, the phagemid DNA was dissolved in 10 μl of distilled water and used for electroporation of *E. coli* TG1 cells (Stratagene, San Diego, CA) as previously described (25). The bacteria were subsequently resuspended in 1

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**Heavy chain $V_H$ combinations used in PCR II**

| pool 1 | pool 2 | pool 3 | pool 4 |
|---|---|---|---|
| MH 1/10 | MH 2/10 | MH 3/10 | MH 4/10 |
| MH 1/11 | MH 2/11 | MH 3/11 | MH 4/11 |
| MH 1/12 | MH 2/12 | MH 3/12 | MH 4/12 |
| MH 1/13 | MH 2/13 | MH 3/13 | MH 4/13 |

| pool 5 | pool 6 | pool 7 |
|---|---|---|
| MH 5/10 | MH 6/10 | MH 7/10 |
| MH 5/11 | MH 6/11 | MH 7/11 |
| MH 5/12 | MH 6/12 | MH 7/12 |
| MH 5/13 | MH 6/13 | MH 7/13 |

**Light chain $V_\kappa$ combinations used in PCR II**

| pool Jκ1 | pool Jκ2 | pool Jκ3 | pool Jκ4 |
|---|---|---|---|
| MH 33/22 | MH 33/23 | MH 33/24 | MH 33/25 |
| MH 34/22 | MH 34/23 | MH 34/24 | MH 34/25 |
| MH 35/22 | MH 35/23 | MH 35/24 | MH 35/25 |
| MH 36/22 | MH 36/23 | MH 36/24 | MH 36/25 |
| MH 37/22 | MH 37/23 | MH 37/24 | MH 37/25 |
| MH 38/22 | MH 38/23 | MH 38/24 | MH 38/25 |
| MH 39/22 | MH 39/23 | MH 39/24 | MH 39/25 |

**Primercombinations used in PCR III**

| | pool Jκ1 | pool Jκ2 | pool Jκ3 | pool Jκ4 |
|---|---|---|---|---|
| pool 1 | MH 26 + 22 | MH 26 + 23 | MH 26 + 24 | MH 26 + 25 |
| pool 2 | MH 27 + 22 | MH 27 + 23 | MH 27 + 24 | MH 27 + 25 |
| pool 3 | MH 28 + 22 | MH 28 + 23 | MH 28 + 24 | MH 28 + 25 |
| pool 4 | MH 29 + 22 | MH 29 + 23 | MH 29 + 24 | MH 29 + 25 |
| pool 5 | MH 30 + 22 | MH 30 + 23 | MH 30 + 24 | MH 30 + 25 |
| pool 6 | MH 31 + 22 | MH 31 + 23 | MH 31 + 24 | MH 31 + 25 |
| pool 7 | MH 31 + 22 | MH 31 + 23 | MH 31 + 24 | MH 31 + 25 |

**FIGURE 2.** Summary of primer combinations used for PCR II and III.

ml of SOC medium (2% tryptone; 0.5% yeast extract; 10 mM NaCl; 25 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$; 20 mM glucose) and incubated for 1 h at 37°C. After incubation, bacteria from one library were plated on large TYE plates containing ampicillin (100 μg/ml) supplemented with 1% glucose (TYE/ap/glu; Ø = 145 mm) and incubated overnight 37°C. The titer of the library was determined by plating 10-fold dilutions ($10^{-2}$-$10^{-7}$) of the electroporated bacteria on TYE/ap/glu plates. The bacteria grown were scraped off the plates with 4 ml of 2 × TY/ap/glu. This primary stock was stored at −20°C.

From the primary stock, 5 μl was used to inoculate 50 ml of 2 × TY/ap/glu. The culture was shaken at 37°C until the $OD_{600}$ reached 0.5. Then, 10 ml was removed and superinfected with $10^{11}$ pfu of VCS-M13 at 37°C for 30 min without shaking. Cells were pelleted by centrifugation, subsequently resuspended in 300 ml of 2 × TY/ap and kanamycin (25 μg/ml), and grown overnight at 30°C with shaking (250 rpm). Phage particles were purified and concentrated by polyethylene glycol precipitation (24).

### *Affinity enrichment of C3aR-specific scFvs*

In the first panning round, immunotubes (Nunc, Wiesbaden, Germany) were coated with MBP-C3aR-(173–332) (50 μg/ml in PBS) and incubated overnight at room temperature with gentle rotation. The tube was washed three times with PBS and subsequently blocked with PBS containing 2% nonfat dry milk (MPBS) for 2 h at 37°C. After three times washing with PBS, 6 × $10^{11}$ phages, that is $10^{11}$ phages from each library, were added to 440 μl of MPBS (2.2%) and incubated for 2 h at room temperature with gentle rotation. The tube was washed 10 times with PBS/Tween-20 (0.1%) and 10 times with PBS. Bound phages were eluted with 1 ml of HCl/glycine (0.1 M; pH 2.2; plus 1% BSA) for 10 min at room temperature. Phages were immediately neutralized with 187.5 μl of Tris/HCl (1 M; pH 9.1) and used to infect *E. coli* TG1 cells. The numbers of phages before and after panning were determined by plating infected *E. coli* Tg1 cells onto TYE/ap/glu plates.

During the next panning rounds, the stringency of the selection conditions was increased as follows. In panning rounds 2 and 3, 10 μg/ml Ag were used. In round 4, the Ag concentration was reduced to 10 ng/ml, and panning was performed in a microtiter plate. In panning rounds 3 and 4, both the tube and the microtiter plate were washed 20 times with PBS/-Tween-20 (0.1%) and 20 times with PBS.

### *Fingerprint analysis*

To analyze the diversity of the scFv libraries during the selection process, individual clones were isolated after each panning round. The scFv DNA of a particular clone was amplified by PCR using primers JK 61 (which sits within the c-Myc peptide fused C-terminal to the $V_L$ chain sequence) and JK 77 (which sits within the pelB sequence). The PCR fragment of the scFv gene was digested using the frequent cutter endonuclease *Bst*NI as previously described (27).

### *Generation and partial purification of scFvs*

*E. coli* strain HB2151 transfected with plasmid pHEN1 and scFv Ab was grown in 2 × TY/amp supplemented with glucose (0.1%) until the $OD_{600}$ reached 0.9. Protein expression was started by adding IPTG to a final concentration of 1 mM. After overnight culture at room temperature with shaking (200 rpm), bacteria were centrifuged (15 min, 11,325 × *g*, 4°C). The supernatant (SN) was concentrated in two steps using the ProVario-3 filtration and concentration system (Filtron, Karlstein, Germany). All steps were performed at 4°C. First, the SN was filtrated through an OMEGA membrane (Filtron) (0.16 μm). The filtrate was subsequently concentrated from 1 l to 50 ml by filtration through a second OMEGA membrane (10 kDa) and was finally dialyzed three times against PBS. The concentrated scFvs were quantified by densitometry using Herolab EasyImage software (HeroLab, Weisloch, Germany).

### *Rescue of phage or soluble scFvs from individual phagemid clones*

Phage were rescued from single ampicillin-resistant colonies of infected *E. coli* TG1 cells using the helper phage VCSM13 as described by Marks et al. (27). To produce soluble scFv Abs, *E. coli* HB 2151 cells were infected with monoclonal phage expressing a particular scFv fragment. The bacteria were grown in 2 × TY/ap supplemented with 0.1% glucose to $OD_{600}$ 0.9 at 37°C. Subsequently, IPTG was added to a final concentration of 1 mM, and bacteria were grown overnight at room temperature with shaking (250 rpm). SN containing either scFv phages or soluble scFvs were used for ELISA analysis.

### *C3aR-(173–332) specific ELISA*

MBP-C3aR-(173–332) (10 μg/ml, 50 μl/well) was coated overnight on a polystyrene microtiter plate (Greiner), rinsed three times in PBS, and saturated in MPBS (2%) for 2 h. All steps were performed at room temperature. After washing three times with PBS, 25 μl of 10% MBPS was added followed by the addition of 100 μl of phages or scFvs. To compare the binding affinity of different phage Abs, the phage number was adjusted to 3 × $10^9$/100 μl. After incubation for 90 min, plates were washed three times with PBS/0.05% Tween-20, three times with PBS, and subsequently incubated with 100 μl of a 1/1000 dilution of a rabbit anti-M13 serum (28). Before use, the antiserum (1/500 diluted in 4% MPBS) was incubated with 2.5 ml of a cytoplasmic solution of *E. coli* BL21 cells overnight at room temperature to prevent cross-reaction of the antiserum with *E. coli* proteins in the Ag preparation. When scFvs were added, plates were incubated with mAb 9E10 (100 μl/well) to detect the c-Myc peptide fused C-terminal to the $V_L$ chain as previously described (21). Plates were washed three times with 50 mM Tris/0.15 M NaCl, pH 7.5; 100 μl of alkaline phosphatase-conjugated goat anti-rabbit polyclonal Ab (Amersham, Braunschweig, Germany) diluted 1/2000 in 50 mM Tris buffer/0.15 M NaCl, pH 7.5, was added; and the plates were incubated for 90 min. To detect scFvs, 100 μl/well peroxidase-conjugated anti-mouse Ab (Sigma, Munich, Germany) diluted 1/1000 in 50 mM Tris buffer/0.15 M NaCl, pH 7.5, was added and incubated for 60 min. Plates were either developed with 100 μl of alkaline phosphatase substrate solution or, in the case of scFvs, with 100 μl of peroxidase substrate solution as previously described (28, 29).

### *Cell culture conditions*

The rat basophilic leukemia cell-lines (RBL-2H3) stably transfected with either the hC3aR cDNA or the hC5aR cDNA (30) were grown as described previously (18, 31). The culture conditions used for the human mast cell line HMC-1 were previously described (10).

### *Isolation of human PMNs and mononuclear cells*

Highly purified PMNs (with an erythrocyte contamination of <5% and an eosinophil contamination of 1–4%) and mononuclear cells (i.e., lymphocytes and monocytes) were prepared by density centrifugation from citrate-anticoagulated blood of healthy human donors using Polymorphprep (Nycomed, Oslo, Norway) according to the manufacturer's instructions. Usually 1 to 2 × $10^7$ cells were obtained from 40 ml of citrate-anticoagulated blood. The mononuclear cells were collected from the upper cell layer; the neutrophils were obtained from the lower layer. The cells were washed twice with PBS, counted, and resuspended in PBS/BSA (1%) to

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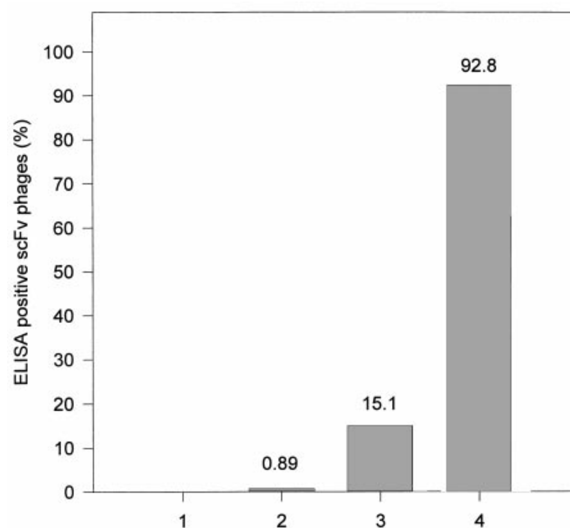


**FIGURE 3.** Number of positive phages through rounds of panning. After each cycle, between 106 and 126 different phage clones were assessed for binding to MBP-C3aR-(173–332) using an ELISA. Arabic numerals at the *bottom* of the columns give the number of cycles.

$5 \times 10^6$ cells/ml. Cells were assayed immediately after isolation. Purification of human platelets was performed exactly as described for guinea pig platelets (32).

*Immunofluorescence microscopy studies*

All steps were performed in single wells of a 96-well microtiter plate. Wells were blocked with 250 $\mu$l of PBS supplemented with 1% BSA (PBS-BSA) for 2 h at room temperature. Purified cells or cell lines were washed once in 50 ml of PBS-BSA and were finally adjusted to a density of $5 \times 10^6$ cells/ml. One hundred microliters of cells were transferred to each well, and the plates were sealed (Dynatech Microtiter System, Denkendorf, Germany). Cells were pelleted by centrifugation for 4 min at $514 \times g$ at room temperature (Minifuge RF, Heraeus Sepatech, Osterode, Germany). The SN was discarded. One hundred microliters of phage or 10 $\mu$g/ml scFvs (100 $\mu$l) were added to the cells. In competition binding assays 1 $\mu$M purified C3a (Advanced Research Technologies, La Jolla, CA) was added along with the scFvs. Cells were incubated for 60 min on ice (all remaining incubation and centrifugation steps were performed at 4°C). Cells were pelleted by centrifugation and were washed twice with 150 $\mu$l/well PBS-BSA. The pellet was resuspended in 100 $\mu$l of PBS-BSA containing mAb 9E10 (1/1000), and the mixture was incubated for 90 min. When scFv phages were used, a rabbit anti-M13 antiserum (1/1000 in PBS-BSA) was added. After washing twice in PBS-BSA, pellets were resuspended in 100 $\mu$l of anti-mouse TRITC/anti-rabbit TRITC (Dianova; 1/100 in PBS-BSA) and incubated for 30 min in the dark. Cells were washed twice with PBS, resuspended in 10 $\mu$l of mounting fluid (PBS/glycerol (50%)) and examined visually on a Zeiss III RS immunofluorescence microscope (Zeiss, New York, NY).

*Flow cytometric analysis of RBL-2H3 cells and the human mast cell line HMC-1*

For flow cytometric measurements cells were treated as described for the immunofluorescence microscopy studies with the following modifications: 1) PBS was used for each washing step; 2) for detection of scFvs, 100 $\mu$l of FITC-labeled anti-mouse antiserum (Becton Dickinson, Heidelberg, Germany; diluted 1/10 in PBS-BSA) was used; and 3) after the last wash cells were resuspended in 150 $\mu$l of PBS/formaldehyde (1%). Cells were assessed in the flow cytometer FACScan and stored as list mode data for subsequent analysis using CellQuest software (Becton Dickinson).

*DNA sequencing*

DNA from single colonies was prepared using standard protocols and sequenced by PCR in a volume of 50 $\mu$l using the following primers: JK61 (a 5′ primer that anneals to the pelB leader sequence), JK75 (a 3′ primer that anneals to the linker sequence), JK76 (a 5′ primer that anneals to the linker sequence), and JK77 (a 3′ primer that anneals to the geneIII sequence; Table I). Sequencing was performed with the Sequenase PCR product sequencing kit and Sequenase version 2.0 DNA polymerase (U.S. Biochemical Corp., Cleveland, OH) according to the manufacturer's instructions.

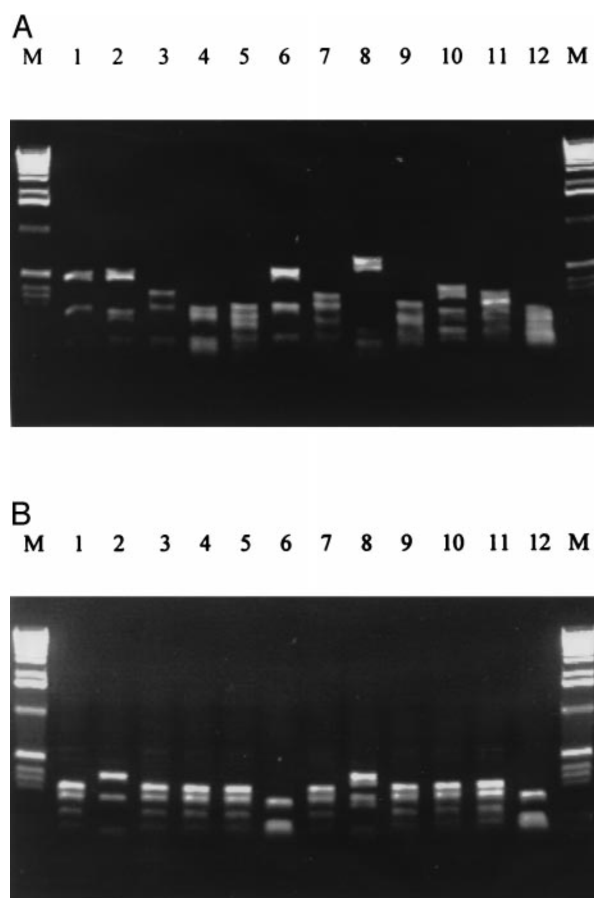


**FIGURE 4.** *Bst*NI fingerprinting of clones before and after panning. Twelve clones before panning (*A*) and after the fourth panning round are depicted. M, marker.

*Peptide synthesis and epitope mapping*

Peptides were synthesized as arrays of N-terminally acylated and C-terminally covalently immobilized products on cellulose sheets derivatized with $\beta$Ala-$\beta$Ala dipeptide anchors by the spot synthesis technique as previously described (33), using a model ASP 222 spotting robot (ABIMED Analysen-Technik GmbH, Langenfeld, Germany). Binding of the scFvs to peptide spots was assayed as previously described with a slight modification (34). After scFv binding, mAb 9E10 was used as the secondary Ab. For detection, an alkaline phosphatase-conjugated goat anti-mouse Ab was used. Peptide arrays included overlapping pentadecapeptides, with an offset of three amino acid residues, spanning the second extracellular loop of the C3aR from positions 173 to 332.

*Measurement of $[Ca^{2+}]_i$*

Measurement of the change in $[Ca^{2+}]_i$ in fura-2/AM-loaded cells was performed exactly as described previously (35). Fluorescence data were converted to $[Ca^{2+}]_i$ by the formula of Grynkiewicz et al. (36).

# Results

*Construction of the scFv combinatorial libraries*

Seven primers specific for mouse $V_H$ subgroups I to V and seven primers specific for mouse $\kappa$-chain subgroups I to VI were constructed. From each subgroup, sequences available in the Kabat database (37) were aligned, and the frequencies of codons encoding the first N-terminal eight amino acids were determined. Best fit

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A

| Group | Clone | CDR 1 | CDR 2 | CDR 3 |
|---|---|---|---|---|
| I | 3G7 | S H G V S | V I W G D G N T K Y H S A L I S | H Y Y K Y A N Y A M D Y |
| | 3F7 | N * * * * | * * * * * * T * * * * * V * * * | * * * * * D * F * L * * |
| II | 2A1 | N Y A M S | S I S S G G T T Y Y P D S V K G | P A S G N D V G F A Y |
| | 4H12 | * * * * * | * * * * * * * * F * * * * * * * | * * * * * * * * * * * |
| | 2E12 | X X X X X | * * * * * * S * * * * * * * * * | * P Y * * * * * * * * |
| | 3H4 | * * * * * | * * * * * * N * . . * * * * * * | * P N * * * * * * * * |
| | 4G2 | X X X X X | * * * * * * V * * * * * * * * * | * P * * * Y * V * * * |
| | 3D7 | * * * * * | * * * * * * S * * * * * * * * * | * P * * * Y * * * * * |
| III | 3A6 | D T Y M H | M I D P A N D N A I Y D P K F Q G | T G D P N R Y F D V |
| | 3H8 | X X X X X | X X X X X X X X X X X X X X X X * | * * * * * * * * * * |
| | 3C6 | * * * L * | * * * * * * * D T V * * * * * * * | * * * S * * * * * * |

B

| Group | Clone | CDR 1 | CDR 2 | CDR 3 |
|---|---|---|---|---|
| I | 3G7 | K A S Q N V G T N V A | S A S Y R Y S | Q Q Y N S Y P F T |
| | 3F7 | * * * * * D * * * * * | * * * * * * R | * * * * * * * * * |
| II | 2A1 | R S S Q S I V H S N G N T Y L E | K V S N R F S | F Q G S H V P P T |
| | 4H12 | * * * * * * * * * * * * * * * * | * * * * * * * | * * * * * * * * * |
| | 2E12 | * * * * N * * * * * * * * * * * | T * * * * * * | T * * * * * * * * |
| | 3H4 | * * * * T * L * * * * * * * * * | T * * * * * * | T * * * * * * * * |
| | 4G2 | X X X X X X X X X X X X X X X X | T * * * * * * | T * * * * * * * * |
| | 3D7 | * * * * * * V * * * * * * * * * | * * * * * * * | * * * * * * * * * |
| III | 3A6 | K S S Q S L L N S R T R K N Y L A | W A S T R E S | K Q S Y N L W T |
| | 3H8 | X X X X X X X X X X X X X X X X X | * * * * * X * | * * * * * * * * |
| | 3C6 | * * * * * * * * * * * * * * * * * | * * * * * * * | * * * * * * * * |

**FIGURE 5.** *A*, Deduced protein sequences of heavy chain CDR1-CDR3. *B*, Deduced protein sequences of light chain CDR1-CDR3. X = positions that have not been sequenced or that could not be determined. Roman numerals depict the three different groups due to sequence homologies. The EMBL Nucleotide Sequence Database accession numbers are: Clone 3G7 heavy chain = AJ222590, 3G7 light chain = AJ222591. Clone 3F7 heavy chain = AJ222592, 3F7 light chain = AJ222593. Clone 2A1 heavy chain = AJ222594, 2A1 light chain = AJ222595. Clone 4H12 heavy chain = AJ222596, 4H12 light chain = AJ222597. Clone 2E12 heavy chain = AJ222598, 2E12 light chain = AJ222599. Clone 3H4 heavy chain = AJ222600, 3H4 light chain = AJ222601. Clone 4G2 heavy chain = AJ222602, 4G2 light chain = AJ222603. Clone 3D7 heavy chain = AJ222604, 3D7 light chain = AJ222605. Clone 3A6 heavy chain = AJ222606, 3A6 light chain = AJ222607. Clone 3H8 heavy chain = AJ222608, 3H8 light chain = AJ222609. Clone 3C6 heavy chain = AJ222610, 3C6 light chain = AJ222611.

primers were designed to match the majority of codons of a subgroup with a maximum of two ($V_H$ primers) or three ($V\kappa$) degenerations. To amplify the mouse repertoire, a three-step PCR approach was performed similar to that described by Marks et al. (27), except that a two-fragment PCR assembly process was used (Fig. 1). In PCR I, heavy and light chain sequences were amplified using variable region-specific primers combined with constant region-specific primers, i.e., $\gamma$1 or $\gamma$2a in the case of the heavy chain, and $\kappa$ in the case of the light chain (Fig. 1). In PCR II, purified DNA fragments of PCR I were used to amplify the $V_H$ and the $V_L$ regions using joining region-specific primers (Fig. 1). In PCR III, $V_H$ and $V_L$ fragments were fused to scFvs by overlap extension of linker segments introduced at the 3′ end of the $V_H$ fragment and at the 5′ end of the $V_L$ fragment during PCR II. In addition, restriction sites were introduced for cloning into the phagemid vector pHEN1.

### *Selection and characterization of the C3aR Abs*

Six combinatorial Ab phage libraries from three BALB/c mice were generated. Each library comprised either the Ig$\gamma$1 or the Ig$\gamma$2a heavy chain repertoire combined with the $\kappa$ light chain repertoire. The sizes of the libraries ranged from $4.8 \times 10^5$ to $6.9 \times 10^6$. From each library $10^{11}$ phages were mixed and incubated in four iterative cycles with MBP-C3aR-(173–332). After each round of panning, between 106 and 126 clones were randomly picked and assessed for binding to MBP-C3aR-(173–332). As shown in Figure 3, none of the clones rescued from panning round 1 bound to the Ag. However, during progressive panning rounds, the number of positive clones increased from nearly 1% after round 2 and 15% after round 3 to >90% after round 4.

DNA fingerprinting of the clones recovered after the first round of panning, using *Bst*NI, revealed a heterogeneous restriction pattern, indicating that numerous unique Abs were present in this population. By the third round of panning, only three restriction patterns were detected (Fig. 4). Abs with the same restriction pattern were found to have a similar sequence, with only a few point mutations within the framework or the complementarity-determining regions (CDRs; Fig. 5). More than 50% of all clones belonged to group II (6 of 11). Three of the remaining clones belonged to group III, and two clones belonged to group I.

Phage clones expressing MBP-C3aR-(173–332)-specific scFvs were used to infect cells of the *E. coli* nonsuppressor strain HB2151. Infected bacteria were induced with IPTG to express soluble scFv Abs. The SN of each individual clone was tested for reactivity with MBP-C3aR-(173–332). In Figure 6 the binding results of both phage Abs and soluble scFvs with MBP-C3aR-(173–332) are depicted. The scFvs of clones 3G7 and 3F7 (group I) gave very strong signals. In fact, from clone 3G7 both scFv phage and the soluble scFvs exhibited the highest values of all clones tested. Clones 3G7 and 3F7 differed at six positions in the heavy chain and at two positions in the light chain CDRs (Fig. 5). From group II, scFvs of clones 2A1 and 4H12 gave strong signals in the ELISA. The reactivity of the phage Abs of these clones, however, differed substantially, i.e., the ELISA signal obtained with clone

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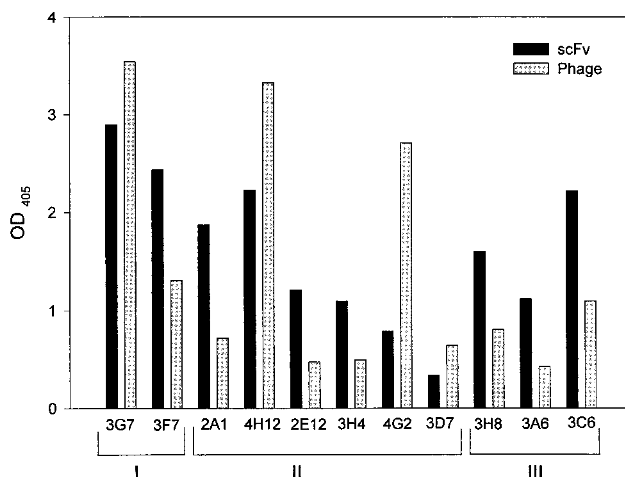


**FIGURE 6.** Comparison of ELISA signals obtained from phage Abs or soluble scFvs. The Roman numerals delineate the three different groups of clones that were tested. To compare the affinities of individual clones, phage numbers were adjusted to $3 \times 10^9$ for each clone.

4H12 was 5 times higher than the signal obtained with clone 2A1. Sequence comparison of both clones revealed only one Y58F replacement within the CDR2 of clone 4H12. Both clones shared the same light chain. The majority of the phage Abs of the remaining clones of group II (2E12, 3H4, and 3D7) gave only faint signals in ELISA, except clone 4G2, which gave a very strong signal (Fig. 6). This increased reactivity of clone 4G2 toward MBP-C3aR-(173–332) is probably due to amino acid replacements within CDR2 (at position 56) and CDR3 (at position 100J) of the heavy chain, two amino acid substitutions that are distinct from the other clones of group II. From group III, phage Abs of clone 3C6 exhibited the strongest signal. The differences in reactivity between clones 3C6 and 3A6 must be attributed to the heavy chains, since both clones shared the same light chain. Indeed, these clones differed at five positions within the CDRs.

Representative clones from each group were selected for immunofluorescence microscopy studies and flow cytometric analysis, i.e., clone 3G7 from group I, clone 2A1 from group II, and clone 3C6 from group III.

### *The scFvs bind to RBL-2H3 cells stably expressing the C3aR and to the human mast cell line HMC-1*

The scFvs were selected by binding to a fusion protein. To determine whether the scFvs recognize the C3aR on cells, we performed both immunofluorescence microscopy studies and flow cytometric analysis using C3aR-transfected RBL-2H3 cells and the human mast cell line HMC-1, which naturally expressed the C3aR (4, 10, 11). To evaluate the specificity of C3aR binding, both binding to C5aR-transfected RBL-2H3 cells and that to nontransfected RBL-2H3 cells were investigated. The histograms shown in Figure 7 demonstrate that scFv 3G7 binds solely to the C3aR-transfected RBL-2H3 cells, not to C5aR-transfected or nontransfected cells. The same results were obtained for scFvs 2A1 and 3C6. In addition, strong staining was observed for the HMC-1 cell line. As a negative control, each cell type (RBL-2H3 C3aR, RBL-2H3 C5aR, RBL-2H3, and HMC-1 cells) was incubated with a control scFv (directed against a cell surface Ag of CHO cells). No specific staining could be detected with this scFv. The same results were obtained in immunofluorescence microscopy studies (data not shown). However, when phage Abs instead of scFvs were used for immunostaining, a high background was observed for C5aR-transfected or nontransfected RBL-2H3 as well as for HMC-1 cells.

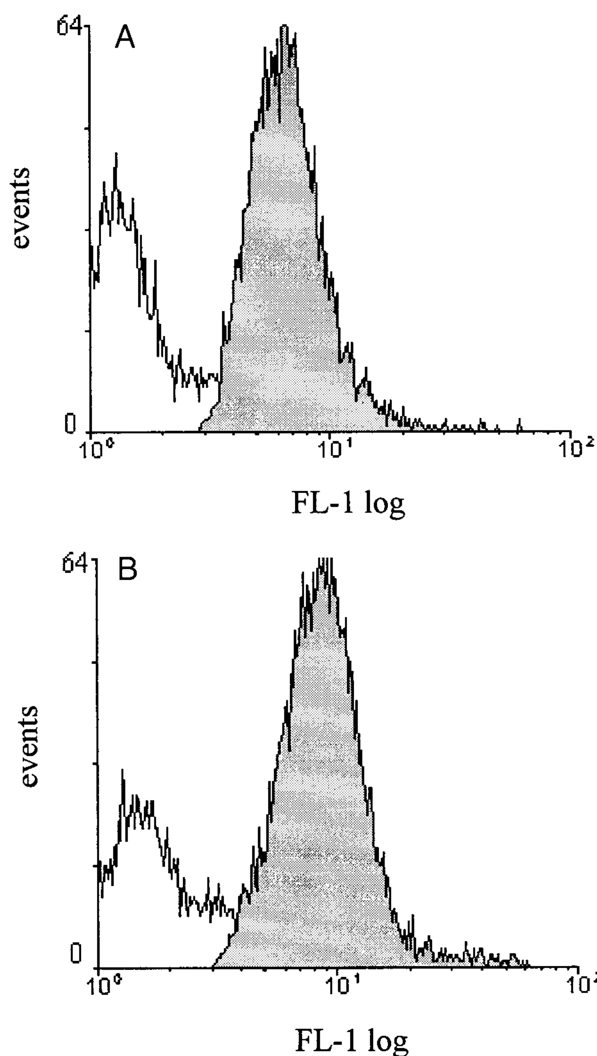


**FIGURE 7.** Histograms of binding of C3aR scFvs to both RBL-2H3 cells transfected with the hC3aR (*A*) and the human mast cell line HMC-1 (*B*). C3aR-transfected RBL-2H3 cells (dashed area) and C5aR-transfected RBL-2H3 cells were incubated with scFv 3G7 and subsequently mixed. Human mast cells were incubated with either scFv 3G7 (dashed area) or a control scFv. Data from one typical experiment of three performed is given. For each cell type, one typical experiment of a total of three performed is depicted.

### *C3aR is expressed on human neutrophils and monocytes, but not on lymphocytes, platelets, or erythrocytes*

Peripheral blood leukocytes were purified from citrate anticoagulated blood using Polymorphprep. This procedure results in two cell layers, the lower of which contains the neutrophils with a contamination of 1 to 4% eosinophils and 2 to 5% erythrocytes. In the upper layer, mononuclear cells are enriched, i.e., monocytes and lymphocytes contaminated with platelets. Purified cells from both layers were stained by the DiffQuick method (Merz + Dade, AG, Düdingen, Switzerland) and examined by both phase contrast and immunofluorescence microscopy (Fig. 8). In Figure 8*A* a typical DiffQuick staining of cells isolated from the lower cell layer is depicted, showing only neutrophils and some contaminating erythrocytes. In the phase contrast view (Fig. 8*B*), the polymorphonuclear structure of the nucleus is visible. All neutrophils that can be seen in the phase contrast view exhibited strong staining in the immunofluorescence view (Fig. 8*C*). No immunofluorescence signal was obtained from erythrocytes. The DiffQuick staining of the

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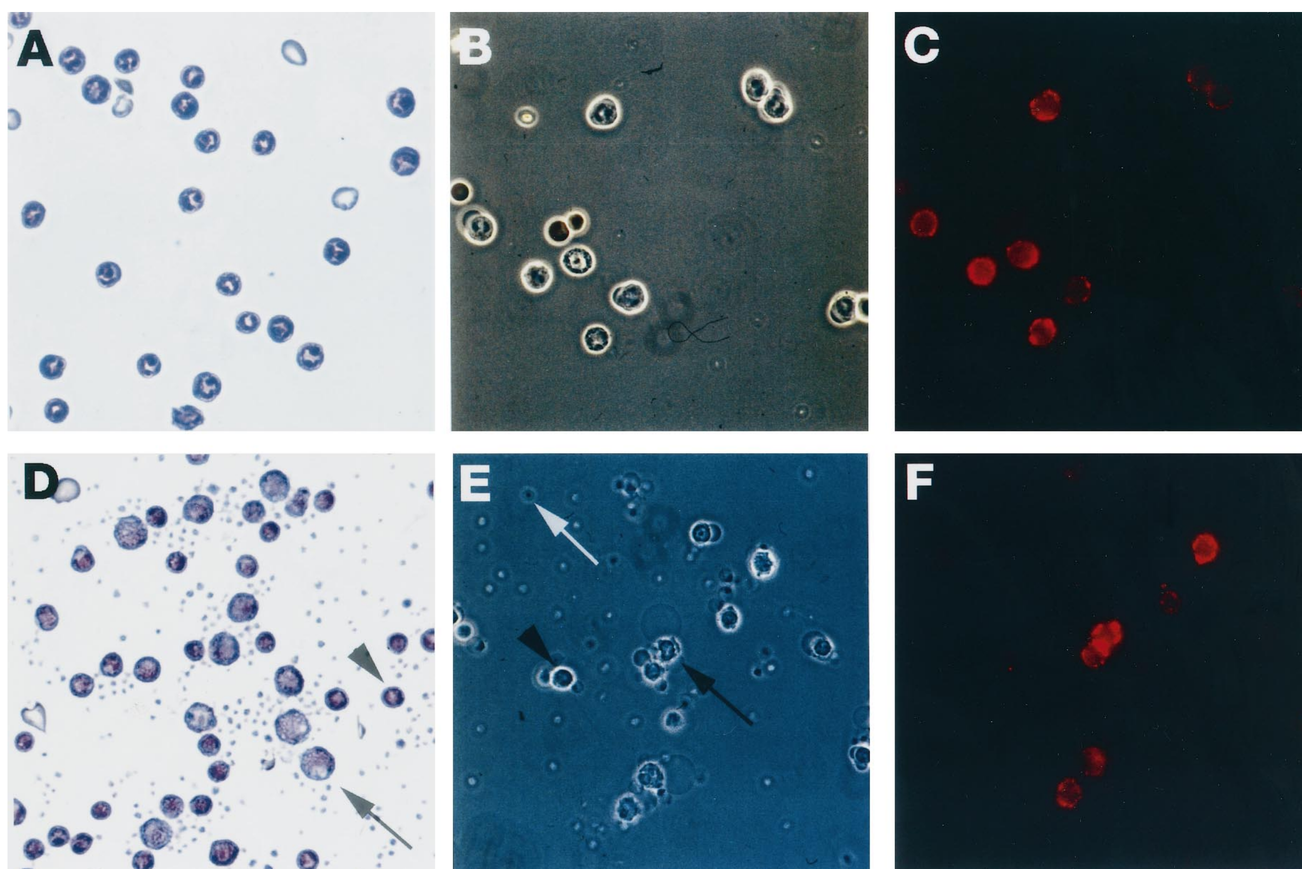


**FIGURE 8.** The scFvs bind to human neutrophils and monocytes, but not to lymphocytes, platelets, or erythrocytes. Peripheral blood cells were purified using Polymorphprep. *A*, DiffQuick stain of purified PMN. *B*, Phase contrast view of purified PMN. *C*, scFv immunostaining of purified PMN. Please note that every PMN shown in *B* gives a positive signal, whereas the erythrocytes are not stained. *D*, DiffQuick staining of purified monocytes (long black arrow), lymphocytes (short black arrow), and platelets. *E*, Phase contrast view of purified monocytes (long black arrow), lymphocytes (short black arrow), and platelets (white arrow). *F*, scFv immunostaining of monocytes, lymphocytes, and platelets. Only the monocytes were recognized by the scFvs, whereas no immunofluorescence signal was obtained from lymphocytes or platelets.

upper cell layer revealed three different cell populations, i.e., monocytes, lymphocytes, and platelets (Fig. 8*D*). In the immunofluorescence view, only a few cells were stained (Fig. 8*F*). Examination of the cells by phase contrast microscopy demonstrated that the monocytes, but not the lymphocytes or platelets, were C3aR positive (Fig. 8*E*). To verify that platelets were not stained by the scFvs, this cell population was also purified from platelet-rich plasma. In this preparation platelets were contaminated by 2% erythrocytes. Again, no staining was observed (data not shown).

### *C3aR scFvs recognize two different epitopes of the second extracellular loop*

To assess whether the clones belonging to the three different groups recognize a common or different epitopes on the second extracellular loop of the C3aR, a peptide scanning analysis was performed. Fifteen-mer peptides spanning the whole sequence of C3aR-(173–332) were synthesized with an offset of three amino acid residues. As shown in Figure 9 the scFvs 3G7 and 3C6, belonging to groups 1 and 3, respectively, reacted with three different peptides from positions 179 to 199, i.e., peptides P3 to P5 (Fig. 9, *A* and *B*). The consensus sequence of these peptides is from positions 185 to 193. The scFv 2A1 belonging to group 2 was positive for three peptides from positions 212 to 232, i.e., peptides P14 to P16, with a consensus motif from positions 218 to 226 and for peptide P5 (positions 185–199).

### *The C3a-C3aR interaction is not inhibited by the C3aR scFvs*

To analyze whether the scFvs are C3aR agonists, we tested their ability to directly stimulate $Ca^{2+}$ mobilization in RBL-2H3 C3aR cells. Conversely, to determine whether the scFvs are C3aR antagonists we tested the ability of the scFvs to inhibit C3a-stimulated $Ca^{2+}$ mobilization from RBL-2H3 C3aR cells.

First, the scFvs were incubated with the C3aR-transfected RBL-2H3, either individually or in combination (clone 3G7 + 2A1 or clone 3C6 + 2A1). The scFvs did not appear to function as receptor agonists, as we did not observe any increase in $[Ca^{2+}]_i$ (data not shown) from the cells challenged with the scFvs. Then, the scFvs were added to the cells either 5 min before addition of the C3a stimulus or simultaneously with C3a. None of the scFv Abs affected C3a-stimulated $Ca^{2+}$ mobilization in RBL-2H3 C3aR cells regardless of whether they were applied alone or in combination (Fig. 10). In addition, we tested the antisera obtained from the three different mice used for the generation of the libraries (diluted 1/10). Again, no inhibition of the functional response was observed, indicating that the N-terminal part of the second extracellular loop is probably not involved in ligand binding. We also tested the ability of C3a to reduce the fluorescence signal obtained by the different scFvs in immunofluorescence microscopy using C3aR-transfected RBL-2H3 cells. No detectable difference was observed between samples with or without competing C3a (data

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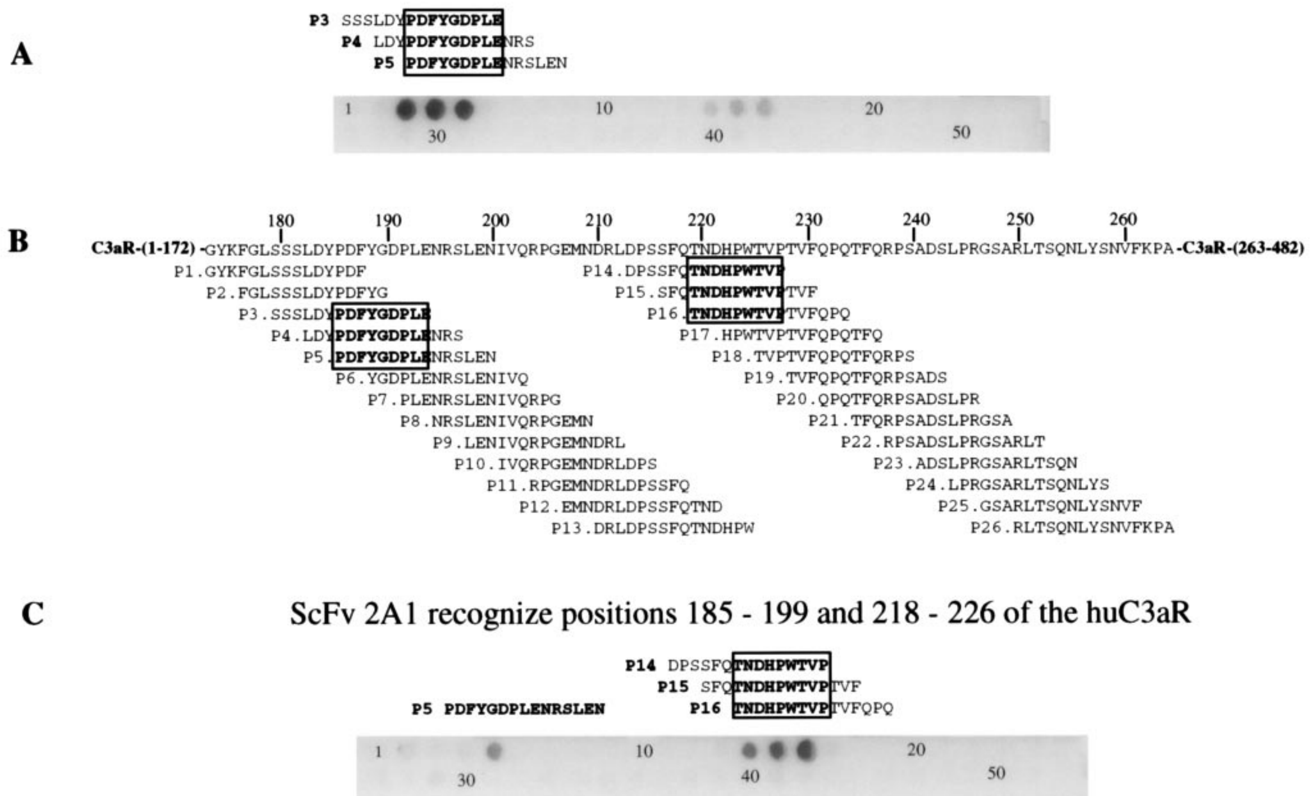


**FIGURE 9.** Epitope mapping of scFv 3G7 (*A*; group I) and scFv 2A1 (*C*; group II) by SPOTscan analysis. Fifteen-mer peptides, which span the whole sequence of the second extracellular loop of the C3aR, i.e., C3aR-(173–332), were spotted on a cellulose sheet, i.e., 50 different peptides with an offset of three residues. For better orientation, the position of every 10th peptide spot is depicted in *A* and *C* by Arabic numerals. In *B*, both the sequence of the second extracellular C3aR loop from positions 173 to 262 and the overlapping 15-mer peptides (P1–P26) are depicted. Peptides P27 to P50, spanning C3aR 263–332, have been omitted, since none of the scFvs bound to peptides within the region (as shown in *A* and *C*). The consensus motifs recognized by the scFvs are indicated both in boldface and by shaded boxes. In *A*, peptides P3 to P5 are depicted (with the consensus motif in boldface and with shaded boxes) corresponding to the three dark spots recognized by scFv 3G7. The three faint spots in *A* (peptides P14–P16) are due to incomplete stripping of the membrane. The scFv 3C6 (group III) showed exactly the same binding pattern as that described for scFv 3G7 (data not shown). In *C*, the reactivity of scFv 2A1 with the C3aR-derived peptides is shown. Four dark spots can be seen, marking peptide P5 (on the *left*) and peptides P14 to P16 (on the *right*). The sequences of peptides P14 to P16 (with the consensus motif indicated in boldface and by shaded boxes) are shown on the *top* of the three dark spots.

not shown). The fact that a threefold molar excess of C3a (1 $\mu$M vs 300 nM scFvs), which binds with high affinity to the C3aR ($K_d$ 1 nM), did not displace the scFvs from the receptor implies that the binding site of C3a and that of the scFvs are in distinct regions.

## Discussion

The hC3aR has recently been cloned by us and others (16, 17, 18). The cDNA clone encodes a protein with 482 amino acids and a molecular mass of 53,864 Da. In comparison, the C5aR contains 350 amino acids with a calculated molecular mass of only 39,320 Da. The difference in size between these two anaphylatoxin receptors is due to the difference in the length of the second extracellular loop, which comprises 175 amino acids in the C3aR, but only 22 amino acids in the C5aR. This loop is unique within the large seven-transmembrane domain receptor superfamily. In fact, no homology to any known protein was found when scanning the SwissProt databank. This feature renders this part of the receptor an interesting candidate for the production of Abs, minimizing the probability of cross-reactivities of such Abs to other proteins.

We decided to clone Ab fragments from combinatorial phage libraries, since the generation of C5aR-specific mAbs using hybridoma technology had only limited success. The approach we used to generate anti-C3aR scFvs, immunization with a C3aR second extracellular loop fusion protein, has successfully been used to generate polyclonal Abs to this receptor (9, 17). Traditional hybridoma technology allows for only a limited sampling of the immune repertoire. In contrast, cloning of the repertoire in *E. coli* potentially allows for a much more extensive survey of the immune response, essential for the selection of rare Abs (21). We constructed combinatorial scFv phage libraries using primers designed to match the V region of murine heavy and light chain subgroups (37). To cover a maximum of sequences with a minimum of different primers, up to two degenerations were introduced in each of the seven $V_H$ and $V_L$ primers. In contrast to the primers described by Huse et al. (38), which cover only 58.5% of the available $V_H$ sequences at a homology level ≥82% (39), the newly designed primers cover 99% of both the $V_H$ and the $V_L$ sequences with a homology level >86%. Optimized primer sequences for mouse scFv repertoires have been described previously (39). However, since no degenerations were used, 10 or nine different $V_H$- or $V_L$-specific primers had to be designed to match the $V_H$/$V_L$ genes with high homologies. In consequence, more PCR reactions had to be performed to construct the libraries.

We found the newly designed primers effective in PCR cloning of a panel of different mAbs, i.e., neoepitope-specific mAbs against both hC3a and hC5a (40), a mAb against elongation factor

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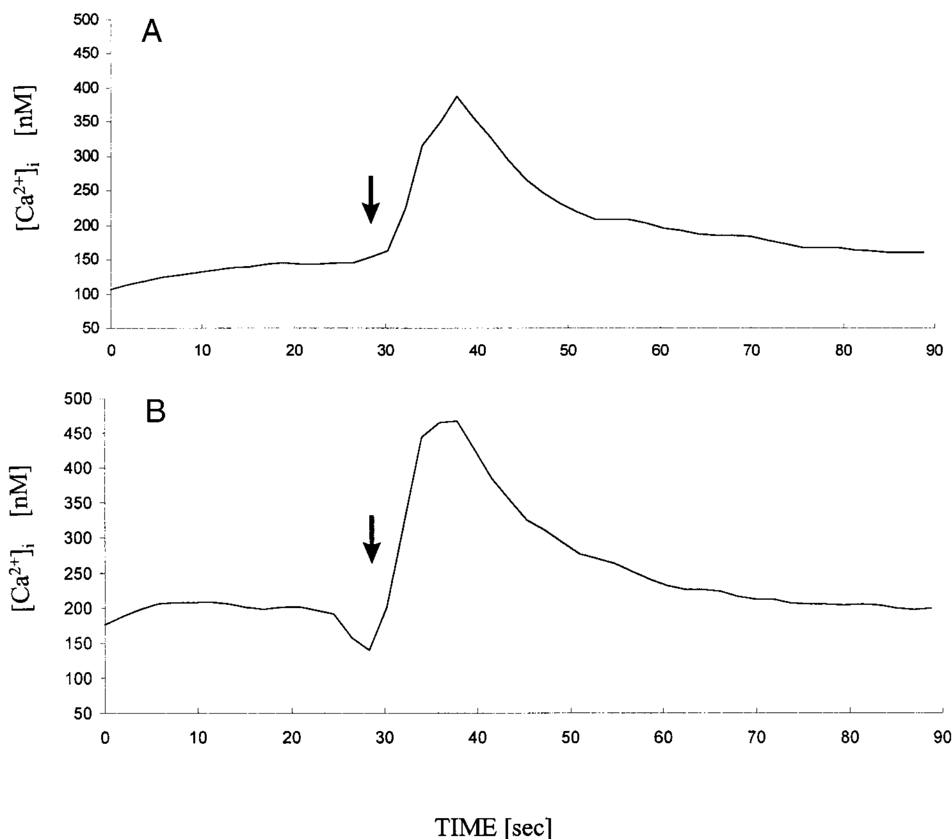


**FIGURE 10.** $[Ca^{2+}]_i$ mobilization from C3aR-transfected RBL-2H3 cells induced by application of *A*) C3a (1 nM), or *B*) C3a (1 nM) plus scFv 3G7, 2A1, or 3C6 (300 nM). The scFvs were applied either alone or in combination (as described in the text). The time of application of the stimuli is indicated by arrows.

Tu (41), and a mAb against the type B polysaccharide of *Haemophilus influenzae* (42). Here we used the primers to clone the Igγ1, Igγ2a, and Igκ repertoire of mice immunized with the second extracellular loop of the C3aR. After four subsequent rounds of panning, almost all phage Abs bound to MBP-C3aR-(173–332) as detected by ELISA. DNA fingerprinting of clones picked after the fourth panning cycle revealed three different restriction patterns. However, the digestion patterns were not equally distributed. More than 50% of all clones belonged to one particular pattern. Eighteen different clones were sequenced, seven of which were found to be identical. The restriction pattern exactly matched the result of the sequencing, so that the remaining 11 clones were subdivided into three groups (named groups I–III). To assess whether differences in sequence correlated with differences in binding specificity, a peptide scanning analysis was performed. Here we found that scFvs belonging to groups I and III bound to the same stretch of amino acids located at positions 185 to 193 of the C3aR, although the CDRs from both heavy and light chain differed considerably in length and composition. This epitope is right next to one of the two potential sites for *N*-linked glycosylation, i.e., positions 9 and 194. The fact that the scFvs recognize the native C3aR on neutrophils and monocytes indicates that either glycosylation does not interfere with binding of the scFvs to the receptor or that position 194 is not glycosylated, at least not on these cells. The six clones belonging to group II reacted with both an epitope located 25 amino acids downstream of the first epitope, spanning positions 218 to 226 and a stretch of amino acids from positions 185 to 199. The recognition of a discontinuous stretch of amino acids indicates that the epitope recognized by the scFvs from group II is probably a conformational one (43). No scFvs that bound to the loop between positions 226 and 332 were found. Surprisingly, none of the selected scFvs or antiserum from the immunized mice inhibited the interaction of C3a with its receptor. This result strongly suggests that the N-terminal part of the second extracellular loop of the C3aR is not involved in the ligand-receptor interaction. One possible function of the loop might be discrimination between C3a and C5a binding. Both molecules are very similar in structure; in fact, a hybrid anaphylatoxin, comprising C5a-(1–69) and the C-terminal pentapeptide of C3a, is able to interact with both the C3aR and the C5aR, at least in the guinea pig system (44). In this regard, C5a/C3a receptor chimeras will be useful tools to test this hypothesis. Another possibility is that the C3aR may have an additional function, beyond its role as an anaphylatoxin receptor. The large extracellular domain could be involved in cell-cell interactions or may serve as a binding domain for another, yet unknown, ligand, e.g., from the rapidly growing family of chemokines. Further studies are needed to elucidate the physiologic role of this up to now enigmatic loop.

After the successful selection of scFv phages specific for MBP-C3aR-(173–332) the expression of soluble scFvs was the major concern. Theoretically, the scFvs should be transported into the periplasm by the pelB leader sequence and subsequently released into the SN. However, as shown in Figure 6, considerable differences in expression were observed. The scFvs from clones in group I were released in high amounts into the SN, whereas the yield from most of the clones in group II (clone 2E12, 3H4, 4G2, and 3D7) was poor. Interestingly, much higher yields were obtained from two other clones in group II, i.e., clones 2A1 and 4H12.

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Recently, we and others have shown that C3a is able to induce both chemotaxis and $[Ca^{2+}]_i$ release from human mast cells (4, 11). In addition, C3aR have been demonstrated on the mast cell line HMC-1 by competitive binding studies (10). Using the selected scFvs, C3aR expression on HMC-1 cells could be confirmed.

Using C3aR-specific scFvs, we could demonstrate C3aR-positive cells at the single cell stage. Preparations of peripheral blood leukocytes obtained using Polymorphprep are contaminated with erythrocytes, platelets, and eosinophils that may affect both $[^{125}I]$C3a binding and functional studies. However, combining three different methods, i.e., DiffQuick cell staining, phase contrast microscopy, and immunofluorescence microscopy, binding of C3aR specific scFvs to individual monocytes and neutrophils could be detected.

Similar results were obtained in a parallel study, showing that neutrophils and monocytes express the C3aR. In that study, functional assays and Northern blot hybridization on highly purified peripheral blood leukocytes, i.e., neutrophils, eosinophils, monocytes, and B and T lymphocytes were combined with flow cytometric analysis using a polyclonal rabbit C3aR second extracellular loop antiserum (9).

The published data concerning the presence of C3aR on human platelets are inconclusive. While Polley and Nachmann (45) reported activation of human platelets by both C3a and C3adesArg, others have convincingly demonstrated that human platelets do not express the C3aR (46–48). Our data confirm the results obtained in the latter studies and provide additional proof that no specific C3aR occurs on human platelets.

In addition, we found no C3aR expression on lymphocytes. These data are in accordance with the results of a recent study, in which we found no C3aRs on unchallenged, circulating T and B cells at either the protein or the mRNA level (9). On the other hand, Northern hybridization revealed C3aR mRNA in lymph nodes (16–18). The key to resolve these apparently inconsistent results may be to look at the status of lymphocyte activation. It is well known that Ag-activated B or T lymphocytes differ substantially in both their receptor and cytokine expression compared with resting lymphocytes.

Indeed, recent data suggest C3aR expression in activated tonsil-derived B cells (13). However, since both C3a and C3adesArg exhibited similar suppressive effects in these cells, it is questionable whether these effects are receptor mediated or due to unspecific ionic interactions with the cell surface.

Finally, we found that C3aR are not expressed on human erythrocytes. These cells do express a variety of different complement receptors, such as decay accelerating factor (DAF) (CD55), homologous restriction factor (HRF) and homologous restriction factor 20 (HRF20) (CD59), and complement receptor 1 (CD35) (49).

In summary, we have generated a panel of C3aR-specific scFv fragments from combinatorial Ab phage libraries that bind to two distinct regions within the large extracellular loop of the C3aR. They do not inhibit the binding or functional activity of C3a to its receptor, indicating that the N-terminus of the second extracellular loop is most likely not involved in ligand binding. In immunofluorescence studies the existence of C3aR on human neutrophils and monocytes could be demonstrated. No C3aR were detected on human lymphocytes, platelets, or erythrocytes.

These well-defined Ab fragments offer the opportunity to study C3aR expression in different tissues under both physiologic and pathophysiologic conditions and may help elucidate the roles of C3a and its receptor in the immune network.

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