Immunoglobulin V_H Usage during Primary Infection of Rhesus Monkeys with Chimeric Simian-Human Immunodeficiency Viruses

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It has been suggested that naive immunoglobulins encoded by the V_H3 gene family interact aberrantly with human immunodeficiency virus type 1 (HIV-1) gp120 via a superantigenic epitope, causing initial expansion and eventual depletion of V_H3-expressing B cells. However, this possibility has not been prospectively assessed during an AIDS virus infection. We determined V_H family usage in rhesus monkeys during primary infection with chimeric viruses expressing HIV-1 envelopes on a simian immunodeficiency virus (SIV_{mac}) backbone (SHIVs). Four SHIVs with different envelopes and pathogenicities were studied. V_H family usage was prospectively assessed in peripheral blood mononuclear cells and lymph node cells of these monkeys by a semiquantitative PCR technique. In the first months following SHIV infection, a period of intense viral antigenemia, representation of various V_H families increased or decreased for individual monkeys, but no single V_H family was consistently altered. In particular, the average representation of V_H3-bearing B lymphocytes did not change. This observation suggests that the envelope glycoprotein of HIV-1 does not selectively expand or deplete the V_H3 repertoire of primate B cells during acute AIDS virus infection, contrary to predictions of the gp120 superantigen hypothesis.

B-lymphocyte dysfunction has long been known to occur in human immunodeficiency virus type 1 (HIV-1)-infected humans. Nonspecific B-lymphocyte activation contributes to hyperimmunoglobulinemia (37). Moreover, defective B-cell responses to T-cell-independent antigens are observed (3), which presumably are not attributable to defective T-cell help. Impaired B-cell responses to mitogens (38) are not corrected when B cells of HIV-1-infected donors are provided with helper T cells from uninfected donors (23). HIV-1-infected individuals are at increased risk for monoclonal gammopathy and for B-cell lymphoma, which sometimes produce antibodies reactive with HIV-1 viral proteins (1). The mechanisms accounting for these immunopathogenic features of HIV-1 infection remain uncertain. Reports of disproportionate numbers of V_H3-expressing B cells and secreted V_H3 immunoglobulin (Ig) in HIV-1-infected individuals at early and late stages of HIV-1 infection have led to speculation that the HIV-1 envelope may contain a superantigen that causes an early expansion and eventual depletion of V_H3-expressing B lymphocytes during infection (5, 6, 11, 12), possibly contributing to B-cell dysfunction. Indeed, the HIV-1 gp120 exterior glycoprotein has been reported to interact aberrantly with Igs encoded by the V_H3 heavy-chain variable-region gene family, binding to framework regions in a superantigen-like fashion (4, 15, 18).

However, studies examining V_H family repertoire changes associated with HIV-1 infection have been limited in several important respects. All have been cross-sectional population studies, rather than the prospective, serial examinations of individuals that would be needed to demonstrate the evolution

of V_H-specific repertoire changes. A genetic and functional characterization of the viruses infecting the evaluated individuals has not been carried out, making it impossible to determine if viral isolates differ in their abilities to recruit B cells expressing Igs of selected V_H families. Finally, it is possible that V_H repertoire changes would be most dramatically affected during periods of intense viremia and high antigen load. This virologic state occurs during the period of primary infection. It has, however, proven to be difficult to identify infected individuals during this period of primary infection.

Nonhuman primate models of lentivirus infections offer significant advantages over studies with humans in illuminating AIDS pathogenic events. The simian immunodeficiency virus (SIV)-infected macaque has proven to be a particularly powerful model for studying primary AIDS virus infections (33, 34). Since the Ig V_H genes in macaque monkeys are very similar to their human homologs (27), this model should be useful for exploring the effect of an AIDS virus infection on the Ig V_H repertoire. However, SIV envelope glycoproteins have limited serologic cross-reactivity with HIV-1 envelope glycoproteins, indicating that significant differences in the interaction of these molecules with antibodies will exist. Consequently, the SIV envelope glycoprotein might not effectively model the putative effect of HIV-1 gp120 on V_H3 B cells.

Chimeric viruses expressing HIV-1 envelope proteins on a SIV_{mac} backbone have recently been shown to infect rhesus macaques, replicating to high virus loads and eliciting long-lasting virus-specific humoral and cellular immunity (32) and causing AIDS-like illness (31). These viruses enable us to explore the in vivo effects of the putative HIV-1 gp120 B-cell superantigen in a nonhuman primate model. To examine the possibility that the HIV-1 envelope induces V_H family-specific alterations in immunity, we defined the changes that occur in

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the expressed V_H repertoire of rhesus monkeys during primary infection with chimeric SIV-HIVs (SHIVs).

MATERIALS AND METHODS

SHIV plasmid construction and preparation of virus stocks. The design and preparation of SHIV-89.6 and SHIV-HXBc2 stocks are described elsewhere (32). In brief, the *env*, *tat*, and *rev* genes of the HIV-1 clone HXBc2 (36) were introduced into an infectious molecular clone of SIV_{mac239} (30). The resulting virus was altered by site-directed mutagenesis to create a functional *vpv* gene. SHIV-89.6 was generated by cloning a fragment containing the *env* and *rev* genes of the HIV-1 89.6 isolate (9) into the *KpnI*-*Bam*HI sites of SHIV-HXBc2. Chimeric virus clones were transfected into CEMx174 cells and propagated in rhesus monkey peripheral blood mononuclear cells (PBMC). Pooled culture supernatants whose titers were determined were stored in liquid nitrogen and served as virus stocks for use in animal inoculations. While monkeys infected with these SHIVs have maintained stable CD4 counts over 2 years, a highly pathogenic strain was obtained after serial passage of SHIV-89.6 and so was termed SHIV-89.6P. Virus stocks of SHIV-89.6P molecular clones KB1 and KB9 were prepared by similar methods (17).

Infection of rhesus monkeys with SHIV. The rhesus monkeys (*Macaca mulatta*) used in this study were maintained at the New England Regional Primate Research Center or the Oregon Regional Primate Research Center in accordance with the guidelines of the Committee on Animals for the Harvard Medical School and the *Guide for the Care and Use of Laboratory Animals* (26a). In preliminary studies, validation testing of the semiquantitative PCR assay was performed with blood from only normal (uninfected) rhesus monkeys. In the principal study, 14 healthy rhesus monkeys were inoculated with SHIV. Four monkeys were infected on day 0 by intravenous inoculation with SHIV-HXBc2 at 400 times the dose yielding 50% infection in a T-cell line in vitro. Four other monkeys were infected with the same dose of SHIV-89.6. Two groups of three monkeys were similarly infected with SHIV-89.6P clone KB1 or KB9. Infection of all animals was confirmed by serologic and/or virologic assay (17, 32).

Monkeys were anesthetized with ketamine-HCl for all blood sampling and biopsies. Venipuncture was performed before infection, on the day of infection, twice weekly until week 8, and monthly thereafter for 4 months. Macaque PBMC were isolated from 5 to 10 ml of EDTA-anticoagulated venous blood by density gradient centrifugation over Ficoll-Paque (Pharmacia) and washed with Hanks balanced salt solution (HBSS). If available, at least 2×10^6 cells were set aside for isolation of mRNA, and the remainder were apportioned for other assays.

All SHIV-inoculated animals became infected, with peak viremia occurring from 10 days to 3 weeks after inoculation. Plasma viral loads during this primary viremia in the monkeys inoculated with the three 89.6-based SHIVs (17, 32) were similar to viral loads seen in monkeys infected with pathogenic SIV_{mac}. Peak plasma viral loads were lower in the SHIV-HXBc2-infected animals. Infection with SHIV-89.6P clone KB1 or KB9 led to precipitous and persistent declines in CD4 cell counts; SHIV-HXBc2 and SHIV-89.6 infection did not cause persistent CD4 lymphocyte loss (17, 32).

The time course of seroconversion in monkeys infected with nonpathogenic SHIVs was documented by enzyme-linked immunosorbent assay (32). Anti-Env antibodies were first detectable 14 to 18 days postinoculation; titers increased during the next 2 to 3 weeks and subsequently contained type-specific neutralizing antibodies. Anti-gp120 antibodies in a cohort of monkeys infected with uncloned pathogenic SHIV-89.6P were documented by radioimmunoprecipitation (31). Thus, monkey B cells were interacting with the HIV-1 envelope.

Lymph nodes from monkeys infected with nonpathogenic SHIV were studied as follows. Lymph nodes were obtained from axillary or inguinal regions before infection and on weeks 1, 2, and 3 following infection. Each site was biopsied only once. To obtain lymph node cells, biopsy specimens were placed in HBSS and disaggregated by teasing with forceps. For histology and immunohistochemistry, lymph nodes were fixed in 4% buffered formalin and embedded in paraffin. Five-micrometer thin sections were cut, deparaffinized, and stained with hematoxylin-eosin or processed for immunohistochemistry. Antigen exposure was facilitated by boiling the sections in a pressure cooker (citrate buffer, pH 6.0) for 5 min (28). Sections were then incubated with a 1:50 dilution of a monoclonal antibody, using L26 (Dakopatts, Hamburg, Germany) to detect CD20⁺ B cells or MIB-1 (Dianova, Hamburg, Germany) to identify proliferating cells (8). Immunostaining was visualized by using the alkaline phosphatase-anti-alkaline phosphatase method (10), with naphthol biphosphate (Sigma, Munich, Germany) and new fuchsin (Chroma K6ngen) as substrates to generate a dark red reaction product. After immunohistochemical processing, the sections were counterstained with hemalaun.

Cell lines. Human B hybridomas F54, F71, and HL-6 were provided by Marshall Posner (Boston, Mass.); Ab19 and Ab37 were provided by J. Donald Capra (Dallas, Tex.). The B-lymphoblastoid cell line ML-1 was provided by Ton Logtenberg (Utrecht, The Netherlands). Each cell line was expanded in RPMI 1640 supplemented with 10% fetal calf serum and L-glutamine and washed in HBSS before mRNA isolation.

Immunofluorescent flow cytometry. Monkey PBMC obtained by whole-blood lysis techniques were immunophenotyped by two-color flow cytometry with human leukocyte-specific monoclonal antibodies that recognize monkey CD20 (B1; Coulter Corp., Hialeah, Fla.), a molecule expressed selectively on B cells (35),

and CD3 (FN18; Coulter), a pan-T-cell marker. Absolute cell numbers were calculated from the lymphocyte counts determined with an automated hematology analyzer (T540; Coulter Corp.).

Nucleic acid preparations. mRNA was extracted from 2×10^6 to 10×10^6 PBMC or lymph node cells by using the Quick Prep micro mRNA kit (Pharmacia, Piscataway, N.J.) and stored under ethanol at -70°C . After aqueous reconstitution of mRNA, cDNA was generated by using oligo(dT) primers and avian myeloblastosis virus reverse transcriptase (Promega, Madison, Wis.). cDNA was stored at -20°C . The cDNA from 10^5 to 10^6 cells was used for each PCR.

PCR primer design. Ig V_H family-specific 5' primers which recognize untranslated leader sequences were adapted from those described by Campbell et al. (7). Primer sequences, with added restriction sites underlined, were as follows: V_H1, ACGCCGTCGACATGGACTGGACCTGGAGGATC; V_H2, ACGCCGTCGACATGGACATACCTTTGTTCCACG; V_H3, ACTAAGTCGACATGGAGTTTGGGTGAGCTGGG; V_H4, ACGCCGTCGACATGAAACCTGTGGTTC TTCC; V_H5, FR1, GAAAAAGCCCGGGAGTCTCTGAA; and V_H6, ACGCCTGACATGTCTGTCTCTCTCTCAT. The V_H5 5' primer used in this study was designed based on FR1 region conserved sequences (13), because the V_H5 leader primer formed primer-dimers with the 3' primer. The same 3' primer, based on a consensus sequence derived from germ line J_H genes, was used for all V_H families: AGGAGAATTCTGAGGAGACGGTGACCAGGGT. As an internal control in some experiments, a separate primer pair was added to amplify the C_κ gene: 5', ACCAAGGTCGACATCAACGAACTGTGGCT; 3', CTGTCTAGCTCTGTGACACTCTCTGGAG. These primers all react with macaque as well as human Ig genes (22, 27). All oligonucleotides were prepared by Operon Technologies (Alameda, Calif.). The 3' primer of V_H and C_κ pairs was directly labeled with [³²P]dATP by using T4 polynucleotide kinase (Promega) and purified over a Chromaspin-10 column (Clontech Laboratories, Palo Alto, Calif.).

PCRs and semiquantitative analysis. All PCRs were performed for 30 cycles (94°C for 20 s, 58°C for 30 s, and 72°C for 30 s) in 50-μl volumes by using the hot-start technique in a Perkin-Elmer GeneAmp 9600 Thermal Cycler. Concentrations of reactants were the same for all V_H families. Five picomoles of one V_H primer and the ³²P-J_H primer were added to each tube, along with 2.5 pmol of C_κ primers. Reaction products were electrophoresed through 6% polyacrylamide and dried onto paper. For quantitation, dried gels were analyzed by direct radioanalytic detection with a Systems 4000 scanner and software (Ambis, San Diego, Calif.). For each specimen, the signal of each V_H family was normalized by comparison with coamplified C_κ, and its representation in the repertoire was expressed as a percentage of the total signal amplified by all the V_H primers for that specimen:

$$V_{H_n} = [(V_{H_n}/C_{\kappa}) / \sum_{i=1}^6 (V_{H_i}/C_{\kappa})] \times 100$$

Potential differences in amplification efficiency for various V_H primer pairs and Ig genes have not been determined, so our values represent a semiquantitative approximation of the actual repertoire distributions. Average baseline variability was calculated separately for each V_H family, based on pooled data from reinfection samples. Deviations from baseline of more than two standard deviations with two or more consecutive specimens were considered significant.

For Fig. 3B, PCR products from the indicated cell lines were digested with either the restriction enzyme *Bam*HI (which recognizes all V_H2 germ line sequences but few V_H3 sequences) or *Stu*I (which recognizes the V_H6 germ line gene but not V_H1 sequences) and analyzed by 6% polyacrylamide gel electrophoresis.

RESULTS

The V_H family repertoire of rhesus monkey B cells following infection with SHIV expressing HIV-1 envelope glycoproteins was evaluated. Fourteen monkeys were inoculated with SHIV isolates: four were inoculated with an isolate expressing Env from the laboratory HIV-1 isolate HXBc2, four were inoculated with an isolate expressing Env from the primary HIV-1 isolate 89.6, and three each were inoculated with a passaged SHIV-89.6P molecular clone, KB1 or KB9.

Alterations in circulating B-lymphocyte counts during primary SHIV infection. To determine whether the initial viremia following SHIV infection was associated with alterations in the circulating B-lymphocyte population, we determined the absolute peripheral blood B-lymphocyte counts of the infected monkeys by flow immunocytometry (32) (Fig. 1). All SHIV infections caused precipitous though transient declines in circulating B-lymphocyte numbers during the period of peak plasma viremia. These B-cell declines began earlier and lasted longer for pathogenic SHIVs. B-cell counts in monkeys infected with nonpathogenic SHIVs rose to roughly 150% of the

FIG. 1. Rhesus monkeys develop transient depletion of CD20⁺ cells (B lymphocytes) in peripheral blood during primary SHIV infection. Cells were phenotyped by two-color immunofluorescent flow cytometry. Values for individual animals are shown. (A) Monkeys infected with SHIV-89.6P clone KB1. (B) Monkeys infected with SHIV-89.6P clone KB9. For data for nonpathogenic SHIVs, see reference 32.

preinoculation values during the second month postinfection before returning to normal. This phenomenon was not seen in monkeys infected with pathogenic SHIVs.

Nonpathogenic SHIV infection stimulates B-lymphocyte expansion in lymph nodes. Lymph nodes were taken from monkeys on days 0, 7, 14, and 21 following infection with nonpathogenic SHIVs to assess the changes that occur in B lymphocytes in secondary lymphoid tissues. (Lymph nodes from the monkeys infected with pathogenic viruses were not available.) The histologic appearances of the B-cell-dependent zones were similar in all lymph nodes that were studied at each time point. During the first week following infection (days 0 and 7), small primary follicles dominated in the cortex (Fig. 2A). A few secondary follicles were present in all animals. However, the majority of the germinal centers were small and inactive, and only a few of them contained proliferating cells (data not shown). The medullary sinuses, from which cells pass into the efferent lymphatics to leave the node, contained less than 16% CD20⁺ cells (data not shown).

At days 14 and 21, the B-cell-dependent zones were expanded (Fig. 2B). In all animals, lymph nodes contained increased numbers of well-developed germinal centers containing many proliferating cells in the dark zone (Fig. 2C). The medullary sinuses showed enhanced cellularity, with an increased proportion of B cells, ranging from 25 to 45% (Fig. 2D). B cells in mitosis were also present in the medullary sinuses (data not shown).

PCR analysis of Ig V_H family repertoire (i) Primer pairs specifically amplify cDNAs of single V_H families. A PCR-based assay was developed to assess changes in the Ig V_H family repertoires of these monkeys following SHIV infection. In this assay mRNA was extracted from PBMC or lymph node cells and reverse transcribed to cDNA, and Ig V_H family members were amplified with V_H family-specific primer pairs. To ensure that the primer pairs selected for use in these studies could

amplify primate Ig cDNA in an Ig V_H family-specific manner, we applied them in an evaluation of the V_H families of a panel of human B-cell hybridomas and B-lymphoblastoid cell lines (Fig. 3A). cDNAs from individual cell lines were amplified by using six Ig V_H family-specific primer pairs. (V_H7 family Igs may be amplified by the V_H1 primer because of sequence similarity in 5' regions of V_H1 and V_H7. However, V_H7 family Igs are very rarely expressed.) V_H1, V_H3, V_H4, and V_H5 PCR products were selectively amplified from these cell lines in this manner. Moreover, primers designed to detect specific V_H family cDNAs did not cross-amplify cDNAs of other V_H families.

In the cases of hybridoma Ab19 and B-lymphoblastoid cell line ML-1, PCR products were amplified with more than one primer pair. To determine whether this represented cross-amplification of more than one Ig V_H family or whether each signal represented a unique Ig message, we digested each PCR product with restriction enzymes which yield previously defined V_H family-specific cutting patterns. If the PCR products resulted from cross-family amplification of cDNA from a clonal cell line, the restriction patterns should be identical. As shown in Fig. 3B, the PCR product amplified from cDNA obtained from Ab19 by using V_H2- but not V_H3-specific primers exhibited a gel mobility shift after *Bam*HI digestion. Analysis of cDNA from ML-1 after digestion by *Sst*I gave a similar result, with a gel mobility shift seen only in the PCR product amplified with the V_H6-specific primer pairs. These restriction differences are in accord with published V_H germ line sequences showing a *Bam*HI site conserved in all members of the V_H2 family but not in V_H3 and a *Sst*I site in V_H6 but not in V_H1 sequences. These results indicate that Ab19 and ML-1 are oligoclonal rather than clonal, with cells expressing genes from two different V_H families. Thus, in all cases, the V_H family-specific primers performed as designed and did not amplify cDNA from more than a single V_H family.

(ii) cDNA amplification is proportional to target copy number. To validate our assay for the quantitative determination of the relative expression of V_H families in B-cell populations, we assessed specimens containing mixtures of clonal hybridoma cell lines in known proportions. As an internal control, we included in each tube primers to amplify the Ig κ -chain constant region. Serial dilutions of hybridoma F54 cDNA (V_H4, λ) were mixed with a constant quantity of hybridoma F71 cDNA (V_H1, κ). Amplification of this cDNA mixture with V_H4-specific primers generated a signal which was proportionate to the concentration of cDNA derived from the V_H4-expressing clone. By contrast, amplification of the same cDNA mixture with V_H1-specific primers generated a constant signal, reflecting the concentration of cDNA from the V_H1-expressing clone in the mixture (Fig. 4A). PCR amplification was linear ($r^2 = 0.99$) over the range between 30 and 60,000 cell equivalents of cDNA (Fig. 4B). For samples outside this range, PCR amplification deviated from linearity, and relative cDNA concentrations could not be accurately quantitated.

We also sought to determine the sensitivity of our PCR assay for unstimulated monkey PBMC, in which B cells constituted roughly 5 to 10% of the assessed cell population. We prepared specimens containing serial twofold dilutions of cDNA from rhesus PBMC, with bovine rRNA added to limit nonspecific nucleic acid losses. Amplification of these cDNAs with family-specific primers (Fig. 5A) demonstrated that PCR amplification of V_H targets was linear (r^2 ranging from 0.91 to 0.99) over the range between 2,000 and 250,000 PBMC equivalents of cDNA (Fig. 5B). For samples outside this range, PCR amplification deviated from linearity, and cDNA concentrations could not be accurately quantitated. When no target cDNA

FIG. 2. Immunohistochemistry demonstrates an expansion within B-lymphocyte zones in the lymph nodes of rhesus monkeys during primary SHIV infection. Immunoreactive cells were stained dark red by the alkaline phosphatase–anti-alkaline phosphatase reaction, with hemalaun counterstain. (A) Before infection, small primary follicles contain few CD20⁺ B cells (monkey 123, day 0). Magnification, $\times 20$. (B) After SHIV infection, an enlarged follicle contains chiefly CD20⁺ B cells (monkey 123, SHIV-89.6, day 21). Magnification, $\times 20$. (C) Monoclonal antibody MIB-1 stains numerous proliferating cells within an enlarged follicle (monkey 123, day 21). Magnification, $\times 40$. (D) There is an increased proportion of CD20⁺ cells within the lymph node medullary sinus after SHIV infection (monkey 042, SHIV-HXBc2, day 21). Magnification, $\times 63$.

FIG. 3. PCR amplification of Ig heavy-chain variable-region cDNAs from human cell lines and clones is family specific. (A) Primers can specifically amplify cDNAs from the six major Ig V_H families. B-LCL, B-lymphoblastoid cell line. (B) Restriction enzyme digestion of PCR products from the indicated cell lines demonstrates that the cell lines are oligoclonal. This accounts for the amplification of PCR product by more than one V_H family-specific primer pair in two cell lines evaluated in panel A.

was added, no signal was seen. The lower sensitivity observed with PBMC relative to hybridomas likely resulted from the high mRNA content of Ig-secreting hybridoma cells and the low percentage (5 to 10%) of B cells in rhesus monkey PBMC.

To determine the ability of this PCR-based assay to detect changes in the V_H repertoire in cell populations expressing Igs of a variety of V_H families, we assayed normal monkey PBMC admixed with serial dilutions of a clonal population of hybridoma cells (F71, V_H1) and calculated the V_H repertoires. Normalization to the common reference band provided by C_κ facilitated comparison of the relative abundances of particular V_H family mRNAs present in these mixed populations of B lymphocytes. Additionally, the C_κ band served as an indicator of the efficiency of reverse transcription and PCRs and the

accuracy of sample loading. PBMC alone contained 20% V_H1-bearing cells. After the higher mRNA content of hybridoma cells was taken into account, the increase in V_H1 representation in the Ig repertoire of the combined population correlated well ($r^2 = 0.92$) with the proportion of admixed hybridoma cells, indicating that assay sensitivity was maintained when complex mixtures of V_H mRNA were analyzed.

Alterations in Ig V_H repertoire of rhesus monkeys during primary infection with nonpathogenic SHIV. The relative abundances of mRNAs from the six major V_H families in peripheral blood and lymph node lymphocytes of rhesus monkeys infected with nonpathogenic SHIVs were then analyzed by using this semiquantitative PCR methodology. In specimens obtained from each of eight monkeys before infection, the relative abundances of the V_H family cDNAs were similar. Although determined by semiquantitative methodology, the relative V_H expression agreed quite well with previously reported values for the percentage of the repertoire contributed by each V_H family (14, 16, 19, 20). For example, the V_H family cDNAs were detected in approximate proportion to their germ line copy number. Thus, V_H3 accounted for roughly 40% of the V_H signal, V_H4 accounted for 25%, and the smaller families accounted for relatively less. Small differences were seen in the

FIG. 4. PCR amplification with Ig V_H family-specific primers and reference primers is quantitative in detecting the proportion of each V_H family in a mixed population of lymphoid cells. (A) cDNA from a V_H1, C_κ clone was mixed with serial threefold dilutions of V_H4, C_κ clone cDNA. The mixture was amplified by using either V_H1- or V_H4-specific primer pairs and C_κ primers. Asterisks mark negative control lanes where cDNA was omitted from the PCR. (B) V_H1-primed (circles) and V_H4-primed (rectangles) PCR product quantitation by scanning radioanalytic detection. Regression analysis fitted to the log-transformed signal from amplified V_H4 cDNA shows linear amplification in the range between 30 and 60,000 cell equivalents of cDNA.

FIG. 5. PCR amplification with Ig V_H family-specific and reference (C_k) primers is sensitive and linear in quantitative analysis of rhesus monkey PBMC across 2 orders of magnitude. (A) PCRs with the indicated primer pairs and serial twofold dilutions of rhesus monkey PBMC cDNA were analyzed by autoradiography after polyacrylamide gel electrophoresis. (B) Quantitation of PCR products shown in panel A by scanning radioanalytic detection. Regression analysis fitted to the log-transformed signal from each primer set shows linear amplification in the range between 2,000 and 100,000 PBMC equivalents.

calculated percentages of each family for individual monkeys. No baseline differences in V_H repertoire between the monkeys assigned to each nonpathogenic SHIV infection group were found.

Repeated determinations of the V_H repertoire in the PBMC of each animal during the month before infection showed some baseline variation, with standard deviations averaging 3.0 (V_H1), 1.0 (V_H2), 3.1 (V_H3), 3.2 (V_H4), 1.4 (V_H5), and 1.6 (V_H6). Standard deviations in baseline values for the cohort taken as a whole were larger, because interanimal differences were typically greater than day-to-day variations for an individual animal. Individual baseline standard deviations could not be determined for lymph node samples, because only a single preinfection specimen was obtained from each monkey.

Following inoculation with either SHIV-HXBc2 or SHIV-89.6, individual animals showed fluctuations in the relative abundances of expressed Igs of particular V_H families in the lymphocytes from blood and lymph nodes. In the blood, lymphocytes of three of eight monkeys exhibited changes in the expressed Ig of one or more V_H families that measured at least twice the baseline variation for those families with two consecutive samples. In the lymph nodes, cells of four of eight monkeys showed such changes. Representative images and quan-

titative data for a single monkey (animal 471) are shown in Fig. 6. V_H1, V_H3, and V_H4 were the most highly represented Ig V_H families in all specimens; cDNAs from the smaller families, i.e., V_H2, V_H5, and V_H6, could barely be detected following PCR amplification. Following infection with SHIV-HXBc2, the representation of V_H4 rose twofold in cells from the lymph nodes, from 20 to 40% of the total signal. V_H4 representation in the PBMC of this animal also increased, although to a lesser degree. By comparison, the preinfection baseline variability in V_H4 representation in the PBMC of this animal was only 3.5%. No changes in V_H3 representation in the Ig repertoire were detected in the PBMC or lymph node cells of this animal following infection.

Indeed, changes in V_H3 representation in the Ig repertoire were seen in PBMC from only three of eight monkeys and averaged only 3% above the range of two standard deviations. Moreover, changes in V_H3 representation in the Ig repertoire in the lymph node cells of these animals were not detected, despite active germinal center formation. To determine whether an expansion of V_H3 in the repertoire may occur during the chronic phase rather than the acute phase of nonpathogenic SHIV infection, one monkey from each group was studied for 3 months following virus inoculation. As shown in Fig. 7, the

FIG. 6. V_H4 family representation is increased in the Ig repertoires of blood and lymph node lymphocytes (LNC) of a single rhesus monkey (no. 471) during primary infection with SHIV-HXBc2. (A) Autoradiograph of PCR products following cDNA amplification with V_H family-specific, J_H, and C_k primers, d., day. (B) The percentage of the total V_H signal contributed by each V_H family, normalized to an internal reference provided by C_k, was determined by scanning radioanalytic detection of the gels shown in panel A.

FIG. 7. The expansion of V_H3 -expressing B cells in peripheral blood of two animals during primary SHIV infection is transient. The monkeys were infected with SHIV-HXBc2 (A) or SHIV-89.6 (B). The representation of individual V_H families in the Ig repertoire was determined by semiquantitative PCR for each animal.

percentages of V_H3 -expressing B cells returned to baseline values in both monkeys during this period of evaluation.

Importantly, no consistent pattern of V_H family recruitment was seen in the Ig response of either cohort of monkeys. In particular, V_H3 Igs did not increase as a percentage of the repertoire during primary infection with either nonpathogenic SHIV clone. The percentage of total V_H signal contributed by V_H3 sequences among SHIV-HXBc2-infected monkeys was approximately 38% for PBMC and 39% for lymph node cells; among SHIV-89.6-infected monkeys, V_H3 contributed 40% of the V_H signal in PBMC and 42% in lymph node cells (Fig. 8A and 9).

Alterations in Ig V_H repertoire of rhesus monkeys during primary infection with pathogenic SHIV. While infection with nonpathogenic SHIV did not lead to detectable superantigen-induced V_H family-specific repertoire alterations, the possibility remained that the phenomenon occurs only with AIDS virus strains capable of inducing immunodeficiency disease. To test this hypothesis, we next studied a cohort of rhesus monkeys infected with SHIV variants which reproducibly induce CD4 lymphocyte depletion and immunodeficiency, resulting in opportunistic infections typical of human AIDS (31), i.e., pathogenic SHIVs.

Using the semiquantitative assay described above, we prospectively defined the V_H repertoires in PBMC from six monkeys prior to and following infection with two pathogenic

SHIV-89.6P clones, KB1 and KB9. The usage pattern of V_H family segments (Fig. 8B) was highly similar to those obtained for rhesus monkeys from the other cohorts in our study. Baseline variability was also comparable, within 2 or 3% of that seen in monkeys infected with nonpathogenic SHIVs. No significant differences between groups of monkeys infected with clone KB1 versus clone KB9 were observed, and neither group showed even a trend suggestive of a consistent alteration in V_H3 B-cell frequencies. Indeed, no individual animal in these groups developed significant changes in the percentage of V_H3 B cells during the 3-month study period.

DISCUSSION

In the present report we document changes in B-lymphocyte populations in the peripheral blood and lymph nodes of rhesus monkeys during primary infection with four SHIVs which encode HIV-1 envelope glycoproteins differing in tropism and pathogenicity. Circulating B-cell populations underwent profound early depletion and subsequent reexpansion following infection with all four SHIVs. Importantly, analysis of Ig V_H repertoires in infected monkeys did not detect evidence for a superantigenic mechanism underlying these events.

Increases in the number of circulating B lymphocytes during primary infection with nonpathogenic SHIVs (32) could reflect clonal expansion of B cells, redistribution of B cells, or both. Our demonstration that follicular hyperplasia and B-cell proliferation occur within lymph nodes suggests that these nonpathogenic SHIV infections stimulate B-cell expansion. Such B-cell expansions have been shown to occur in SIV- and HIV-1-infected individuals (21, 37). Moreover, an increased number of B cells were found within lymph node medullary sinuses, presumably in the process of leaving the lymph nodes. Therefore, B-lymphocyte expansion within the lymph nodes could contribute to the elevation of B-cell counts and the alterations in V_H usage by circulating B cells that we observed in the peripheral blood during nonpathogenic primary SHIV infection.

In view of reports suggesting the existence of a B-cell superantigen within HIV-1 gp120, we sought to determine whether the B-cell changes seen in SHIV-infected monkeys were associated with alterations in Ig gene usage. A viral superantigen binding to framework epitopes on Igs derived from a single V_H gene segment could selectively activate B cells bearing Ig derived from that particular V_H segment. Whether activation triggers clonal expansion or apoptosis, it could introduce a selection bias. Depletion and repopulation of the B-lymphocyte pool in blood, as observed in all SHIV-infected monkeys, could amplify the effects of such a V_H -specific bias. Therefore, we serially assessed representation of the six major Ig V_H families in PBMC and lymph node cells from SHIV-infected macaques by using a semiquantitative PCR-based assay. We found that the relative expression by B cells of various V_H family Igs did indeed change in individual monkeys after virus infection. However, no single V_H family was consistently altered in any cohort of SHIV-infected monkeys.

The diversity of shifts in V_H family usage observed in SHIV-infected monkeys conflicts with reports from several laboratories of V_H3 -specific changes in the Ig repertoire in humans infected with HIV-1 (2, 4–6, 11, 12, 25). As the present experiments represent the first in vivo study of a putative gp120 B-cell superantigen in nonhuman primates, it is possible that experimental peculiarities in the study could account for our inability to detect a systematic alteration in the Ig repertoire of SHIV-infected monkeys.

HIV-1 strain differences have generally been overlooked in

FIG. 8. No consistent change in Ig V_H family representation occurs in PBMC of rhesus monkeys during primary infection with SHIV. The representation of individual V_H families in the Ig repertoire was determined by semiquantitative PCR for each animal separately, and then the values were averaged. The cohorts were infected with SHIV strains as indicated. (A) Nonpathogenic SHIV-HXBc2 ($n = 4$) and SHIV-89.6 ($n = 4$). (B) Pathogenic SHIV-89.6P clones KB1 ($n = 3$) and KB9 ($n = 3$). Bars indicate the cohort averages, and error bars show one standard deviation.

studies of biased V_H family usage during infection. Indeed, there has been no characterization of the infecting virus in any of the studies of B-lymphocyte V_H family usage in humans. Conceivably, the envelope glycoproteins expressed by the HXBc2 and 89.6 SHIVs may differ in some important way from the envelopes previously studied. However, an envelope derived from the same HIV-1 isolate as HXBc2 (clone BH10 [29]) has been reported to bind V_H3 Ig (4). Moreover, to maximize the generalizability of our results, we assessed the immune sequelae of infection of rhesus monkeys by four different SHIV clones, each with a different pathogenicity and tropism, representing both laboratory-propagated and primary HIV-1 isolates.

It may be supposed that an HIV-1 gp120 superantigen might interact differently with human and rhesus monkey Igs. However, several lines of evidence suggest that this is unlikely. First, monkey Igs are quite similar to their human homologs. Sequences of expressed antibodies from macaques exhibit between 93 and 97% homology to a human sequence in framework regions of V_H3 (27), and Ig germ line homologies may be even higher (22). Serologic reactivities of rhesus monkey and human Igs are sufficiently similar that rhesus monkey anti-HIV-1 gp120 antibodies can even share an idiotypic marker, termed 1F7 (24), associated with human anti-HIV-1 antibodies (26). Second, it has been reported that the putative gp120 superantigen binds in vitro to murine, rabbit, monkey, and

chimpanzee Igs, as well as human Ig, suggesting no species restriction of this interaction (39).

In contrast to previous studies with humans, our experiments focused on B-cell repertoire changes during very early stages of infection. Prior studies have detected anomalous V_H3 expression by lymphocytes of subjects who were recent HIV-1 seroconverters and by those of individuals infected with HIV-1 for less than 1 year (5, 12). We cannot rule out the possibility that V_H3-specific perturbations in Ig repertoire might develop later in the clinical course of a SHIV infection of rhesus monkeys. On the other hand, it seems more likely that B-cell changes should be greatest during primary infection, when an intense viral antigenemia drives a transient depletion and subsequent expansion of the B-lymphocyte population. Indeed, profound B-lymphocyte population shifts were observed in all 14 monkeys in the present study, but none showed even a trend towards an increasing frequency of V_H3 B cells in up to 3 months of follow-up postinfection. Moreover, three of six monkeys infected with pathogenic SHIVs had CD4 counts of below 200 by the study's end, an indication that they had developed advanced immunodeficiency, without alterations in V_H3 expression.

The V_H repertoire analyses described in the present study were performed with mRNAs extracted from unselected lymphocyte populations, without regard for antigen specificity or heavy-chain isotype. It is possible that evaluation of selected

FIG. 9. No consistent change in Ig V_H family representation in lymph node cells of rhesus monkeys occurs during primary infection with nonpathogenic SHIVs. The representation of individual V_H families in the Ig repertoire was determined by semiquantitative PCR for each animal separately, and then the values were averaged. The cohorts were infected with SHIV-HXBc2 or SHIV-89.6, as indicated. Bars indicate the cohort average ($n = 4$), and error bars show one standard deviation.

B-cell subsets in monkeys (e.g., B cells producing anti-Env antibodies) could reveal V_H-specific repertoire alterations. However, the results of the present study suggest that any such perturbation would have to be restricted to a minor fraction of the B-cell population. This conclusion is consistent with our finding that fewer than 1% of normal CD20⁺ human or macaque PMBC respond to recombinant HIV-1 gp120 (HXBc2 or 89.6, at concentrations of up to 10 μ g/ml), using Indo-1 and flow cytometry to measure calcium mobilization (data not shown). By contrast, the multimember V_H3 gene family generally accounts for 40 to 70% of B cells in humans and monkeys (14, 16, 19).

In summary, the HIV-1 envelope glycoprotein did not appear to selectively expand or deplete the V_H3 repertoire of macaque B cells during primary SHIV infection. This result challenges the notion that a gp120 superantigen disrupts B-lymphocyte populations during HIV-1 infection.

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