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Immunology

A novel strategy for generating monoclonal antibodies from single, isolated lymphocytes producing antibodies of defined specificities

(PCR/antibody-forming cells/V_H and V_L genes/immunoglobulin/plaque assays)

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ABSTRACT **We report a novel approach to the generation of monoclonal antibodies based on the molecular cloning and expression of immunoglobulin variable region cDNAs generated from single rabbit or murine lymphocytes that were selected for the production of specific antibodies. Single cells secreting antibodies for a specific peptide either from gp116 of the human cytomegalovirus or from gp120 of HIV-1 or for sheep red blood cells were selected using antigen-specific hemolytic plaque assays. Sheep red blood cells were coated with specific peptides in a procedure applicable to any antigen that can be biotinylated. Heavy- and light-chain variable region cDNAs were rescued from single cells by reverse transcription–PCR and expressed in the context of human immunoglobulin constant regions. These chimeric murine and rabbit monoclonal antibodies replicated the target specificities of the original antibody-forming cells. The selected lymphocyte antibody method exploits the *in vivo* mechanisms that generate high-affinity antibodies. This method can use lymphocytes from peripheral blood, can exploit a variety of procedures that identify individual lymphocytes producing a particular antibody, and is applicable to the generation of monoclonal antibodies from many species, including humans.**

The enormous diversity of immunoglobulin antigen-binding regions is generated by a series of unique genetic and cellular mechanisms that operate during lymphocyte development and immune responses. It permits the isolation of antibodies that bind an unlimited range of molecular conformations (1). The hybridoma technique (2) enabled the reproduction of specific monoclonal antibodies (mAbs) and revolutionized the exploitation of antibodies in research, industry, and medicine. However, in general, the hybridoma technology is restricted to the generation of rodent mAbs. Moreover, it results in the immortalization of only a small fraction of the specific antibody-forming cells available in an immunized animal. Newer techniques based on the screening of libraries of randomly recombined immunoglobulin heavy and light chain cDNAs (3, 4) are restricted by practical limits to the size of libraries and the requirement for the antibody to be properly folded and expressed in bacteria. Furthermore, the generation of these libraries disrupts the pairing of light and heavy chains that were somatically mutated and coselected in single cells *in vivo* during immune responses; thus, combinatorial techniques fail to fully exploit the immense power of the immune system to generate high-affinity antibodies.

We report here a conceptually distinct method for generating mAbs that overcomes these limitations. It involves first identifying within a large population of lymphoid cells a single lymphocyte that is producing an antibody with a desired specificity or function, and then rescuing from that lymphocyte the genetic information that encodes the specificity of the antibody (Fig. 1). The selected lymphocyte antibody method (SLAM) permits the reproduction of the high-affinity antibodies generated during *in vivo* immune responses in multiple species.

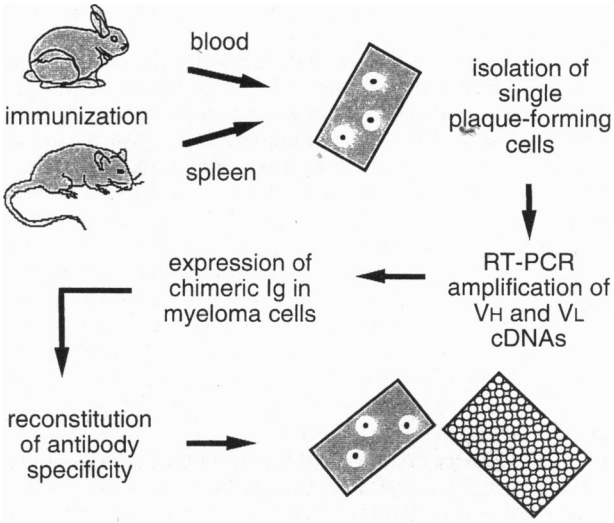

FIG. 1. Strategy for cloning immunoglobulin V_H and V_L cDNAs from single cells producing specific antibodies.

Over 30 years ago, Nossal and Lederberg (5, 6) pioneered the use of micromanipulation techniques to analyze the specificity of antibodies secreted by single cells. Techniques that permitted screening of large populations of cells to directly identify single cells that produced antibody of a particular specificity followed, first identifying cells producing antibodies specific for a bacterial antigen by their adherence to the relevant bacteria (7) and, subsequently, identifying cells producing antibodies specific for heterologous erythrocytes by formation of hemolytic plaques (8). The hemolytic plaque assay has since been modified to detect cells producing antibodies specific for a wide range of antigens that can be attached to erythrocyte surfaces. Although these methods allowed identification of single antibody-forming cells (AFCs) and analysis of the specificity of the antibodies they produced, the AFCs died rapidly, precluding the generation of clones that would produce mAbs (9). Our method provides a means to clone cDNAs that encode the specificity of the antibody produced by such a single cell. The SLAM strategy thus harnesses the power of techniques like plaque assays for screening large numbers of cells for AFCs producing specific antibodies, allowing the generation of mAbs with desired characteristics.

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Abbreviations: SLAM, selected lymphocyte antibody method; AFC, antibody-forming cell; hCMV, human cytomegalovirus; SRBC, sheep red blood cells; IL-3, interleukin 3.

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MATERIALS AND METHODS

Peptides. Peptides were generated by solid-phase synthesis by Ian Clark-Lewis (The Biomedical Research Centre). One peptide incorporated the sequence NETIYNTTLKYGDVVGV corresponding to amino acid residues 68–84 of gp116 of human cytomegalovirus (hCMV), with the C-terminal addition of the sequence GGKKC to aid solubility and to facilitate coupling to the keyhole-limpet hemocyanin used as a carrier for immunizations. Another control peptide corresponded to residues 608–626 of the gp58 protein of hCMV. A third peptide incorporated the sequence CTRPNNNTRKSIRIQRGPGRAFVTIGKIGKIGNMRQAHC, corresponding to the sequence of the V3 loop (residues 301–336) of the gp120 protein of HIV-1 (10, 11). The sequence QYIKANSKFIGITELKK, representing a "universal" T-cell epitope derived from tetanus toxoid (12), was added to the amino terminus of peptides used for immunization. For coating of erythrocytes, the peptides were modified by the addition of a biotin group at the amino terminus.

Oligonucleotides. Oligonucleotides were synthesized using an Applied Biosystems model 391 DNA Synthesizer, PCR-MATE. Specific primer sequences are given in Table 1.

Immunizations. Mice were immunized s.c. with the hCMV gp116 peptide conjugated either to keyhole limpet hemocyanin (KLH; 50 μg of peptide–KLH conjugate in complete Freund's adjuvant) followed on days 7, 19, 26, and 47 by 25 μg i.p. and s.c., or to a peptide incorporating the V3 loop and the tetanus toxoid T-cell epitope (100 μg in complete Freund's adjuvant), followed on days 15 and 73 by 25 μg i.p. and s.c. in incomplete Freund's adjuvant. For the production of anti-erythrocyte antibodies, mice were immunized i.v. with 1×10^8 sheep red bloods cells (SRBCs) and again 3 days later with 1×10^5 SRBC i.p. Rabbits were immunized with 300 μl of packed SRBCs i.v. and again 9 days later with 500 μl of packed SRBC i.v.

Identification and Isolation of Single AFCs Using Hemolytic Plaque Assays. Five days after the final immunizations, spleen cells (murine) or peripheral blood cells (rabbit) were harvested and screened for the production of specific antibodies using hemolytic plaque assays with native SRBCs or SRBCs coated with relevant peptides. Mononuclear cells were purified from peripheral blood (rabbit) by density centrifugation using Nycoprep 1.077 Animal (Nycomed, GIBCO) as described by the manufacturer. For the detection of cells producing anti-peptide antibodies, SRBCs were first derivatized with biotin by treatment with Sulfo NHS Biotin (Pierce) at 50 μg/ml in phosphate-buffered saline (pH 8.5) for 2 hr and then were washed, reacted for 2 min with streptavidin at 1 mg/ml (Sigma), and washed again. Streptavidin-coated SRBCs were reacted for 2 min with the respective monobiotinylated peptides and washed. For plaque assays, the cell population containing AFCs was suspended in tissue-culture medium containing the peptide-coated SRBCs (2.5% vol/vol) and supplemented with a dilution of anti-IgG antiserum (rabbit anti-mouse, 1 in 400; goat anti-rabbit, 1 in 400) previously shown to be optimal for "enhancing" the formation of plaques by antibodies of the IgG class and, as a source of complement, guinea pig serum (5% vol/vol) that had been previously absorbed with an equal volume of SRBC. Drops (3 μl) of these mixtures were placed on glass microscope slides under a layer of warm paraffin oil (BDH) (13) and were incubated at 37°C for 30–60 min. Plaques of lysed erythrocytes were identified using an inverted microscope. The central AFC in the plaque was isolated by micromanipulation using an inexpensive and simple device: a hand-held micropipette pulled from a glass capillary and attached by rubber tubing to a 1-ml tuberculin syringe .

mRNA Purification from Single Cells. Single cells were transferred to microfuge tubes and the mRNA was purified as follows: 50 μl of proteinase K (10 mg/ml) and 150 μl of lysis buffer (10 mM Tris, pH 7.4/0.1 M NaCl/1 mM EDTA/0.5% SDS) were added and the mixture immediately vortexed for 1 min. Tubes were incubated for 1 hr at 37°C. Forty-five microliters of 2.5 M NaCl was added to the mixture along with 50 μg of oligo(dT) cellulose (Pharmacia) resuspended in 50 μl of binding buffer (10 mM Tris, pH 7.4/0.5 M NaCl/1 mM EDTA/0.5% SDS) and then incubated on a rotator for 16 hr. The oligo(dT) cellulose was washed twice with 300 μl binding buffer and twice with 200 μl wash buffer (10 mM Tris·HCl, pH 7.4/0.1 M NaCl). mRNAs were eluted by resuspension of the oligo(dT) cellulose in 15 μl of 10 mM Tris·HCl (pH 7.4; ref. 14) and incubation for 5 min at room temperature.

Reverse Transcription–PCR Amplification. cDNA was synthesized from single cell-derived mRNAs using Moloney murine leukemia virus–reverse transcription (GIBCO) and random hexamer primers (25 pmol) (Pharmacia) or oligo(dT_{24}). In the primary PCR amplifications, murine variable region cDNAs were amplified (40 cycles: 94°C, 45 sec; 50°C, 1 min; 72°C, 2 min; 5 sec extension/cycle) with *Taq* polymerase (Promega) and 250 μM of each dNTP (Pharmacia). Separate tubes were used for V_H and V_κ chain amplifications, each tube containing one-half of the cDNA generated from single cells. The sense primers were based on a consensus of the FR1 region of V_κ (mV_κS-RE, Fig. 2*A* and Table 1) or the FR1 region of V_H (mV_HS, Fig. 2*A* and Table 1). Antisense primers corresponded to CK (mCkAS, Fig. 2*A* and Table 1) or to a consensus of the CH1 regions of Cγ genes (mCH1 AS, Fig. 2*A* and Table 1). Seminested PCR was performed on 1 μl of primary product (30 cycles: 94°C, 45 sec; 55°C, 1 min; 72°C, 2 min; 5 sec extension/cycle) using the same V_κ or V_H sense primers and antisense primers corresponding to a consensus of the respective J_κ (mJ_κAS, Fig. 2*A* and Table 1) or J_H regions (mJHAS, Fig. 2*A* and Table 1).

For rabbit AFC, primary PCR amplifications (40 cycles: 94°C, 45 sec; 60°C, 1 min; 72°C, 2 min; 5 sec extension/cycle) were performed as above, but using sense primers corresponding to the leader region of V_κ (rV_κS, Fig. 2*A* and Table 1) or the 5′ untranslated region of V_H (rV_HS, Fig. 2*A* and Table 1). Antisense primers corresponded to regions in the respective Cκ 3′ untranslated region (rCκAS, Fig. 2*A* and Table 1) or CH1 of Cγ genes (rCH1AS1, Fig. 2*A* and Table 1). Seminested PCR (40 cycles: 94°C, 45 sec; 58°C, 1 min; 72°C, 2 min; 5 sec extension/cycle) was performed on 1 μl of the product of primary amplification of light chain cDNA using the same V_κ sense primer and an antisense primer corresponding to a Jκ consensus sequence (rJKAS, Fig. 2*A* and Table 1). To facilitate ligation, PCR was continued for 20 cycles using Vent polymerase (94°C, 45 sec; 50°C, 1 min; 72°C, 2 min; 5 sec extension/cycle) and versions of the same V_κ sense primer (rV_κS-RE, Table 1) and J-region antisense primer (rJKAS-RE, Fig. 2*A* and Table 1), which added the restriction sites *Eco*RV and *Sal*I, respectively. Nested PCR was performed on 1 μl of the primary product of amplification of V_H cDNA (40 cycles: 94°C, 45 sec; 58°C, 1 min; 72°C, 2 min; 5 sec extension/cycle) using a sense primer corresponding to V_H1 leader sequence (rV_H1S-RE, Fig. 2*A* and Table 1) and an antisense primer corresponding to an inner region of CH1 (rCH1AS2, Fig. 2*A* and Table 1). To facilitate ligation, PCR was performed for a further 20 cycles using Vent polymerase (94°C, 45 sec; 50°C, 1 min; 72°C, 2 min; 5 sec extension/cycle) using the same sense primer based on the V_H1 leader sequence and an anti-sense primer based on the J_H region (rJ_HAS-RE, Fig. 2*A* and Table 1), which added the restriction sites *Eco*RV and *Nhe*I, respectively.

Expression of Cloned V_H and V_L cDNAs. PCR products were electrophoresed in 2% agarose and bands of the predicted size were isolated. Murine V_κ fragments were ligated to an in-frame murine interleukin 3 (IL-3) leader sequence through the *Hin*dIII site added by the sense primer. To facilitate ligation of light chain cDNAs into the human expression vector PAG4622 that contained C_κ (15), generously provided by Sherie Morrison (University of California, Los Angeles), V_κ cDNAs (bearing at their 5′ ends the IL-3 leader-encoding region) were further modified using Vent polymerase (New England Biolabs) and a sense primer based on the murine IL-3 leader sequence (mIL3S-RE, Fig. 2*A* and Table 1) and an antisense primer based on Jκ2 (mJκ2AS-RE, Fig. 2*A* and Table 1), which added the restriction sites *Eco*RV and *Sal*I, respectively.

Table 1. Nucleotide sequence of oligonucleotide primers used in PCR reactions

Name	Nucleotide sequence
mV_κ S-RE	5′-TACCTCGAGATGCAAGCTTCCAGA(TG)GTGACAT(CT)(GT)TGATGACCCAGTCTC-3′
mCK AS	5′-ATCGTCGACAGATCTCTAACACTCATTCCTGTTGAAGCTCTTGAC-3′
mJ_κ AS	5′-CGTTTCAGCTCGAGCTTGGTCCCAGCACCGAACGTG-3′
$mJ_\kappa 2$ AS-RE	5′-GCAGTCGACTTACGTTTTATCTCGAGCTTGGT-3′
mV_H S	5′-G(AG)GGT(CG)CA(AG)CT(GT)(GC)(TA)G(GC)AGTC(AT)GG-3′
mV_H2 S-RE	5′-ATACCTCGAGATGCAAGCTTCCGAGGTCCAACTTGTGGAGTCTGG-3′
mCH1 AS	5′-TCTTGT(CT)CA(CA)CT(TC)(GA)GT(CG)(CT)TGCT(GT)GC(CT)G-3′
mJ_H AS	5′-TGAGGAGACGGTGACCGTGGTCCCGGGGCCCCAG-3′
mJ_H2 AS-RE	5′-AGTGCTAGCTGAGGAGACGGTGACCGTGGTCCCT-3′
rV_κ S	5′-ATTGTCGACATGGACACGAGGGCCCCCACT-3′
rV_κ S-RE	5′-TCGGATATCCACCATGGACACGAGGGCCCCCACTCAGCTGCTG-3′
rC_κ AS	5′-ATTGTCGACGCTCTAACAGTCACCCCTATTGAAGCT-3′
rJK AS	5′-ACCACCTCGGATCCTCCGCCGAAA-3′
rJK AS-RE	5′-GCAGTCGACTTACCTTTGACCACCACCTCGGTCCCTCCGCCGAA-3′
rV_H S	5′-CTGCAGCTCTGGCACAGGAGCTC-3′
rV_H1 S-RE	5′-TCGGATATCCACCATGGAGACTGGGCTGCGCTGGCTTCTC-3′
rCH1 AS1	5′-CTCCGGGAGGTAGCCTTTGACCAGGCA-3′
rCH1 AS2	5′-TGGGAAGACTGATGGAGCCTTAGGTTGCCCT-3′
rJ_H AS-RE	5′-AGTGCTAGCTGAAGAGACAGTGACCAGGGTGCC-3′
mIL-3 S-RE	5′-TCGGATATCCACCATGGTTCTTGCCAGCTCTACCA-3′

"RE" indicates incorporation of restriction site (underlined in sequence).

Murine V_H fragments were modified by introduction of a 5′ *Hin*dIII site by PCR amplification using Vent polymerase and a sense primer based on a V_H2 FR1 region (mV_H2S-RE, Table 1) and the mJH AS antisense primer. This product was ligated through the *Hin*d III site to an in-frame leader sequence from murine IL-3. To facilitate cloning of heavy chain cDNAs into the expression vector PAH4604 containing human Cγ1 (15), generously provided by Sherie Morrison, V_H fragments were further PCR-modified with Vent polymerase using the IL-3 leader sense primer (mIL3 S-RE, Fig. 2*A* and Table 1) and an antisense JH2 primer (mJH2 AS-RE, Fig. 2*A* and Table 1), which added the restriction sites *Eco*RV and *Nhe*I, respectively.

Because the rabbit cDNA fragments already encoded the respective immunoglobulin leader sequences, addition of the IL-3 leader sequence was not required. Murine and rabbit V_κ and V_H cDNAs were digested with *Eco*RV/*Sal*I and *Eco*RV/*Nhe*I, respectively, gel-purified, and then ligated into the PAG4622 or PAH4604 vectors.

C653 myeloma cells were cotransfected by electroporation (200 V, 960 μF) with 10 μg of each construct as described (15) and selected in SmM L-histidinol. Clones were screened by ELISA for human immunoglobulin secretion. Selected clones were used to generate conditioned medium from which chimeric antibodies were purified by affinity chromatography using protein A.

ELISA. Plates were coated with the respective synthetic peptide or recombinant protein (10 μg/ml in PBS with 0.5% NaN_3) and after blocking with 0.5% skim milk powder in PBS (blocking buffer), were washed three times with blocking buffer. Protein A-purified chimeric antibodies were added to the plates with or without the addition of competing peptides. Plates were washed three times with blocking buffer, and then developed using an affinity-purified horseradish peroxidase-conjugated goat anti-human immunoglobulin antiserum.

Antibody Affinity Measurements. Binding assays were performed using a BIAcore biosensor (Pharmacia Biosensor). Approximately 5000 response units of full-length recombinant gp120 (Intracel, Cambridge, MA) were immobilized to the carboxymethylated dextran matrix of a Pharmacia Biosensor CM 5 sensor chip through solvent-exposed lysines. Amine coupling immobilization was carried out at 25°C with a constant flow rate of 5 μl/min using HBS running buffer (10 mM

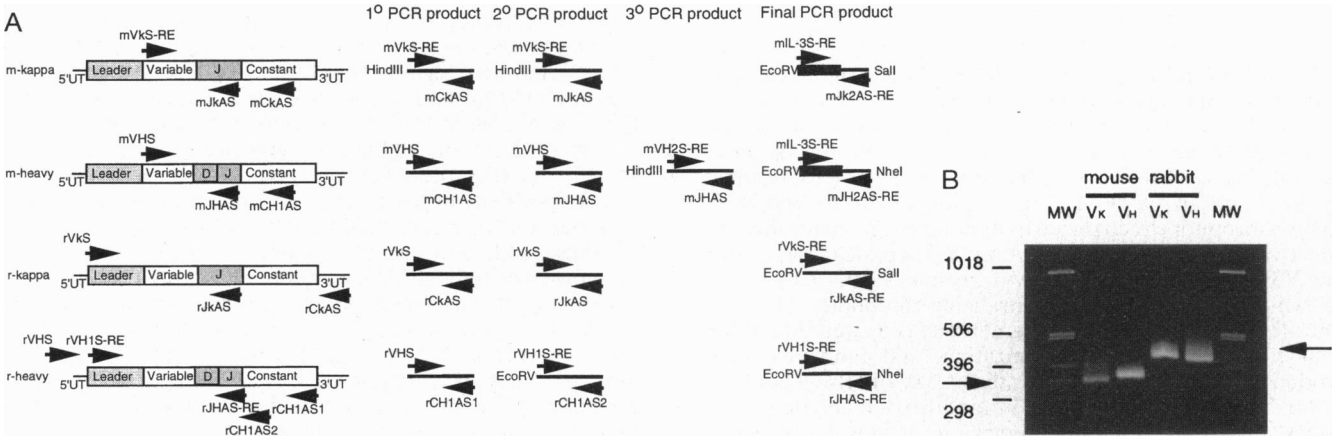

FIG. 2. PCR amplification of immunoglobulin V_H and V_L cDNAs from single murine and rabbit lymphocytes. (*A*) Schematic representation of the approximate locations of annealing of oligonucleotide primers to the kappa and heavy chain immunoglobulin cDNAs in PCR amplifications from single rabbit (r-kappa, r-heavy) and murine (m-kappa, m-heavy) lymphocytes (scheme not to scale). Primer sequences are given in Table 1. (*B*) Agarose gel electrophoresis and ethidium bromide staining of PCR-amplified immunoglobulin variable region cDNAs from a single mouse and a single rabbit SRBC-specific AFC. Shown are bands of ≈350 bp (murine) and 430 bp (rabbit), corresponding to V_H cDNAs, and of ≈340 bp (murine) and 430 bp (rabbit), corresponding to V_κ cDNAs. Control reverse transcription–PCR reactions performed in the absence of template cDNAs failed to amplify any potential contaminating immunoglobulin sequences (data not shown).

Hepes, pH 7.4/150 mM NaCl/3.4 mM EDTA, with 0.005% BIAcore P20 surfactant). Carboxyl groups on the sensor chip surface were first reacted with 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride/0.05 M *N*-hydroxysuccinimide for 6 min to form reactive esters. Recombinant, full-length gp120 was dissolved at 15 μg/ml in 10 mM sodium acetate (pH 4.5) and injected for 6 min followed by a 6 min injection of 1 M ethanolamine hydrochloride to block unreacted *N*-hydroxysuccinimide esters. The chip was then washed for 2 min with 100 mM H_3PO_4 to remove any noncovalently bound protein.

For the binding analysis and determination of the binding constants, the chimeric anti-HIV gp120 V3 loop mAb was injected over the immobilized gp120 surface at concentrations ranging from 10 μg/ml to 0.6 μg/ml. All binding experiments were performed at 25°C with a flow rate of 5 μl/min in HBS running buffer. After 12 min of dissociation, the sensor chip surface was regenerated with a 10 μl injection of 100 mM H_3PO_4. The binding curve for the interaction followed first-order kinetics according to the equation:

$$dR/dt = k_a C R_{max} - k_a C R_t - k_d R_t$$

where dR/dt is the overall binding rate, k_a is the apparent association rate constant, k_d is the apparent dissociation rate constant, R_t is the measure of bound protein at time t [measured in response units (R)], R_{max} is the maximum binding capacity of the immobilized protein surface (in RU), and C is the concentration of the injected antibody. The calculated values for dR/dt derived from each experiment performed at different concentrations (C) of the antibody were plotted against the corresponding Rt values. The linear plot yields a slope equivalent to $k_aC + k_d$, and a y intercept equivalent to k_aCR_{max}. The slopes from these curves plotted against the concentration of antibody tested gives the association rate constant ($M^{-1}\cdot s^{-1}$).

The dissociation rate constant was determined after the sample injection was terminated with buffer. Thus, the concentration for the analysis becomes zero (C = 0) and therefore:

$$-dR/dt = k_d R_t \text{ (for } C = 0).$$

The dissociation constant can then be obtained from the following linearized equation:

$$\ln R_{tl}/R_{to} = k_d(t_o - t_l)$$

where R_{to} is the response 20 sec after the termination of mAb injection and Rt_l is the response at a later time point. A linear plot of $\ln R_{tl}/R_{to}$ versus $(t_o\text{-}t_l)$ reveals the dissociation constant s^{-1}.

RESULTS

Amplification of V_H and V_L cDNAs from Single Antigen-Specific Lymphocytes. Rabbits or mice were immunized as described above with SRBCs or viral peptides chosen because they were known to include epitopes that were the target of neutralizing antibodies. One peptide incorporated residues 68–84 of gp116 of hCMV, the region of gp116 known to contain an epitope recognized by antibodies that neutralize the infectivity of this virus (16, 17). A second peptide incorporated the V3 loop of gp120 of HIV-1 (residues 301–336), again known to be a target of neutralizing antibodies (10, 11). Murine spleen or rabbit peripheral blood cells were harvested 5 days after the final immunization, and individual cells producing antibodies specific for the hCMV or HIV-1 peptides or for SRBCs were identified in modified hemolytic plaque assays as described. This modified plaque assay was an efficient method of screening for specific AFCs. For example, from mice immunized with the HIV-1 gp120 peptide, 70,000–90,000 cells forming gp120 peptide-specific plaques could be identified per spleen. Individual cells were plucked and transferred to tubes using a micropipette. Polyadenylylated mRNA was isolated from each single cell and reverse transcription–PCR was performed (Fig. 2) using sense primers annealing to variable region sequences and antisense primers annealing to constant or joining region sequences (Table 1).

In the case of the murine cells, degenerate V_H and V_κ sense primers were designed to recognize the FR1 regions of most of the respective V_H and V_κ genes in the GenBank data base. V_H cDNAs of the predicted sizes were successfully amplified from one of the three cells isolated that produced antibodies specific for the gp116 peptide, three of the five cells isolated that produced antibodies specific for the V3 loop of gp120, and two of the six cells isolated that produced antibodies specific for SRBC. Cloning and sequencing of these cDNAs confirmed their identities as nongermline murine V_H fragments, each unique and unrelated to any potentially contaminating V_H-bearing cDNAs in the laboratory. V_κ cDNAs of the predicted sizes were successfully amplified from two of the three isolated cells producing antibodies specific for gp116, three of five cells isolated producing antibodies to the V3 loop of gp120, and from four of the six single cells producing antibodies specific for SRBC. Sequencing of three of these V_κ cDNAs confirmed their identities as unique murine V_κ sequences.

In the rabbit, there is preferential usage of the V_H1 variable region gene and diversity appears to be largely generated by a mechanism involving gene conversion (18). Therefore, we elected to base our strategy for amplification of V_H cDNA on the use of nested, nondegenerate sense primers designed to recognize the 5′ untranslated region and the leader sequence of V_H1 (19). The sense primer for amplification of V_κ was a nondegenerate oligonucleotide designed to recognize the leader sequences of V_κ cDNAs in the GenBank database. V_H cDNAs of the predicted size were successfully amplified from three of the 11 isolated rabbit anti-SRBC AFCs isolated from peripheral blood. cDNA sequencing of these bands confirmed their identity as nongermline rabbit V_H fragments, unique and unrelated to any possible contaminating rabbit V_H-bearing cDNAs in the laboratory. V_κ cDNAs of the predicted sizes were successfully amplified from six of the 11 single rabbit lymphocytes and cDNA sequencing confirmed that they were unique.

Reconstitution of Antigen-Binding Specificities. V_H and V_κ cDNAs were cloned and expressed in mouse myeloma cells as chimeric antibodies with the respective human IgG1 or kappa constant regions (15). The resulting murine and rabbit chimeric antibodies had the specificities of the respective parental antibodies. Thus, in a specific ELISA assay, the chimeric antibody derived from the plaque-forming cell secreting antibodies specific for the gp116-derived peptide bound the hCMV gp116 peptide (Fig. 3). Likewise, the chimeric antibody derived from the plaque-forming cell secreting antibodies specific for the HIV-1 gp120 peptide bound the HIV-1 gp120 V3 loop peptide. Moreover, the anti-HIV gp120 V3 loop chimeric mAb not only bound the V3 loop peptide but also bound specifically (Fig. 3) and with high affinity (Table 2) to full-length recombinant HIV-1 protein. Thus, the k_A of the interaction between the chimeric anti HIV-1 V3 loop mAb and recombinant full-length HIV-1 gp120 antigen was calculated to be 1.76×10^9 M^{-1} by BIAcore analysis (Table 2).

The specificity of these reactions was confirmed by demonstrations that the inclusion in the ELISA assay of soluble peptides resulted in specific inhibition of binding. Thus, binding of the anti-HIV-1 V3 loop chimeric antibody to either the V3 loop peptide or to recombinant gp120 was inhibited only by the V3 loop peptide and not by peptides from the gp116 or gp58 proteins of HCMV (Fig. 3). Likewise, the binding of the anti-gp116 peptide chimeric antibody was only inhibited by the homologous peptide and not by the peptide from gp58 of HCMV. A chimeric antibody incorporating V_H and V_L cDNAs derived from a SRBC specific AFC showed no activity in these ELISA assays.

Myeloma cells transfected with chimeric antibodies derived from rabbit or murine cells producing anti-SRBC specific antibodies but not untransfected myeloma cells generated hemolytic plaques (Fig. 4). Purified chimeric antibodies incorporating V_H and V_L from an AFC that secreted antibodies

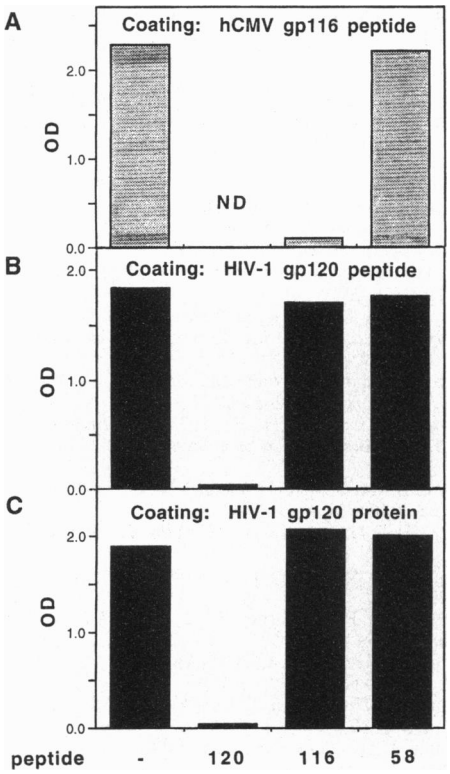

FIG. 3. ELISA analysis of antigenic specificity of chimeric antibodies derived from AFC specific for peptides from hCMV-gp116 or HIV-gp120. Shown are results of ELISA assays on plates coated with (*A*) the peptide from hCMV gp116, (*B*) the V3 loop peptide from HIV-1 gp120, or (*C*) recombinant HIV-1 gp120 protein. Protein A-purified chimeric antibodies incorporating V_H and V_L cDNA from a murine AFC specific for the peptide corresponding to residues 68–84 of gp116 of HCMV at 150 μg/ml (shaded bars) or from a murine AFC specific for the V3 loop of HIV-1 gp120 at 20 μg/ml (solid bars) were added to wells with or without 100 μg/ml of competing peptides corresponding to residues 68–84 of HCMV gp116 (indicated as "116"), the V3 loop of HIV-1 gp120 (indicated as "120"), or residues 608–626 of HCMV gp58 (indicated as "58"). A purified chimeric antibody incorporating V_H and V_L cDNA from an SRBC-specific AFC (20–150 μg/ml) failed to generate a signal in all three ELISA assays. ND, not determined.

specific for SRBCs bound sheep erythrocytes as detected by immunofluorescence and agglutination studies (Fig. 4).

DISCUSSION

We report here the cloning and expression of immunoglobulin variable region cDNAs of unknown sequence from single cells that produce antibodies specific for a given target. The fact that useful frequencies of successful amplification of V_H and V_κ cDNA from single AFCs were obtained in both mice and rabbits—two species in which V_H genes are used in very different ways—suggests that the SLAM strategy will be applicable to many species. The only requirement is the availability of general data on immunoglobulin sequences.

A key advantage of SLAM is that it allows the screening of very large populations of AFCs for the identification of the rare AFC that produce antibodies with a very specific, desired characteristic (e.g., affinity, specificity, catalytic activity, etc.). The screening of large numbers of lymphoid cells can be facilitated by prior enrichment of AFCs from peripheral blood

Table 2. BIAcore apparent kinetic rate constants (k_a and k_d) and affinity constant (k_A) for the binding of the chimeric anti-HIV gp120 V3 loop mAb to immobilized recombinant full-length HIV gp120 protein

$k_a(M^{-1}\cdot s^{-1}) \times 10^5$	$k_d(s^{-1}) \times 10^{-4}$	$k_A(M^{-1}) \times 10^9$
4.28 ± 0.034	2.58 ± 0.05	1.76

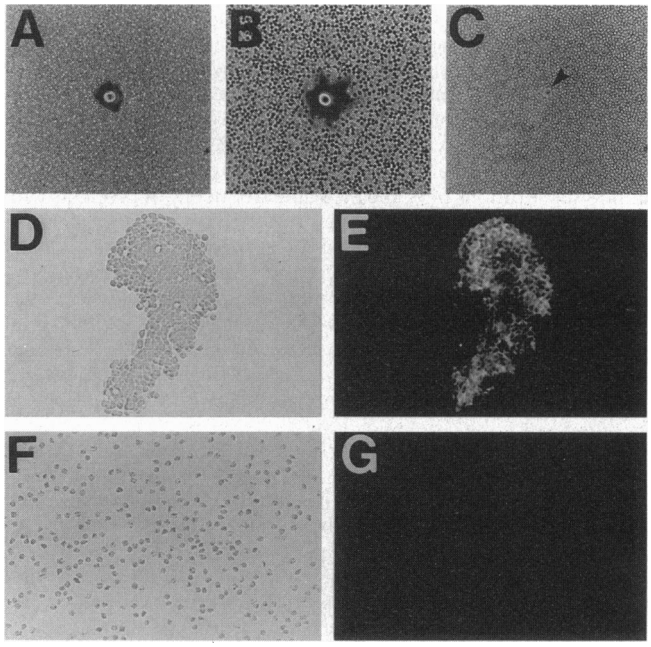

FIG. 4. Reconstitution of anti-SRBC specificity in chimeric antibodies generated using V_H and V_L cDNA from rabbit or mouse SRBC-specific AFC. Shown are hemolytic plaques generated by myeloma cells transfected with constructs directing the expression of chimeric antibodies incorporating V_H and V_L cDNAs cloned from SRBC-specific AFC of mouse (*A*) or rabbit (*B*) origin, but not by a nontransfected myeloma cell (*C*). Protein A-purified chimeric antibody generated using V_H and V_L cDNAs from a rabbit SRBC-specific AFC was incubated with SRBCs that, after washing, were allowed to react with fluorescein isothiocyanate (FITC)-labeled goat anti-human Ig. (*D*) The agglutination and positive immunofluorescent staining (*E*) of SRBCs, not seen with SRBCs reacted with FITC-labeled goat anti-human Ig alone (*F* and *G*).

or lymphoid tissues by physical or immunological methods (20). The hemolytic plaque assay provides one versatile method for screening large numbers of cells for the presence of AFCs specific for virtually any antigen. A variety of chemical and immunological methods have been used to couple to the surface of the erythrocytes a diversity of antigens such as organic molecules (21), which have been used to successfully identify anti-hapten AFCs, as well as peptides (22), proteins (23–26), and carbohydrates (27–32). Unfortunately, the methods used have not been readily adaptable from one antigen to another, particularly for protein or peptide antigens. In contrast, our use of a streptavidin bridge to link sheep erythrocytes to antigens derivatized with biotin has general applicability to proteins, carbohydrates, and organic molecules to which biotin groups can be added using a range of commercially available reagents. Plaque assays have also been developed that use as their targets nucleated cells or bacteria (33). Such modified assays can be used to detect mAbs to unknown cell-surface antigens. Individual lymphocytes producing desired antibodies can also be identified and isolated by the specificity of their cell-surface immunoglobulin, for example, using the fluorescence-activated cell sorter and fluorochrome-coupled antigen (34) or magnetic separation and magnetic particles coated with a specific antigen (35). Further innovations in screening techniques that identify single cells producing antibodies with particular properties, such as catalytic activity, should further enhance the utility of SLAM in the generation of mAbs with highly specialized characteristics and functions.

Recently, Lagerkvist *et al.* (36) reported the use of a similar strategy to clone and express variable region cDNAs from antigen-specific human lymphocytes isolated using antigen-coated magnetic beads. Others have reported the amplification of immunoglobulin cDNAs from single hybridoma cells (37, 38) or from single B lymphocytes of unknown antigenic specificity (39)

or that use a particular V gene (40, 41). While we have used mRNA as the starting point for the rescue of V_H and V_L cDNAs, the same information may be obtained by amplifying genomic DNAs (39, 40). Estimates for the frequency of errors during PCR amplification of immunoglobulin sequences suggest a low overall error rate (40–42). Moreover, because the nature of the genetic code is such that point mutations tend to produce conservative amino substitutions (43), the probable frequency of products with altered biological activity is even lower. BIAcore affinity measurements of the chimeric anti-HIV gp120 V3 loop mAb show antibodies with high affinities can be rescued from single lymphocytes generated from an immune response. Rabbits produce high-affinity antibodies and rabbit mAbs are thus likely to exhibit higher affinities than those reported for murine mAbs. Higher affinity may be a significant advantage for certain purposes, for example, the generation of more efficient catalytic antibodies (44).

SLAM should be particularly useful for the generation of human mAbs. Medical applications necessitating repeated administration of mAbs require human mAbs, because the administration of rodent antibodies to humans provoke immunological reactions (45). SLAM provides a simple alternative to the "humanization" of rodent mAbs (46) or library strategies (3, 4) and complements the use of transgenic mice (47, 48) for generating human mAbs. SLAM should be of particular value for the generation of monoclonal versions of antibodies generated in the course of a human immune response or disease. One week after vaccination of humans, cells producing antibodies specific for *Haemophilus influenzae* type-b capsular polysaccharide (27) or for other bacterial polysaccharides (28–31), reach ≈0.1% of the peripheral blood mononuclear cells. Hemolytic plaque assays have also been used to detect cells producing antibodies specific for a range of viruses such as rubella, CMV, mumps, herpes simplex virus, and respiratory syncytial virus within populations of cells from human peripheral blood (49). We have shown here that AFCs isolated from the peripheral blood can serve as useful sources of mRNAs for the generation of mAbs using SLAM.

SLAM may not be useful for obtaining human mAbs to self antigens, where mechanisms of immunological tolerance largely prevent the generation of antibody responses to self-antigens. Nonetheless, immunological tolerance is not absolute, particularly at the level of the B lymphocyte, and SLAM may be useful in generating antibodies to a variety of human antigens such as human cytokines. For example, neutralizing antibodies to tumor necrosis factor α (50), interferon γ (51), and interleukin 1α (52) have been detected in patients following inflammatory diseases or treatment with recombinant proteins.

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