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Two Neutralizing Human Anti-RSV Antibodies: Cloning, Expression, and Characterization

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Abstract

Background: Respiratory syncytial virus (RSV) infection is a major problem in the newborn and aging populations. Fully human monoclonal antibodies with the ability to neutralize RSV could have a major impact on the immunotherapy of the disease. The generation of human antibodies has been difficult because there exists no general way to activate B cells against an antigen of choice in vitro.

Materials and Methods: Human spleen cells from individuals exposed to RSV were used to repopulate SCID mice. Hu-SCID mice were boosted with RSV fusion (F)-protein and subsequently developed B cell tumors. The tumors were removed and cultured and subcloned in vitro, using a feeder layer of CD154-expressing T cells. Two of these tumors produced the antibodies designated RF-1 and RF-2. VL genes were isolated by standard PCR techniques, however, it was necessary to use high-temperature reverse transcriptase to clone the VH genes.

Results: RF-1 and RF-2 VH genes were both found to be closely related members of the VH2 family. Vk genes originated from the VK III family. RF-1 and RF-2 recombinant antibodies expressed in CHO cells (cRF-1 and cRF-2) were found to have affinities for RSV F-protein of 0.1 nM and 0.07 nM, respectively, and both were able to neutralize several A and B subtypes of RSV.

Conclusion: The technique of immortalizing human B lymphocytes, by passage in SCID mice and expression as recombinant antibodies in CHO cells, provides a method by which high-affinity human antibodies can be developed for immunotherapy of viral diseases.

Introduction

The development and therapeutic use of fully human monoclonal antibodies has been an elusive goal for many years, as currently there is no general way to activate human B cells against a human antigen of choice. The most common immunotherapeutic approach is to utilize antibodies from other non-human species that have been engineered to reduce their immunogenicity (e.g., chimeric or humanized murine antibodies) (1–3). Alternatively, a closely related species, the macaque, has been used to produce PRIMATIZED® antibodies. Macaques are able to mount a high-affinity response to human antigens (4,5), and their immunoglobulin genes share a high degree of homology with human immunoglobulin sequences.

Phage display libraries, generated from naive human lymphocytes, have recently been employed and offer the possibility of identifying and selecting human antibodies against a human antigen of choice (6–8). The antibodies successfully developed by this technique, however, are few

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and often require further manipulation to generate acceptable affinities.

One area where the generation of wholly human monoclonal antibodies (MAbs) with immunotherapeutic potential has seen some success is in infectious diseases. Antigen-specific B cells, generated through natural infection or immunization, can be isolated as a potential source for the generation of human antibodies. Cells from such donors are commonly reactivated with a selected antigen or mitogen ex homine and the activated B cells immortalized. Alternatively, B cells generated from virally infected humans have been used successfully to generate human antibodies via phage display technology (9) or antibodies have been selected from synthetic repertoires (10).

Previously, we have shown that B cells from an immune donor producing antibodies to a predetermined viral antigen could be immortalized by boosting donor spleen cells during passage in a severe combined immunodeficiency disorder (SCID) mouse (11). The target antigen in this case was the fusion protein (F-protein) from respiratory syncytial virus (RSV). Antigen-specific titers in the human spleen (hu-SPL)-SCIDs approached 2×10^6, and the subsequent immortalization process was highly efficient. In addition, the monoclonal antibodies isolated by this means were of high affinity and highly effective in in vitro functional inhibition tests. Two Epstein-Barr virus (EBV)-transformed B cell lines producing antibodies, RF-1 and RF-2, against the RSV fusion protein were established (11).

EBV-transformed B cells lines, however, have inherently low and unstable antibody production, and therapeutic MAbs produced by virally transformed human cells would need extensive viral removal procedures if used in humans. Here we show that cloning of these tumor cell populations and isolation of their immunoglobulin (Ig) variable (V) region genes allows the construction of recombinant, fully human anti-RSV antibodies that can be expressed at high levels in CHO cells. The two antibodies described, however, were unusual in their resistance to cloning by standard polymerase chain reaction (PCR) amplification techniques that have been successfully used for many other human Ig V regions. It was therefore necessary to resort to a different technique to isolate the anti-RSV V region genes. The cloning, sequence, and significance of these genes is discussed here.

Materials and Methods

Generation of Anti-RSV Monoclonal Antibodies

Establishment of the two B cell lines producing the antibodies RF-1 and RF-2 has been described elsewhere (11,12). Briefly, human spleen cells were primed with RSV F-protein in vitro prior to transfer into a SCID mouse. The resulting hu-SPL-SCIDs were subsequently boosted with F-protein in adjuvant and developed B cell tumors in their spleens. Two such hu-SPL-SCIDs with high anti-F-protein IgG titers were sacrificed, B cell tumors from these animals removed, and the cells teased into a single-cell suspension in RPMI tissue culture medium containing 10% fetal bovine serum (FBS). F-protein-specific clones, RF-1 and RF-2, were established through limiting dilution clonings (see below).

RSV F-Protein

F-protein was prepared by the method described by Walsh et al. (13). Briefly, HEp-2 cells infected with the Long A strain of RSV were lysed using a buffer of phosphate-buffered saline (PBS) containing 1% (v/v) Triton X-100 and 1% (w/v) sodium deoxycholate. F-protein was purified from the crude cell lysate on an affinity column of a murine monoclonal anti-F antibody (Mab 858-1, Chemicon International, Temecula, CA) coupled to CNBr-activated Sepharose-4B (Pharmacia, Uppsala, Sweden, Cat. No. 17-0430-01) using standard procedure described by the manufacturer. The column was washed extensively with lysis buffer and purified F-protein was eluted from the column with 0.1 M glycine, pH 2.5, containing 0.1% (w/v) sodium deoxycholate. The eluate was neutralized immediately with 1 M Tris, pH 8.5, and dialyzed extensively against PBS. Following the removal of detergent on an Extracti-D gel column (Pierce, Rockford, IL, Cat. No. 20346), F-protein concentration was determined using a BCA kit assay (Pierce, Cat. No. 23225) according to the manufacturer's recommendation. Finally, the solution was sterilized by gamma irradiation, $\geq$10,000 rad at a rate of $\geq$1000 rads/min.

Limiting Dilution Cloning of EBV-Transformed Cells

To assure monoclonality and simplify gene cloning, the EBV-transformed cells producing RF-1 and RF-2 were single-cell cloned by limiting dilution using the mouse thymoma line EL-4 B5 (14) as feeder cells (15). EL-4 B5 cells were grown in Iscove's modified Dulbecco's medium

(IMDM) (Irvine Scientific, Santa Ana, CA) supplemented with L-glutamine (Gibco-BRL, Gaithersburg, MD), nonessential amino acids, sodium pyruvate (both from Sigma, St. Louis, MO), gentamycin, and 10% FBS (both from Gibco-BRL). Exponentially growing cells were collected, washed in fresh media, and irradiated with 2500 rads. These cells were plated out at a concentration of 5×10^4 cells/well in a 96-well plate in growth medium. Exponentially growing RF-1 or RF-2 cells were plated out onto the irradiated EL-4 B5 cells at various concentrations ranging from 0.33 to 30 cells/well. The wells with growing cells were tested for the presence of human anti-RSV fusion protein antibodies by ELISA (see below). Cells from selected wells were cloned again by limiting dilution at no more than 0.33 cell/well. Those cells, from which the daughter cells were all shown to produce anti-RSV F-protein antibodies, were considered single-cell clones. They were subjected to RNA extraction for cloning of the antibody coding genes.

Isolation of mRNA

To isolate mRNA, 5×10^6 freshly cultured RF-1 or RF-2 tumor cells were used in the Microfast Track mRNA Isolation Kit (Invitrogen, San Diego, CA). Cells were initially washed twice with cold PBS, pelleted by centrifugation ($1500 \times g$, 3 min), and lysed with the lysis buffer supplied with the Microfast Track Kit. mRNA was coprecipitated with glycogen with 70% ethanol and the resulting precipitate was stored at −20°C in 70% ethanol until use. Prior to PCR amplification, aliquots of the mRNA ethanol suspension were centrifuged at $10,000 \times g$, 10 min, and the precipitate dried under vacuum (Speed Vac). Dried mRNA was then dissolved in 10 μl DEPC water.

Reverse Transcriptase Reactions

mRNA was reverse transcribed into single-stranded cDNA using one of two methods. In the first method, mRNA was combined with 50 pmoles of oligo $(dT)_{15}$ primer and reverse transcribed using Superscript reverse transcriptase (RT) (Gibco/BRL) (16). In the second method, mRNA was reverse transcribed using thermostable rT*th* DNA polymerase (Perkin Elmer/Cetus, Foster City, CA) according to the manufacturer's recommendations. rT*th* DNA polymerase has two capabilities; it can reverse transcribe RNA efficiently in the presence of $MnCl_2$ at elevated temperatures and, it possesses DNA polymerase activity for use in PCR when manganese ions are chelated and magnesium is added.

PCR Primers for VH and VL Cloning

Sets of primers specific for different V region heavy-chain (VH) and light-chain (VL) families were used for PCR amplification (Table 1). RF-1 and RF-2 were both previously determined to possess κ light chains using anti-κ or anti-λ reagents in an ELISA assay.

The 5′ and 3′ heavy chain primers contained *Sal*1 and *Nhe*1 restriction sites, respectively. The 5′ and 3′ light chain primers contained *Bgl*II and *Bsi*W1 sites, respectively. The primers were designed to be degenerate in certain positions to facilitate priming of V region family members with polymorphisms at these sites. Primers specific for either heavy or light Ig constant regions were used as internal controls.

PCR Amplification

cDNA produced by the SuperScript RT method was divided into aliquots and amplified by PCR using 5 U of Pfu polymerase (Stratagene, San Diego, CA) and 50 pmoles of 5′ and 3′ primer. The reaction mixtures were subjected to 40 rounds of amplification using the following protocol; 93°C for 1.5 min, 54°C for 2.5 min, and 72°C for 3 min, followed by a final incubation of 72°C for 5 min. cDNA produced by the rT*th* DNA polymerase method was divided into aliquots as above and amplified by PCR according to the manufacturer's directions. The rT*th* DNA polymerase functions as a DNA polymerase after the $MnCl_2$ present in the cDNA reaction is chelated and $MgCl_2$ is added. The reaction mixtures were incubated at 70°C for 15 min and 95°C for 3 min, followed by 35 cycles of amplification at 95°C for 1 min and 60°C for 1 min.

Ten microliters of the reaction mixture was run on an agarose gel to check for amplified fragments of the correct theoretical size (350 bp). All PCR reactions were set up in duplicate to ensure that any potential errors introduced by the polymerase reaction could be detected by DNA sequencing of the amplified fragments.

Cloning PCR Amplified Material into a Mammalian Expression Vector

The PCR reactions were extracted with phenol/chloroform and then digested with the appropri-

Table 1. Synthetic oligonucleotide primers used for PCR amplification of immunoglobulin heavy- and κ light chain variable region genes from cell lines RF-1 and RF-2[a]

Family	PCR Primers
	5′ heavy-chain variable region primers (*Mlu*1)
VH1	5′-$(AG)_{10}$ ACG CGT G(T/C)C CA(G/C) TCC CAG GT(G/C) CAG CTG GTG-3′
VH2	5′-$(AG)_{10}$ ACG CGT GTC (T/C)TG TCC CAG GT(A/G) CAG (C/T)TG (C/A)A9-3′
VH3	5′-$(AG)_{10}$ ACG CGT GTC CAG TGT GAG GTG CAG CTG-3′
VH4	5′-$(AG)_{10}$ ACG CGT GTC CTG TCC CAG GTG CAG-3′
VH5	5′-$(AG)_{9}$ ACG CGT GTC TGT GCC GAA GTG CAG CTG GTG-3′
	3′ heavy-chain variable region primer (*Nhe*1)
	5′-$(AG)_{10}$ GCC CTT GGT GCT AGC TGA GGA GAC GG-3′
	5′ κ-variable region primers (*Bgl*II)
VK1	5′-AT CAC AGA TCT CTC ACC ATG GAC ATG AGG GTC CCC GCT CAG-3′
VK2	5′-AT CAC AGA TCT CTC ACC ATG AGG CTC CCT GCT CAG-3′
VK3	5′-AT CAC AGA TCT CTC ACC ATG G(G/A)G (A/T)CC CC(T/A) GC(T/G) CAG CT-3′
VK4	5′-AT CAC AGA TCT CTC ACC ATG GTG TTG CAG ACC CAG GTC-3′
	3′ κ-variable region primer (*Bsi*WI)
	5′-$(AG)_{10}$ TGC AGC CAC CGT ACG TTT GAT TTC CA(G/C) CTT-3′

[a]Underlined nucleotides are restriction endonuclease sites used in the cloning of the amplified product.

ate restriction enzymes for cloning into complementary sites in the vector. The PCR reactions containing VH and VL fragments were cloned sequentially into the mammalian expression vector NEOSPLA5 (17). The NEOSPLA5 vector contains unique VH and VL cloning sites with separate cytomegalovirus (CMV) promoter and enhancer elements driving transcription of the heavy- and light-chain genes. VH fragments were cloned upstream of a human γ 1 constant (C) region heavy-chain gene (allotype, Gm1a, Gm1z) present in the vector. VL genes were cloned upstream of human CK region (allotype, Km3), also present in the vector. Restriction sites were designed for cloning of V region genes that allowed natural amino acid sequences at the cloning sites. NEOSPLA5 also contains the dominant selectable marker neomycin transferase (*NEO*) gene. Expression of the *NEO* gene was intentionally impaired in two ways: the translation initiation sequence was weakened, and an intron, containing heavy and light Ig genes and a dihydrofolate reductase (DHFR) gene, was introduced within the *NEO* gene. Therefore, identification of G418 clones producing high levels of cRF-1 (RF-1 produced in CHO cells) or cRF-2 (RF-2 produced in CHO cells) antibody is limited only to those clones that produce high levels of NEO mRNA. High-level production of NEO mRNA requires integration into a transcriptionally active site on the CHO genome. Thus, only clones integrated into active sites will be selected in G418. NEOSPLA5 additionally contains the DHFR gene for amplification in methotrexate. NEOSPLA5 clones containing both heavy- and light-chain inserts from two independent PCR reactions were sequenced (Model 373A, Applied Biosystems, Foster City, CA) to ensure that the DNA sequences obtained were identical. This eliminated the concerns that the polymerase used in the amplification reaction had introduced errors.

Expression and Production of Anti-RSV Antibodies

NEOSPLA5 constructs containing RF-1 or RF-2 Ig genes were restricted with the enzyme Pac 1 to separate two halves of the plasmid: that containing the bacterial replication and selection elements and that containing those elements required for mammalian expression. Cleaved vector was then electroporated into CHO (DG44)

cells, as previously described (18), and cells were selected in G418 (400 μg/ml) containing media. Transfected cells were cloned at a frequency of 40,000 cells/well in flat-bottomed 96-well tissue culture plates. This ensured, by previous calibration, a statistical frequency of <1 G418-resistant cell per well and clonal density per plate. Wells that survived G418 selection were assayed by ELISA for the production of human immunoglobulin after approximately 4 weeks. Positive clones were scaled up to T25 flasks, after which, culture supernatant was assayed for the presence of Ig and specific binding to RSV F-protein (see below).

Binding of Recombinant Antibodies to RSV F-Protein

BINDING DETERMINED BY ELISA. Determination of human IgG concentration and anti-F activity was performed by ELISA. Immulon I plates (Dynatech Labs, Chantilly, VA) were coated overnight with goat anti-human Ig (Southern Biotechnology Associates, Birmingham, AL, Cat. No. 2040-01) (0.05 μg/well) or F-protein (0.05 μg/well) in 0.1 M bicarbonate buffer, pH 9.5, and blocked with PBS containing 1% (v/v) FBS. Serial dilutions of hu-SPL-SCID sera, culture supernatants, or purified antibodies were incubated on the plate. Bound human IgG was revealed by the subsequent addition of goat anti-human IgG-horseradish peroxidase (HRP) (Southern Biotechnology Associates, Cat. No. 2010-05) and OPD (o-phenylenediamine dihydrochloride) substrate (Sigma, Cat. No. P8287). A commercially available myeloma protein (IgG1, k) (Binding Site, San Diego, CA, Cat. No. BP078) of known concentration was used as control for IgG determination. A selected high anti-RSV titered human serum was used as a positive control in the anti-F-protein ELISA.

Scatchard Analysis

The dissociation constants (Kds) for cRF-1 and cRF-2 binding to F-protein were determined by competing a fixed amount of labeled antibody with various amounts of nonlabeled antibody. The results were plotted as Scatchard plots and Kds determined as the reciprocal of the slope of the curve multiplied by −1 (19). We used an approach developed by IGEN (Gaithersburg, MD, ORIGEN® Analyzer), using chemiluminescence. Briefly, a fixed amount of F-protein, labeled with biotin, using a commercial kit according to the manufacturer's instructions (Amersham, Cat. No. RPN 2202), was incubated with a predetermined amount of ruthenium-labeled antibody according to IGEN's protocol. The concentration of labeled antibody gave approximately 50% occupancy of all antigen sites. Nonlabeled antibody was added as a competitor at various concentrations, ranging between 1/10× and 100× the concentration of labeled antibody. After 2 hr at room temperature, excess avidin-labeled DYNA-Beads (DYNAL, Oslo, Norway, Cat. No. 112.06) were added. Within 5 to 20 min, the amount of ruthenium bound to the beads was determined using a chemiluminescence enzymatic reaction. From the amount of chemiluminescence detected, the amount of labeled antibody bound to the beads was calculated based on the specific activity determined earlier.

Virus Neutralization

Determination of virus neutralization titers were performed as described by Walsh et al. (20). One laboratory B-type strain, CH18537, four A wild-type and four B wild-type strains were used to assess the neutralizing capacity of anti-F-protein human MAb, RF-2. Serial dilutions of cRF-2 and a standard human serum exhibiting anti-RSV neutralizing activity (ARF) were preincubated with virus (50–100 pfu) for 30 min at room temperature in 100 μl minimal Eagle's medium (MEM)/well of a microtiter plate. HEp-2 cells (5×10^4/well) were added in 100 μl MEM and incubated for 3 days at 37°C, 5% CO_2. The plates were washed, fixed with acetone, and air dried, and RSV antigen was detected by ELISA using a mouse MAb. The neutralization endpoint was determined arbitrarily as the dilution that reduced antigen production by 50% compared to control wells, which contained no antibody. Viral neutralization was performed in the absence of complement.

Results

Single-Cell Cloning of EBV-Transformed Cells

Antibody production from EBV-transformed cells continuously decreases and ultimately ceases (21). To immortalize the antibody production, it is therefore necessary to clone the Ig-coding genes before the cells cease production. Although cloning requires monoclonal donor cells, EBV-transformed cells are notori-

Table 2. Limiting dilution cloning of RF-1[a] and RF-2[b]

No. Cells/ Well	No. Wells	Anti-F Wells *n* (%)	Wells with Growth *n* (%)
RF-1			
30	48	48 (100)	48 (100)
10	48	48 (100)	48 (100)
3.3	96	27 (28)	68 (71)
1.1	192	17 (9)	112 (58)
0.38	384	18 (5)	116 (30)
RF-2			
30	40	15 (37.5)	40 (100)
10	120	22 (18)	120 (100)
3.3	120	9 (7.5)	102 (85)
1.1	120	1 (0.83)	50 (41.6)
0.33	180	5 (2.8)	30 (16.7)

[a]Irradiated EL4-B5 cells were plated out at 5×10^4 cells/well in a flat-bottomed 96-well plate. Approximately 24 hr later, RF-1 cells in exponential growth were plated out on the feeder layer at the described concentrations. After 2–3 weeks, the wells were scored for growth and for presence of anti-F activity.

[b]Cloning of RF-2 by limiting dilution. Done as described above.

ously difficult to clone. Isoelectric focusing gel electrophoresis of protein A–purified preparations of the two anti-F-protein antibodies, RF-1 and RF-2, was performed. It indicated that at least two populations of antibodies existed in the RF-2 preparation and there was a strong possibility of oligoclonality in the RF-1 preparation (not shown). By using the mouse thymoma line EL-4 B5 (14) as feeder layer, we expanded single cells by limiting dilution. First, 5×10^4 EL-4 B5 cells/well were plated out in a microtiter plate. Then, the EBV-transformed cells were plated out on top of the EL-4 B5 cells at various concentrations, starting at 0.33 cells/well. The supernatant was tested for the presence of human IgG and for antigen-specific IgG. Cells that produced RF-1 and RF-2 (see Table 2) were cloned in this manner. Essentially, all RF-1 cells plated out at 0.33 cells/well grew up. Of these, approximately one-sixth produced anti-F-protein reactive antibody. Approximately 50% of the RF-2-containing wells grew up. Of these, approximately one-sixth produced anti-F-protein-specific antibody. The clones selected for further analysis both produced IgG1, kappa antibodies.

PCR Amplification of RF-1 and RF-2 Tumor Cell cDNA Produced by Superscript RT

LIGHT CHAINS. Using families of VK primers described in Materials and Methods, a band of approximately 350 kb was successfully amplified from both RF-1 and RF-2 cell lines. This was the expected size for the VL genes. The primer giving the strongest band on an agarose gel was the VkIII primer in both cases. Control C region primers produced a band of 350 bp for the CK region, demonstrating the integrity of the cDNA under amplification reaction conditions.

HEAVY CHAINS. PCR amplification of material produced in a reverse transcriptase reaction using Superscript RT at 37°C with the sets of heavy-chain VH family primers described in Materials and Methods failed to produce bands on an agarose gel. This was the case for mRNA isolated from both RF-1 and RF-2. Control mRNA derived from several other cell lines was successfully amplified under the same conditions, producing a band of approximately 350 bp, which corresponded to the amplified VH gene. This indicated that the primers were probably not the reason for the lack of amplification of RF-1 and RF-2 VH genes.

Control primers designed to amplify the heavy-chain CH1 region also failed to amplify the RF-1 and RF-2 cDNA. These same primers amplified the appropriate control fragment from a cDNA preparation from a different cell line. The failure of control primers as well as V region primers to amplify the appropriate region suggested the failure of Superscript to produce cDNA in the RT reaction, even though light-chain cDNA was successfully amplified.

PCR Amplification of cDNA from RF-1 and RF-2 Tumor Cell Lines Produced by Thermostable rTth DNA Polymerase

Because the heavy-chain CH1 region control primers as well as V region primers were unable to amplify a fragment from RF-1 and RF-2 mRNA, this suggested that Superscript RT was unable to produce cDNA from RF-1 and RF-2. By contrast, thermostable rT*th* DNA polymerase was capable of transcribing mRNA from RF-1 and RF-2, as evident from the PCR bands of approximately 350 bp, the expected size for an ampli-

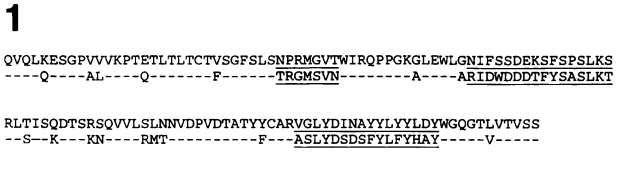

Fig. 1. Amino acid sequence of the heavy-chain variable region from RF-1 (above) compared to RF-2 (below). CDR regions are underlined.

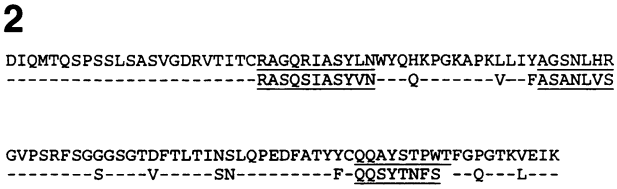

Fig. 2. Amino acid sequence of the light-chain variable region from RF-1 (above) compared to RF-2 (below). CDR regions are underlined.

fied heavy-chain V region. The 5′ primer giving the strongest amplification was the VH2 family–specific primer in both RF-1 and RF-2. Thereafter, the VH region genes were cloned into NEOSPLA5 vector for subsequent expression in CHO cells.

Analysis of V Genes from RF-1 and RF-2

RF-1 and RF-2 clones containing VH and VL inserts were sequenced. The DNA sequence of the VH genes from both RF-1 and RF-2 are shown in Figure 1. Comparative analysis of VH genes with immunoglobulin database sequences was performed using the DNAPLOT program from Althaus and Mueller (22). RF-1 and RF-2 VH gene sequences showed that they were both from the VH2 heavy-chain gene family, confirming the preferential amplification by VH2 primers by PCR. Further analysis suggested that RF-1 VH originated from the V2-26/DP-26+ germ-line sequence and RF-2 VH sequence from the DP-28/VH2-MC1 germ-line V gene. Both these germ-line genes are closely related. Both RF-1 and RF-2 genes showed a high degree of homology with one another; however, comparison of their complementarity determining regions (CDR) 3 regions showed distinct differences (Fig. 1). Both RF-1 and RF-2 VH genes used separate D region genes (DA5 and D2, respectively) and have different extents of N region addition, thus giving rise to distinctly different CDR3 regions. The same JH4 gene appears to have been used by both RF-1 and RF-2.

VL genes from at least two independent PCR reactions from both RF-1 and RF-2 were sequenced. The sequence of the VL genes is shown in Figure 2. Both VL genes were from the κ III family, which confirmed the results obtained using different VK gene family primers and they were highly homologous with one another.

Further analysis of the VL genes suggested, in fact, that they had both originated from the same germ-line gene (DPK9/012) but utilized different J region genes. RF-1 utilized JK1 and RF-2 used JK2. Although RF-1 and RF-2 apparently used the same V gene, giving almost identical frameworks, the CDR regions, particularly CDR2 and CDR3, differed significantly.

Expression Levels of RF-1 and RF-2 MAbs by CHO Transfectants

CHO transfectants selected in G418 were assayed by ELISA for antibody production. The highest-producing cRF-1 clones were expanded and subjected to two rounds of methotrexate-induced amplification, first with 5 nM, then 50 nM methotrexate. The highest-producing cRF-2 clones were expanded and subjected to three rounds of methotrexate amplification at 5 nM, 50 nM, and 500 nM methotrexate. Each round of amplification was performed by cloning at limiting dilution and identifying the highest producers. The cRF-1-producing clone after two rounds of amplification had a specific cellular productivity of 45 pg/cell/day. The cRF-2-producing clone after three rounds of amplification had a specific cellular productivity of 42 pg/cell/day.

Binding Characteristics and Affinity of Recombinant cRF-1 and cRF-2 Produced by CHO Cells

AFFINITY MEASUREMENTS. The binding of both RF-1 and RF-2 to F-protein was strong and specific. Half-maximal responses to F-protein were achieved at concentrations of 0.5 and 2 ng/ml, respectively (Fig. 3A,B), whereas there was no binding to a series of control proteins (11). The discrepancy between the apparent binding activities of B cell–produced RF-2 (purified prior to single-cell cloning) and CHO cell–produced cRF-2 is likely due to the presence of contaminating non-F-protein-specific antibodies, as indicated by isoelectric focusing gels (not shown). The Kds of cRF-1 and cRF-2 for binding to purified F-

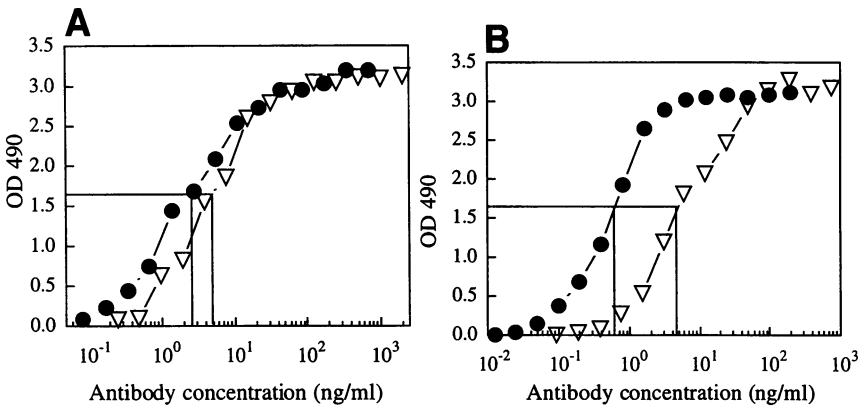

Fig. 3. Binding curve of monoclonal antibody RF-1 and cRF-1 (A) and RF-2 and cRF-2 (B) to RSV F-protein. (A) Purified RSV fusion protein was used as capture in an ELISA to determine binding activity of B cell–produced RF-1 (▽) versus CHO cell–produced cRF-1 (●). Direct binding of a dilution series of the two antibodies was visualized via HRP-labeled secondary antibodies. (B) Purified RSV fusion protein was used as capture in an ELISA to determine binding activity of B cell–produced RF-2 (▽) vs. CHO cell produced cRF-2 (●). Direct binding of a dilution series of the two antibodies was visualized via HRP-labeled secondary antibodies.

protein was determined by Scatchard plots (Fig. 4). The Kd of cRF-1 was determined to be 0.1 nM, whereas the Kd of cRF-2 was determined to be 0.07 nM. This correlates well with the value determined on the B cell–produced MAbs, prior to gene cloning, that were measured as 0.1 nM and 1 nM, respectively, where the latter was an oligoclonal population.

VIRAL NEUTRALIZATION STUDIES. To determine whether the antibodies had virus neutralizing activity, they were subjected to an in vitro neutralization assay: live virus was preincubated with MAb prior to its addition to infection-susceptible target cells. The outcome of the assay was measured by ELISA as the amount of antibody required to decrease viral antigen in the cells by 50%, after a given incubation time. An adult human serum exhibiting anti-RSV neutralizing activity (ARF) was used as a positive control. The titer of this serum represented one taken from the top 20% of such available sera.

Both cRF-2 and ARF serum were able to inhibit viral infection of both the A and B subtypes. The average cRF-2 concentration needed for neutralization of A and B subtypes was 0.139

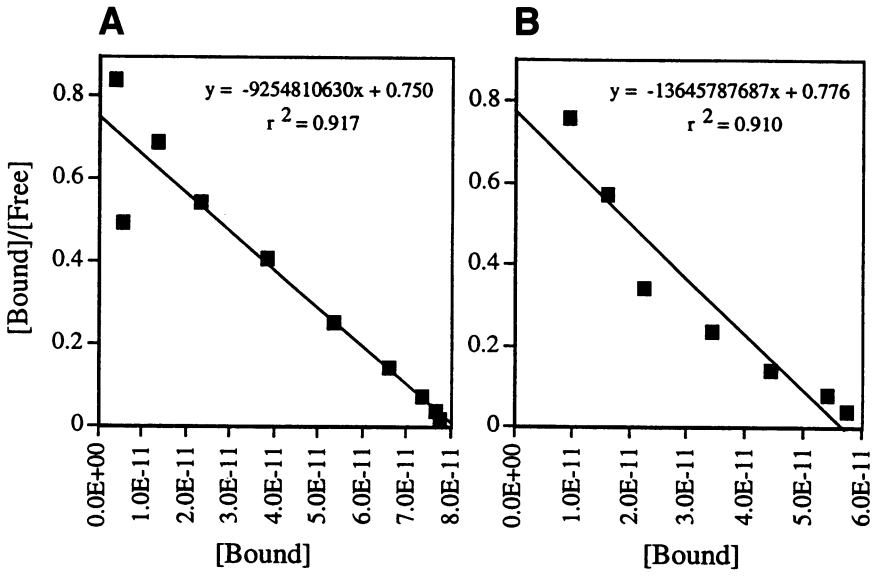

Fig. 4. Scatchard analysis of cRF-1 and cRF-2 binding to F-protein. Binding of a fixed amount of ^{125}I-labeled antibody with various amount of nonlabeled competing antibody, from 1/10× to 100×, was used to generate Scatchard plots (bound versus bound/free) for each of both antibodies, CHO–produced cRF-1 (A) and CHO–produced cRF-2 (B).

Table 3. Neutralization of RSV infection of HEp-2 cells by cRF-2 antibodies[a]

RSV Virus Strain	cRF-2 (μg/ml)	ARF (μg Ig/ml)
B7	0.470	25
B84	1.881	25
B61	0.235	18
B146	0.470	13
CH18537	0.470	25
A148	0.235	25
A226	0.058	13
A18	0.029	6.3
A15	0.235	25

[a]Numbers show the concentration in μg/ml of antibody required to cause 50% neutralization of RSV infection of Hep-2 cells in vitro. cRF-2 was used at a stock concentration of 6 mg/ml in the absence of complement. ARF is adult human serum with an RSV neutralization titer in the top 20% of all adult anti-RSV sera. Ig concentration of this serum corresponds to 20 mg Ig/ml.

ug/ml and 0.705 ug/ml, respectively (Table 3). On the basis of total IgG content, ARF serum gave neutralization values of 17.3 ug/ml and 21.2 ug/ml for A and B subtypes, respectively. This represents a 124-fold better neutralization ability of cRF-2 over anti-RSV serum Ig for subtype A isolates, and a 30-fold increase over subtype B. cRF-1 did not effectively inhibit virus infection in vitro in the absence of complement (data not shown), indicating that it does not bind a neutralizing epitope.

Discussion

The methodology described here, together with previous publications (11,12), demonstrates the usefulness of the SCID mouse system for the isolation of B cell lines producing human antibodies. Although both anti-RSV antibodies described here were of the IgG1 isotype, there does not appear to be any obvious isotype or subtype selection taking place in the hu-SCID mouse (23). All IgG subtypes are represented in similar ratios to that found in human serum. However, it is possible that specific antibodies in hu-SCID serum might be skewed to a specific subtype, depending on the antigen (12).

The yield of antigen-specific antibodies using this approach has been high. Of five mice tested, all had tumors that sustained production of antigen-specific antibodies (12). However, one of the potentially limiting issues concerning the practical use of this approach is that EBV transformed cells lose production over time (21) and do not clone well. By using the EL-4 B5 cells as feeders, we were able to single-cell clone the EBV-transformed cells, with a recovery yield comparable to that of hybridoma cells; i.e., between approximately 50% and 80%.

Both RF-1 and RF-2 VH genes are from the VH2 family, which is a very infrequently utilized family (24,25). It remains a possibility that VH2 family usage could be due to selection in the SCID mouse; however, this is unlikely. There are no data available reporting on selective use of any V gene family over another in the SCID mouse.

The V genes were isolated and expressed as recombinant IgG1 forms with a defined allotype. Cloning and expression of recombinant human antibodies and expression in CHO cells did not result in any loss of antigen-binding affinity. It is, in fact, an advantage to produce antibodies in a dedicated CHO system rather than as hybridomas because of stability, high production levels, and low viral load.

The technique described here represents a way of immortalizing B cell clones prior to Ig gene cloning and expression in CHO cells. Both RF-1 and RF-2 mRNA were resistant to amplification by standard PCR conditions. However, they were easily amplified using a thermostable RT. An alternative method for isolation and selection of human Ig genes is phage display technology. In this system, RNA is isolated from a pool of human B cells, converted to cDNA, and expressed as protein on the surface of bacteriophage. Since the standard conditions of PCR amplification usually employed for phage display technology are similar to those for cloning into mammalian expression vectors, it is likely that the VH2 genes described here would have been missed. The literature seems to support this, as antibodies derived from VH2 genes are infrequently reported in the literature (24–26; J. Marks, personal communication). Thus, VH2 may be underrepresented because of resistance to common cloning methods. If VH2 genes are preferentially used to target specific antigens and these genes resist cloning using standard PCR methodology, then potentially useful antibodies may be lost. The resulting repertoire might be skewed to sequences that match the primers

and/or the reverse transcriptase used rather than reflect the relative abundance, affinity, and/or activity of antibodies represented in vivo. Whether this is specific to the two VH2 genes described here or whether it represents a general feature of VH2 genes is unknown. There may be a secondary structure associated with VH2 mRNA that precludes their reverse transcription without the use of a high-temperature RT. In a system such as the one described above, where antigen is used to stimulate and expand B cells, genes that may be difficult to clone can be more easily rescued.

Fully human antibodies with the capability of neutralizing RSV could have a major impact on the immunotherapy of this disease. The current method for treatment of RSV is by intravenous Ig (IVIG) (27), of which only a small fraction is RSV-specific. The use of a recombinant MAb would result in a more specific therapy. It would have less variability and more defined effector functions than IVIG preparations. Data from our in vitro studies show a 124-fold better neutralizing titer for strain A RSV than the equivalent amount of Ig from a high-titer anti-RSV serum. The activity of cRF-2 also compares favorably with two anti-F-protein MAbs: one in preclinical development (28) and another currently in the clinic (29). Thus, a high specific neutralizing activity would result in a lower effective dose. This, combined with the commercial advantage of a CHO-producing clone that may potentially produce 500 mg/L in a bioreactor, makes cRF-2 administration an economically feasible method for the treatment of RSV infections.

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