CLONAL ANALYSIS OF A HUMAN ANTIBODY RESPONSE

Quantitation of Precursors of Antibody-producing Cells and Generation and Characterization of Monoclonal IgM, IgG, and IgA to Rabies Virus

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The maturation of the specific antibody response to foreign antigens (Ags)[1] has been thoroughly investigated in experimental animals (1–8). A first in vivo exposure to Ag induces a "primary" antibody response constituted mainly of IgM with relatively low affinity for the inducing Ag. A second and any further exposure to the same Ag evoke "secondaries" or "memory" responses that involve mainly IgG with a higher affinity for the Ag. Although study of the human antibody response to some Ags, e.g., tetanus toxoid (TT) and keyhole limpet hemocyanin, has been attempted (9–13), the enormous difficulty in generating human mAbs (14, 15) has hindered the definition of the clonal basis of such responses.

The recent progress made in the generation of human mAb-producing cell lines (14–21) and in the characterization of novel B cell subsets (22, 23) allowed us to investigate the human antibody response to self and exogenous Ags at the clonal level (16–19). In the present studies, we quantitated the circulating B cells committed to the production of antibodies to rabies virus and determined their phenotype in healthy humans before and after multiple administrations of inactivated rabies virus vaccine. Moreover, using EBV transformation and somatic cell hybridization techniques, we constructed 10 cell hybrids secreting IgM, IgG, and IgA mAbs to the virus. We found that in the preimmune B cell repertoire, circulating lymphocytes committed to the production of virus-binding IgM, but not IgG or IgA antibodies, are present in high number. These cells are surface $CD5^+$ and the antibodies they produce are polyreactive and low affinity. After vaccination with inactivated rabies virus, B lymphocytes producing monoreactive high affinity IgG and IgA antibodies to the virus consistently appear in the circulating. Most of these cells are surface $CD5^-$ and account for >10% of the total IgG- and IgA-producing cell precursors, respectively.

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[1] *Abbreviations used in this paper:* Ag, antigen; HDCV, human diploid cell vaccine; Ins, insulin; RNP, ribonucleoprotein complex; TT, tetanus toxoid.

One of the IgG mAbs we generated using these cells efficiently neutralized the virus in vitro and in vivo (Dietzschold, B., P. Casali, Y. Ueki, M. Gore, C. E. Rupprecht, A. L. Notkins, and H. Koprowski, manuscript submitted for publication). Finally, the characterization of the V gene segments of these human mAbs to rabies virus suggested that no relationship may exist in the clonal origin of the (early) low affinity IgM and the (late) high affinity IgG or IgA produced in response to the virus.

Materials and Methods

Vaccination with Inactivated Rabies Virus. The recently introduced human diploid cell vaccine (HDCV) against rabies virus is consistently more immunogenic and safer than the preparations previously used (24). Preventive vaccination with HDCV is required for all laboratory personnel involved in handling rabies virus. Before handling the virus, four healthy laboratory workers (three males and one female, 30–52 yr old) were vaccinated with β-propiolactone-inactivated HDCV (PM-1503-3M strain) (Merieux Immunovax Rabies Vaccine; Merieux Institute, Inc., Miami, FL) according to the recommended schedule. These subjects received one intradermal dose of vaccine on days 0, 7, and 21; three of them received a fourth ("booster") injection on day 142. Venous blood (50 ml) was obtained on day −1 (before vaccination) and on days 7, 14, 28, 52, and 163 after the first injection. One subject (B) provided 450 ml instead of 50 ml of blood on days −1 and 52.

Preparation of PBMC and B Lymphocytes. PBMC were separated from peripheral blood and T lymphocytes were removed as previously decribed (25). The remaining cells contained ~25% B lymphocytes, as defined by (B1) mAb directed to CD20 (Coulter Electronics, Inc., Hialeah, FL), <1% T cells, and variable proportions of monocytes, NK cells, and dendritic cells (25). This PBMC preparation is referred to here as B cells. $CD5^+$ and $CD5^-$ B cells were purified from total B cells using phycoerythrin-conjugated B1 mAb to CD20, FITC-conjugated mAb to $CD5^+$, and a FACS as described in detail (22, 23, 25).

Titration of Total IgM, IgG, or IgA and Antibodies with Various Ag-binding Activities. IgM, IgG, or IgA concentrations were measured using appropriate ELISAs as described (16–18). For titration of IgM, IgG, and IgA to various Ags, plates (Beckman Instruments, Inc., Palo Alto, CA) were coated with 10 μg/ml of purified human IgG Fc fragment (52,000 mol wt), ssDNA (500,000 mol wt), 1 μg/ml of purified TT (110,000 mol wt), or 2.5 μg/ml of recombinant human insulin (Ins) (6,000 mol wt; a gift from Dr. W. R. Fields, Lilly Research Laboratories, Indianapolis, IN) (18). Antibody to rabies virus components was assayed using plates coated with disrupted rabies virus (ERA strain, 5 μg/ml) that had been purified as described (26). The virus glycoprotein (G; 62,000 mol wt), ribonucleoprotein complex (RNP) (nucleoprotein, N, 55,000 mol wt; nonstructural protein, NS, 40,000 mol wt; polymerase, L, 120,000 mol wt), and membrane protein (M, 20,000 mol wt) were readily accessible to antibody on the coated plate. Reference binding curves were constructed as described (16–18). Specific horseradish peroxidase- or alkaline phosphatase-conjugated affinity-purified goat antibodies were used to detect μ, γ, H, and κ, λ L chains (Cappel Laboratories, Malvern, PA, and Sigma Chemical Co., St. Louis, MO), as well as α1 and α2 H chains (The Binding Site Inc., San Diego, CA). Alkaline phosphatase-conjugated specific mouse mAbs were used to detect the different IgG subclasses (ICN ImmunoBiologicals, Lisle, IL). Substrate conversion was measured as absorbance at 492 or 415 nm using an automated ELISA reader (Titertek Multiskan Plus; Flow Laboratories, McLean, VA).

EBV Infection and Culture of B Cells: Determination of Frequencies of Antibody-producing Cell Precursors. Concentrated EBV (5×10^6 transforming U/ml) was prepared as described (25). B cells (up to 5×10^7) were resuspended in a freshly thawed 1.0-ml EBV aliquot and incubated at 37°C for 2 h. After addition of fresh FCS-RPMI (RPMI 1640 medium [Biofluids, Rockville, MD] containing 10% FCS [Gibco Laboratories, Grand Island, NY], L-glutamine [2 mM], penicillin [50 IU/ml], and streptomycin [50 μg/ml]), the cells were distributed in limiting dilution microcultures (96-well round-bottomed plates; Nunc Hazetlon, Denver, PA) in presence of 10^5 syngeneic or allogeneic irradiated (1,800 rad) PBMC as feeders, as described (16–18). After a 4-wk culture, antibody concentration was determined in spent fluids.

The criteria used for determination of positiveness have been reported (18). The precursor frequencies of total and rabies virus-binding IgM-, IgG-, or IgA-producing cells were calculated by analysis according to Poisson distribution (18). The precursor frequency of virus-binding IgM-, IgG-, and IgA-producing cells were expressed as percentages of total IgM-, IgG-, and IgA-producing cell precursors, respectively (16–18).

Human Monoclonal EBV-transformed Cell Lines and Construction of Somatic Cell Hybrids. Cell lines producing antibodies binding rabies virus were established from EBV-infected lymphoblasts by at least three sequential subculturing steps and then stabilized by fusion with F3B6 cells, an Ig nonsecretor, and HAT-sensitive and ouabain-resistant human-mouse hybrid (17–21). The resulting EBV-transformed B cell hybrids were cloned at 0.5 cell/well until all progeny microcultures produced antibody to the virus. This required in general at least three cloning steps. mAbs were prepared from culture fluids as described (18).

Competitive Inhibition Studies and Kinetics of Dissociation of Human mAbs Binding to Solid-phase Ag by Soluble Ag. Aliquots of PBS (0.05 ml) containing 0.05% Tween 20 and 0.1% BSA (PBS-Tween) and 0.2–0.4 μg of mAb were mixed with aliquots of PBS-Tween (0.05 ml) containing 0.1% BSA and increasing amounts (0.1–200 μg) of soluble IgG Fc fragment (3.8×10^{-8} to 7.1×10^{-5} M), ssDNA (4.0×10^{-12} to 8.0×10^{-6} M), Ins (3.3×10^{-10} to 6.6×10^{-4} M), TT (1.8×10^{-11} to 3.6×10^{-5} M), or rabies virus components, including the G (0.003–30 μg), the RNP (0.005–50 μg), and the M (0.002–20 μg) protein. After an 18-h incubation at room temperature, the mixtures were transferred into ELISA plates precoated with IgG Fc fragment, ssDNA, Ins, TT, or rabies virus. After 1-h incubation, the mAb bound to the solid-phase Ag was measured. The Ag-binding activity of each mAb in the presence of free ligand was expressed as percentage of its binding activity measured under identical conditions but in absence of any free ligand (100% binding). Dissociation constant (K_d, g/μl or mol/liter) values were calculated as described (17, 18).

Analysis of mAb V_H and V_κ Gene Segments. DNA probes used in this study were amplified after insertion into the appropriate plasmids or phage and transformation of *Escherichia coli* strains DH5α (Bethesda Research Laboratories, Gaithersburg, MD) or LE392 (Pharmacia Fine Chemicals, Piscataway, NJ). The DNA inserts were prepared from plasmid DNA by using the appropriate restriction enzymes, and these DNA fragments were labeled with deoxycytidine 5'-α-[^{32}P]triphosphate (dCTP) (sp act, 3,000 Ci/mmol; Amersham Corp., Arlington Heights, IL) by random priming oligolabeling (27). The cDNA V_H segment probes used in these studies included: 51P1 (370 bp, V_HI) (28), VCE-1 (310 bp, V_HII) (29), 56P1 (460 bp, V_HIII) (29), 58P2 (405 bp, V_HIV) (29), 83P2 (245 bp, V_HV), identical to V_H251 (30), 15P1 (350 bp, V_HVI) (28), and 20P1 (222 bp, V_HIIIb) (28). The V_κ probes used included: M1-3 (320 bp, V_κI) (31), M607-A (290 bp, V_κII) (31), Humkv 305/P1 (3.1 kbp, V_κIII) (32), and EVJK2 (7.0 kbp, V_κIV) (33). Cellular RNA was extracted from human mAb-producing cell lines by the guanidinium/cesium chloride method (34). Total cellular RNA (10 μg) was slot blotted on Gene Screen Plus membranes (New England Nuclear, Boston, MA) in 50% (vol/vol) deionized formamide and 6% (vol/vol) formaldehyde according to the manufacturer's protocol. Blots were prehybridized for 1 h and then hybridized with different V_H probes at 42°C in 50% (vol/vol) deionized formamide, 5× SSC, 5% Denhardt's solution, 25 mM NaH_2PO_4, pH 6.5, 1% SDS, and 100 μg/ml of denatured salmon sperm DNA. After hybridization, blots were washed at room temperature for 30 min in 2× SSC and 0.1% SDS, and then washed at 65°C in 0.1 × SSC and 0.1% SDS for 30 min. Autoradiography was performed using Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY).

Results

Recruitment of Precursors of Cells Producing IgM, IgG, and IgA Antibodies to Rabies Virus by Inactivated Vaccine. The efficacy of the vaccination procedure was evaluated by measuring the kinetics of the plasma virus-neutralizing activity and the titer of circulating IgM, IgG, and IgA antibodies binding rabies virus after the first, as well as after each further rabies virus vaccine administration, in four healthy human volunteers. Before vaccination, these subjects did not display any significant virus-neu-

tralizing activity, i.e., <2 IU/ml (Fig. 1). Their plasma virus-neutralizing titer, however, rose sharply as soon as 1 wk after the first vaccine injection and peaked, in some cases to >200 IU/ml, 4 wk after the third injection. The sharp rise and subsequent steady level of the plasma virus-neutralizing activity paralleled the high concentrations of the plasma anti-rabies virus antibodies of the IgG, and, to a much lesser extent, of the IgM or IgA class (Fig. 1). The increase of plasma virus-binding IgM was paralleled in magnitude and with similar kinetics profile by an increase in plasma IgM binding ssDNA, TT, and Ins (not shown).

Because of the resting state of most lymphocytes in the normal B cell repertoire, the definition of its diversity demands, as an absolute prerequisite, the use of a polyclonal B cell activator capable of inducing Ig production in each B lymphocyte clone. We have previously shown that EBV is ideally suited for such a purpose in humans, in that it induces transformation and Ig production with equal efficiency in B lymphocytes bearing different surface H chains, i.e., μ, γ, or α, as well as $CD5^+$ and $CD5^-$ B cells (16–21, 25). To quantitate the precursors of cells producing antibodies to rabies virus, we obtained circulating B cells from the same four subjects and at the same times used in the experiments described above. After EBV infection, these cells were immediately plated in limiting dilution microculture assays. After a 4-wk culture, the frequency of cells producing virus-binding IgM, IgG, and IgA was determined. Before any virus injection, the B cells committed to the production of IgM-binding rabies virus constituted in some subjects up to 1.90 and 1.74% of total circulating precursors of cells producing IgM and Ig, respectively (Fig. 2, *A* and *A'*). Their percentages increased two- to threefold 1 wk after the second vaccine injection and returned to the prevaccination values as early as 1 wk after the third vaccine injection (Fig. 2, *A* and *A'*).

In contrast to IgM-producing cell precursors, circulating B cells committed to the production of IgG and IgA to rabies virus were not detectable (<0.005% of the total IgG- and IgA-producing cell precursors, respectively, or <0.0005% of the total Ig-producing cell precursors) in any subject before the primary vaccination (Fig. 2, *B*, *B'*, *C*, and *C'*). However, these lymphocytes were detected in the circulation as early as 1 wk after the first vaccine injection, when B cells committed to the produc-

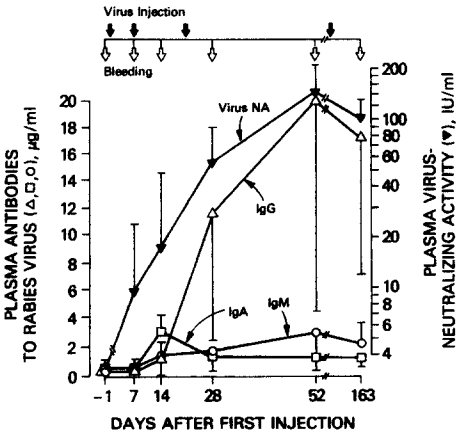

FIGURE 1. Kinetics of appearance of circulating IgM, IgG, and IgA to rabies virus after immunization with inactivated rabies virus vaccine. Rabies virus vaccine was administered intradermally to four healthy subjects on days 0, 7, 21, and 142. Peripheral blood was obtained on days −1, 7, 14, 28, and 163 after the first vaccine injection. Plasma was separated and tested using specific ELISA techniques for rabies virus-binding IgM (○), IgG (△), and IgA (□) concentration, as well as for its in vitro virus-neutralizing titer (▼). Data are mean values ± SD (*vertical bars*).

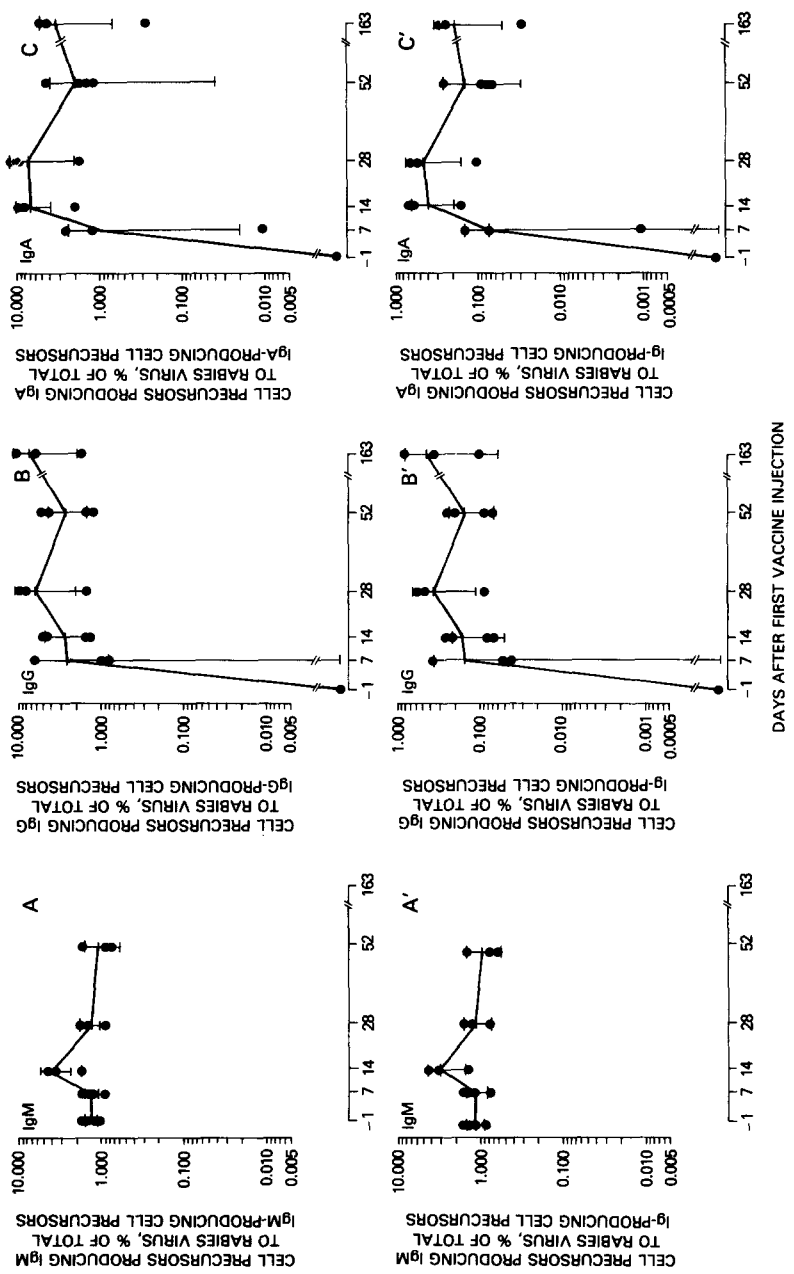

FIGURE 2. Frequency of circulating B lymphocytes committed to the production of IgM, IgG, or IgA binding rabies virus before (day −1), after the first (day 7), second (day 14), third (days 28 and 52), and fourth (day 163) vaccine injection, as determined by limiting dilution analysis of EBV-infected B lymphocytes. (*A*, *B*, and *C*) Frequency of virus-binding IgM-, IgG-, and IgA-producing cells as percent of total IgM-, IgG-, and IgA-producing cell precursors, respectively. Data are mean values ± SD (*vertical bars*) of cell frequencies in four or three healthy subjects. (*A'*, *B'*, and *C'*) Frequency of virus-binding IgM-, IgG-, and IgA-producing cells as percent of total Ig-producing cell precursors. Data are mean values ± SD (*vertical bars*) of cell frequencies in four or three healthy subjects.

tion of anti-rabies virus IgG and IgA accounted for up to 6.3 and 2.5% of total IgG- and IgA-producing cells precursors, respectively (Fig. 2, *B* and *C*), or up to 0.34 and 0.15%, respectively, of total Ig-producing cell precursors (Fig. 2, *B'* and *C'*). Their frequency peaked 1 wk after the third vaccine injection: anti-rabies virus IgG- and IgA-producing cell precursors constituted up to 11.0 and 12.4% of the total IgG- and IgA-producing cell precursors, respectively (Fig. 2, *B* and *C*). In each subject, the number of these cells was reduced by half or two-thirds 4 wk after the third vaccine injection. After the fourth injection, the frequency of B cells committed to the production of virus-specific IgG and, to a lesser extent, IgA antibodies returned to the peak values observed after three vaccine injections (Fig. 2, *B*, *B'*, and *C*, *C'*).

In general, the rise in frequency of the circulating anti-rabies virus IgG-producing cell precursors preceded the increase in plasma titer of the respective antibodies, although this relationship was less marked in the case of anti-virus IgM and IgA antibodies, the plasma concentration of which never reached the values of their IgG counterpart (compare Fig. 2, *B*, *B'*, *C*, and *C'* with Fig. 1). This together with the results of several limiting dilution experiments (not shown) involving the culture of non-EBV-infected circulating B cells from vaccinated subjects suggested that most B cells "committed" to the production of IgG to rabies virus were in fact actually producing these antibodies.

Phenotype of the B Cells Committed to the Production of Antibodies to Rabies Virus. We have previously shown that in the normal human B cell repertoire a major proportion (~10–20% of total B cells) of lymphocytes are committed to the production of polyreactive, mainly IgM, antibodies, that bind a variety of different self and exogenous Ags (18, 19, 22, 23). These antibodies are produced by $CD5^+$ B cells. These lymphocytes account for the high frequency of cells committed to the production of antibodies binding an exogenous Ag before any and after injection with the same Ag. For example, in both subjects never exposed to TT and in those immunized with TT, the vast majority of IgM antibodies binding TT are produced by $CD5^+$ B cells (22, 23). These antibodies display a low affinity for TT and are polyreactive. In contrast, the high affinity monoreactive IgG antibodies to TT found in TT-vaccinated subjects are produced by $CD5^-$ B cells (18, 19, 22, 23). To determine whether an analogous pattern of B cell commitment to low affinity IgM and high affinity IgG antibodies exists in response to rabies virus, we purified $CD5^+$ and $CD5^-$ B cells from one volunteer before and after vaccination, infected these cells with EBV, and cultured them to assess the reactivity of the antibodies produced. Consistent with the B cell subsets segregation pattern observed in the antibody response to TT (22), the virus-binding IgM detected before immunization with rabies virus and the vast majority of those detected 4 wk after the third vaccine injection were produced by $CD5^+$ B cells; the vast majority of the IgG antibodies detected only after vaccination were produced by $CD5^-$ B cells (Fig. 3).

Generation and Characterization of Human mAbs to Rabies Virus. To characterize the antibodies produced in response to rabies virus, we generated, by EBV-transformation and somatic hybridization techniques, eight mAb-producing continuous cell lines using circulating lymphocytes obtained from three subjects at different times during immunization. Because of the overrepresentation of the B cells committed to the production of IgM in the human B cell repertoire (18, 19, 25), when utilizing B cells from immunized subjects, we deliberately biased the selection of rabies virus-

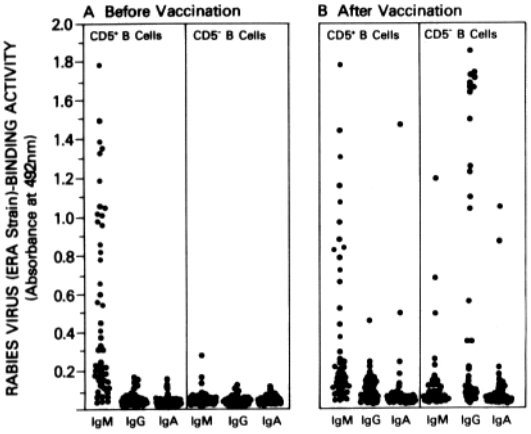

FIGURE 3. Production of rabies virus- binding antibodies by $CD5^+$ and $CD5^-$ B cells before and after vaccination with inactivated rabies virus vaccine. $CD5^+$ and $CD5^-$ B PBL were purified from a subject before vaccination and 52 d after the third vaccine injection. These cells were infected with EBV and cultured in microplates at 250/well in presence of irradiated feeders. After a 4-wk culture, fluids were tested for antibody activity. Each dot represents the concentration of antibody (expressed as absorbance at 492 nm) in the fluid from one microculture well. Approximately 50 microculture wells were assayed in each column.

binding antibody-producing cells in favor of those clones producing IgG or IgA over those producing IgM. Three of these mAb-producing EBV-transformed hybrid cells made IgM, four made IgG, and one made IgA. The features of these eight mAbs were compared with those of two other IgM mAbs produced by EBV-transformed hybrid cells generated from two of the same subjects before primary rabies virus vaccination (Table I). All IgM mAbs (Ab 50, 51, 52, 55, and 59) bound to the RNP complex, in most cases, to its N component. The IgA mAb (Ab 105) and one of the IgG (Ab 56) mAbs also bound to the RNP complex (N component); one of the IgG mAbs (Ab 53) bound to the M protein; and the remaining two IgG mAbs (Ab 53 and 57) bound to the G protein. One (Ab 57) of the two rabies virus G-binding IgG mAbs efficiently neutralized the virus in vitro and in vivo (Dietzschold, B., P. Casali, Y. Ueki, M. Gore, C. E. Rupprecht, A. L. Notkins, and H. Koprowski, manuscript submitted for publication).

To study in further detail the Ag-binding activity of these antibodies, each mAb was tested at different concentrations for binding to solid-phase virus components, as well as to different self Ags (Fc fragment of human IgG, ssDNA, and Ins) and non-self Ags (TT). The binding curves derived from the analysis of the two IgM mAbs generated from cells obtained before immunization and one of the two IgM mAbs generated after completion of the immunization schedule (Table I) are depicted in Fig. 4, *A*, *B*, and *C*. These IgM mAbs were polyreactive and bound in a dose-dependent fashion not only to rabies virus components, but also and with different efficiency, to other Ags included in the assays. Assay of the binding activity of the remaining two IgM mAbs, one generated after the first vaccine injection and one generated after the fourth, gave similar results (not shown). In contrast, the four IgG and one IgA mAbs generated from two fully immunized subjects were monoreactive, binding in a dose-dependent fashion only to rabies virus components, and to none of the other Ags tested. Fig. 4, *D*, *E*, and *F* shows the binding curves derived from the analysis of the IgA mAb and two of the four IgG mAbs; assay of the remaining two IgG mAbs gave similar results (not shown).

The polyreactivity of the IgM as well as the monoreactivity of the IgG and IgA mAbs were confirmed by the results of competitive inhibition experiments, in which

TABLE I

Characteristics of Human mAbs to Rabies Virus Generated before and after Vaccination

mAb	Donor	Time§	Virus binding‖	C_H chain	V_H gene*		C_L chain	V_L gene*		K_d‡				
					Probe	Family		Probe	Family	Rabies virus	Fc fragment	ssDNA	Ins	TT
Ab 50	A	Before	RNP	μ	20P1	IIIb	κ	305/P1	VκIII	10^{-6}	10^{-5}	5.6×10^{-7}	10^{-4}	6.4×10^{-6}
Ab 52	B	Before	RNP	μ	20P1	IIIb	κ	M607-A	VκII	1.7×10^{-6}	3.0×10^{-5}	10^{-6}	10^{-4}	2.5×10^{-6}
Ab 51	A	After first	RNP	μ	20P1	IIIb	κ	305/P1	VκIII	2.4×10^{-6}	10^{-5}	2.5×10^{-7}	8.3×10^{-5}	8.2×10^{-6}
Ab 55	C	After fourth	RNP	μ	20P1	IIIb	κ	305/P1	VκIII	1.0×10^{-6}	ND	10^{-6}	8.3×10^{-5}	2.7×10^{-6}
AB 59	B	After fourth	RNP	μ	20P1	IIIb	λ	ND	ND	1.0×10^{-6}	ND	2.8×10^{-6}	1.2×10^{-4}	3.6×10^{-6}
Ab105	C	After fourth	RNP	α1	15P1	VI	κ	M1-3	VκI	5.8×10^{-9}	-¶	-	-	-
Ab 56	C	After fourth	RNP	γ2	56P1	III	λ	ND	ND	6.5×10^{-9}	-	-	-	-
Ab 53	B	After fourth	M	γ1	20P1	IIIb	κ	M1-3	VκI	1.2×10^{-9}	-	-	-	-
Ab 57**	B	After fourth	G	γ2	51P1	I	λ	ND	ND	1.1×10^{-10}	-	-	-	-
Ab 58	B	After fourth	G	γ2	58P2	IV	κ	305/P1	VκIII	5.0×10^{-9}	-	-	-	-

* Gene probe, cDNA probe hybridizing with cellular RNA.

‡ K_d is expressed in g/μl for rabies virus components and in mol/liter for all other Ags.

§ Time of generation, before, after the first or fourth vaccine injection.

‖ Virus binding, viral component bound by each mAb.

¶ K_d value too high to be calculated (low affinity).

** Ab 57 neutralized rabies virus in vitro and in vivo.

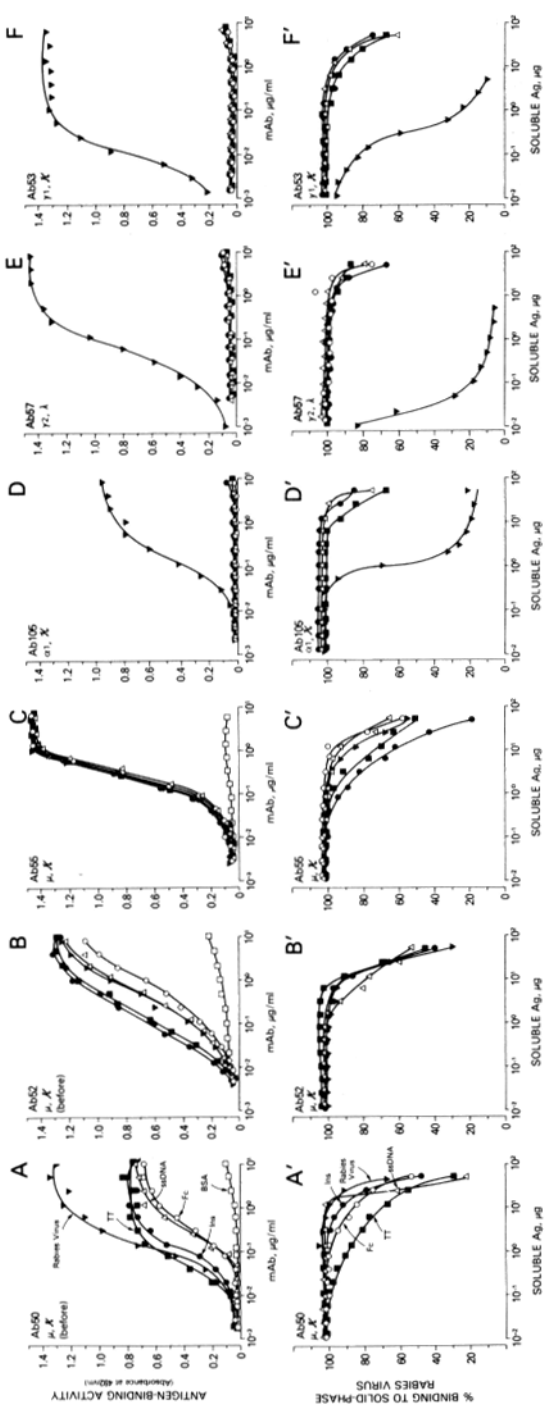

FIGURE 4. (*A–F*) Dose-dependent binding of six different human mAbs to solid-phase rabies virus (▼) and other self and non-self Ags: IgG Fc fragment (○), ssDNA (△), Ins (●), TT (■), or BSA (□). The Ag-binding activity of each mAb is expressed as optical absorbance. (*A′–F′*) Dose-dependent inhibition of the binding of six different human mAbs to solid-phase rabies virus by free homologous or heterologous ligands. The mAbs tested were the same as those of *A–E*. Identical amounts of each mAb were incubated for 18 h with increasing amount of free rabies virus (▼), soluble IgG Fc fragment (○), ssDNA (△), Ins (●), or TT (■). The mixtures were then transferred into ELISA plates precoated with rabies virus. The binding of each mAb to solid-phase Ag in the presence of each different free ligand was measured and is expressed as percentage of the Ag-binding activity determined after incubation of the same mAb in absence of free ligand.

the binding of each mAb to solid-phase rabies virus was measured in the presence of homologous (rabies virus) or heterologous free Ags. The binding of mAb50, mAb52, and mAb55 to solid-phase rabies virus was inhibited, in general inefficiently, by free virus components as well as by different soluble heterologous Ags, including TT, ssDNA, and Ins (Fig. 4, *A'*, *B'*, and *C'*). The converse was also true: the binding of each IgM mAb to solid-phase Fc fragment, ssDNA, Ins, or TT was inhibited not only by the soluble homologous Ag, but also by the soluble heterologous Ags, as well as free rabies virus (not shown). Competitive inhibition analysis of the two remaining IgM mAbs provided similar results (not shown). Unlike the binding of polyreactive IgM mAbs, the binding of the monoreactive IgG and IgA mAbs to solid-phase rabies virus components was inhibited very efficiently and in a dose-dependent fashion by free rabies virus components, but not by any of the other soluble Ags tested. These results are exemplified by the inhibition curves derived from the analysis of mAb105, mAb57, and mAb53 (Fig. 4, *D'*, *E'*, and *F'*).

Competitive inhibition experiments involving pairs (solid and free phase) of homologous ligands allowed for the calculation of the K_d of the mAbs for the different Ags studied. The polyreactive IgM mAbs displayed relatively high K_d values (low affinity) for rabies virus ($>10^{-6}$ g/μl) and for the other ligands tested ($\sim 10^{-4}$–10^{-6} mol/liter) (Table I). In sharp contrast, the monoreactive IgG and IgA mAbs displayed K_d values for rabies virus three to four orders of magnitude lower ($\sim 10^{-9}$–10^{-10} g/μl) (higher affinity) than those displayed by the polyreactive mAbs. The K_d values of monoreactive mAbs for Ags other than rabies virus were too high ($>5.0 \times 10^{-3}$ mol/liter) to be calculated (very low affinity).

V_H and V_κ Gene Segments Usage by the mAbs to Rabies Virus. It has been reported that polyreactive antibodies and certain rheumatoid factors may use selected V_H and V_L segments (35, 36). Probing of the slot-blotted RNA from the 10 different mAb-producing cell lines using seven different [^{32}P]cDNA segments, corresponding to members of the six conventional V_H families (V_HI through V_HVI) and to a segment (20P1) only distantly related to the V_HIII family, V_HIIIb (28, 37), revealed two discrete patterns of V_H gene segment utilization by the polyreactive and the monoreactive mAbs. RNA from all five polyreactive IgM mAb-producing cell lines hybridized with the 20P1 V_H cDNA probe (Table I). In contrast, RNA from only one (mAb 53, an IgG1) of the five monoreactive high affinity mAb-producing cell lines hybridized with the 20P1 V_H cDNA (Table I). The hybridization of RNA from the remaining four monoreactive high affinity mAb-producing cell lines showed that two mAbs, an IgG2 and an IgA1, utilized members of the V_HIII and V_HVI families, respectively (Table I); the other two mAbs, both IgG2, utilized members of the V_HI and the V_HIV gene families. None of the mAbs utilized gene segment members of the V_HII or V_HV gene families. Probing of the slot-blotted RNA from the seven κ^+ mAb-producing cell lines using four different [^{32}P]DNA segments (members of the V_κ I, II, III, and IV families) revealed a preferential utilization of V_κIII family gene segments by the polyreactive IgM antibodies. Three of the four κ^+ IgM mAbs used a gene segment member of the V_κIII family. The remaining mAb utilized a segment member of the V_κII gene family. In contrast, only one of the three κ^+ IgA and IgG mAbs utilized a segment member of the V_κIII gene family; the remaining two utilized members of the V_κI gene family (Table I). None of the mAbs utilized members of the V_κIV gene family.

Discussion

The present studies showed that in humans not previously exposed to rabies virus, a major proportion of circulating B lymphocytes (up to 2% of total Ig-producing cell precursors) are committed to the production of rabies virus-binding IgM, but not IgG or IgA (<0.0005% of total Ig-producing cell precursors). Exposure to rabies virus brings about a dramatic change in the B cell repertoire. After three vaccine injections, B cells committed to the production of IgG and IgA to the virus consistently appeared in high number in the circulation, accounting for up to almost 1% of total Ig-producing cell precursors. In contrast, circulating B cells committed to the production of IgM capable of binding rabies virus increased only by a factor of two to three, accounting for up to ~5% of total Ig-producing cell precursors.

The anti-rabies virus IgG and IgA antibodies displayed a discrete pattern of Ag reactivity when compared with their IgM counterparts. In fact, the IgM mAbs produced by B cells obtained either before or, even, after immunization displayed a low affinity for rabies virus (K_d, $>10^{-6}$ g/μl) and were polyreactive. In contrast, the anti-rabies virus IgG and IgA mAbs produced by B cells obtained after immunization were monoreactive and displayed a high affinity for the virus (K_d, $\sim 10^{-9}$–10^{-10} g/μl). Thus, the virus binding is but one of many Ag-binding activities expressed by the polyreactive IgM antibodies. These likely belong to the broader class of antibodies defined as "natural antibodies" (17–19, 22, 38–44). Their polyreactivity and low affinity suggest that the primary B cell repertoire may be largely Ag independent in its generation, although Ag-induced IgM with higher affinity might have also participated in the rabies virus-induced response. Such antibodies, however, would have been produced by cells present only at very low frequency in the circulation, and perhaps missed by our sampling procedure, i.e., mAb generation.

The present experiments also showed that regardless of the specific immune status of a subject, the B cells committed to the production of polyreactive low affinity IgM binding to rabies virus are surface $CD5^+$. In contrast, in immunized subjects, those committed to the production of monoreactive high affinity IgG are mainly surface $CD5^-$. This is consistent with our earlier observations suggesting that the $CD5^-$ B cell compartment provides, or at least contains, the lymphocytes responsible for a secondary, high affinity "mature" antibody response, as well as high affinity memory B cells (18, 22).

The low affinity polyreactive IgM mAbs selected for binding to rabies virus were restricted both in Ag reactivity and in V gene family utilization. Indeed, all of the IgM mAbs recognized the rabies virus RNP complex, mainly its N component, and all utilized V_H segment members of the same gene family, as determined by positive hypridization of their RNA with the 20Pl cDNA probe. The selective utilization of V_L gene members of the same family (V_κIII) by three of four of the V_κ^+ IgM mAbs further underlines their genetic homogeneity. The 20P1 V_H DNA segment was originally derived from a fetal liver library of expressed V_H segments (28) and is utilized in unmutated configuration in a human IgM mAb with anti-self (Sm) reactivity (37). This V_H segment shares sequence homology with some members of the conventional V_HIII family, although its allocation in the V_HIII family may be questionable and might well constitute a member of a yet to be defined V_H subfamily, tentatively named V_HIIIb (37).

The shift from low to high specificity and affinity in the antibody response to rabies virus coincided with the utilization of a variety of different V_H and V_κ gene segments by most of the participating cell clones. Only one (mAb53, a monoreactive IgG2) of the five high affinity mAbs generated from the immunized subjects used a member of the V_HIIIb gene family. However, unlike the polyreactive low affinity IgM, mAb53 was specific for the viral M, not N component. Thus, unless an improbable gene replacement mechanism (45) was operative in the response to rabies virus, most of the B cells recruited in the late high affinity antibody response were likely the progeny of clones different from those committed to the production of polyreactive IgM antibodies. A similar shift from a V_H gene family restricted primary response to a more diverse V_H gene segment utilization in the mature, secondary, or tertiary response is exemplified by BALB/c mice responding to oxazolone (7). This contrasts with the clonal dominance of a single V_H family in both the primary and secondary immune responses, as exemplified by the response to phosphorylcholine in similar BALB/c mice (3), or by the response to arsonate in A/J mice (6). A comparable pattern of clonal dominance throughout the maturation of the human antibody response may be found when analyzing the specific antibodies generated by immunization with an Ag of different nature, e.g., polysaccharides.

The utilization of a V_HIIIb gene segment by one of the monoreactive high affinity "secondary" antibodies may be compatible with the hypothesis that a minor component of an Ag-driven "mature" antibody response to rabies virus is due to the progeny of cells originally producing polyreactive "autoantibodies" and using selected, e.g., V_HIIIb, gene segments. These cells would undergo an Ag-driven process of clonal expansion and somatic point mutation with simultaneous loss of the surface CD5 molecule; in vitro proliferation of normal $CD5^+$ B cells can be associated with loss of the CD5 expression (23, 46). In A/J mice, B cells producing antiarsonate antibodies after active immunization are thought to be the progeny of virgin B lymphocytes originally making Ig with unmutated V_H gene segments and endowed with multiple "anti-self" reactivity (47). Maturation of the antibody response to arsonate parallels class switch from IgM to IgG, increasing number of point mutations in the Ig V_H gene segments, and loss of "anti-self" reactivity. Sequencing of the gene segments encoding the V regions of the polyreactive low affinity, as well as monoreactive high affinity, antibodies to rabies virus will help to unequivocally identify these genes and to elucidate the possible contribution of Ag-selected somatic point mutations to the affinity of such antibodies.

The inability to easily culture Ag-induced antibody-producing cells and to use them to construct continuous mAb-producing human cell lines has been the major impediment in the study of the human B cell function and repertoire (14, 15). In the present studies, by applying recently developed methods (16–21) to the analysis of lymphocytes obtained from volunteers immunized with rabies virus, we have defined the properties of the B cell clones recruited through the progression of the immune response from polyreactive low affinity to monoreactive high affinity antibodies. Moreover, we have demonstrated that human IgG mAbs of predetermined specificity and high affinity and with, possibly, important biological activities (e.g., virus neutralization) can be systematically generated from the Ag-primed human B cell repertoire. This approach may be used to analyze the specific antibody response to other foreign Ags or self Ags in humans. It also may be used, as shown elsewhere (Dietz-

schold, B., P. Casali, Y. Ueki, M. Gore, C. E. Rupprecht, A. L. Notkins, and H. Koprowski, manuscript submitted for publication), to generate human mAbs for immediate application in vivo.

Summary

We quantitated and characterized the changes in the human B cell repertoire, at the clonal level, before and after immunization with rabies virus. Moreover, we generated 10 monoclonal cell lines producing IgM, IgG, and IgA antibodies to the virus. We found that in healthy subjects, not previously exposed to the virus, nearly 2% of the circulating B lymphocytes were committed to the production of antibodies that bound the virus. These B cells expressed the surface CD5 molecule. The antibodies they produced were polyreactive IgM that displayed a relatively low affinity for the virus components (K_d, 1.0–2.4 × 10^{-6} g/μl). After immunization, different anti-virus (IgG and IgA) antibody-producing cells consistently appeared in the circulation and increased from <0.005% to >10% of the total B cells committed to the production of IgG and IgA, respectively. Most of such B cells do not express CD5 and produce monoreactive antibodies of high affinity for rabies virus (K_d, 6.5 × × 10^{-9} to 1.2 × 10^{-10} g/μl). One of these IgG mAbs efficiently neutralized rabies virus in vitro and in vivo, as detailed elsewhere (Dietzschold, B., P. Casali, Y. Ueki, M. Gore, C. E. Rupprecht, A. L. Notkins, and H. Koprowski, manuscript submitted for publication). Hybridization experiments using probes specific for the different human V gene segment families revealed that cell precursors producing low affinity IgM binding to rabies virus utilized a restricted number of V_H gene segments (i.e., only members of the V_HIIIb subfamily), whereas cell precursors producing high affinity IgG and IgA to rabies virus utilized an assortment of different V_H gene segments (i.e., members of the V_HI, V_HIII, V_HIV, and V_HVI families and V_HIIIb subfamily).

In conclusion, our studies show that EBV transformation in conjunction with limiting dilution technology and somatic cell hybridization techniques are useful methods for quantitating, at the B cell clonal level, the human antibody response to foreign Ags and for generating human mAbs of predetermined specificity and high affinity.

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