Activation and Negative Selection of Functionally Distinct Subsets of Antibody-secreting Cells by Influenza Hemagglutinin as a Viral and a Neo-Self Antigen

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Summary

We have compared transgenic mice that express the influenza virus PR8 hemagglutinin (PR8 HA) as a membrane-bound neo-self antigen (HA104 mice) with nontransgenic (non-Tg) mice for their ability to generate HA-specific B cell responses after primary immunization with PR8 virus. HA-specific, IgM-secreting B cells were induced with similar frequencies in HA104 and non-Tg mice. In addition, a B cell clonotype (C4) that is characteristic of anti-HA immune responses of BALB/c mice was identified among HA-specific IgM hybridomas from HA104 mice. A subset of HA-specific, IgG-secreting B cells that arises rapidly after primary virus immunization in non-Tg mice, however, was substantially reduced in HA104 mice. Likewise, a B cell clonotype (C12) that dominates HA-specific IgG hybridomas generated after primary immunization of non-Tg mice was present at greatly reduced frequencies among hybridomas from HA104 mice. Because HA-specific, IgG-secreting B cells were generated by HA104 mice in response to a mutant HA containing an amino acid interchange in a B cell antigenic site, we conclude that these PR8 HA-specific, IgG-secreting B cells are negatively selected in HA104 mice as a result of their specificity for the neo-self PR8 HA. The findings demonstrate that HA-specific B cells that display distinct phenotypic potentials in non-Tg mice also differ in their susceptibility to negative selection from the primary B cell repertoire of HA104 mice: a subset of B cells that undergo rapid differentiation to become HA-specific IgG antibody–secreting cells (ASC) after activation in non-Tg mice is negatively selected in HA104 mice. By contrast, a subset that gives rise to HA-specific, IgM-secreting ASC persists in the primary repertoire of HA104 mice and can be activated by virus immunization.

During their development, B cells undergo a program of gene rearrangements to generate unique Ig variable (V)[1] region domains that determine their antigenic specificity (1). Signals received by these antigen receptors play a central role in directing the differentiation of B cells into a variety of functionally distinct phenotypes. Under the appropriate conditions, mature B cells respond to receptor-mediated signaling by undergoing proliferation and differentiation to produce antibody-secreting cells (ASC) and/or memory B cells (2, 3). These differentiated B cell populations have distinct functional potentials for postactivation events such as somatic hypermutation and antibody secretion, and they appear to develop along different pathways, as indicated by their discrete anatomical locations, differential expression of clonotypes, and other cell-surface markers (4–10).

Under other conditions, both mature and immature B cells can be inactivated or eliminated by signals transmitted through their antigen receptors (11). By inducing the elimination of autoreactive B cells, receptor-mediated signaling plays an important role in establishing B cell tolerance to self antigens. Studies using transgenic (Tg) mice expressing Ig specific for self antigens have identified a variety of processes that can lead to the inactivation or elimination of self-reactive B cells at different stages of their development (12). These different outcomes can be promoted by differences in the physical form of the self antigen; membrane-bound antigens appear in some model systems to promote more efficient deletion of self-reactive B cells than soluble versions of the same antigen (13, 14), although these distinctions are not clear cut (15). Moreover, B cells that can react with self antigens can, in some circumstances, persist among peripheral B cells (16, 17), and receptor specificity for self antigens may participate in selection of B cells into distinct subsets (18, 19). The factors that direct B cell differentia-

[1] *Abbreviations used in this paper:* ASC, antibody-secreting cell; HA, hemagglutinin; HAU, hemagglutinating units; RT, reverse transcriptase; TBS, Tris-buffered saline; Tg, transgenic; V, variable.

tion into functionally distinct subsets and affect their regulation during development are not yet understood.

We are addressing these issues by analyzing the antigen-specific B cell repertoires that are induced in responses to the influenza virus PR8 hemagglutinin (HA) in BALB/c mice, and in transgenic mice that express the PR8 HA as a membrane-bound neo-self antigen (HA104 mice). The HA is the major glycoprotein on the influenza virus particle which, because of its role in mediating and evading protective immunity to influenza virus, has been extensively characterized in terms of its immunological recognition (20). In the case of the PR8 HA, the locations of four major B cell antigenic sites that are recognized by BALB/c mice have been determined (21). Moreover, the genetic basis for PR8 HA–specific B cell responses of BALB/c mice has been studied in considerable detail (22–28). Although many different VH and VL germline gene segments can be used to generate PR8 HA–specific antibodies (27), these studies have identified B cells that use characteristic VH and/or Vκ gene segments and are reproducibly expressed at different stages of anti-PR8 HA antibody responses of BALB/c mice. For example, a B cell subset termed C12 that recognizes the antigenic site Cb and dominates early primary B cell responses to the PR8 HA has been identified (26). Although they arise quickly after primary immunization, C12 B cells have frequently undergone class switching and they produce IgG antibodies. Moreover, C12 B cells disappear abruptly during the primary response, display limited evidence of somatic hypermutation, and make little or no contribution to secondary responses to the PR8 HA in BALB/c mice (29). A second B cell subset, termed C4, is likewise frequently represented during primary responses (24). C4 B cells recognize the antigenic site Sb; unlike C12 B cells, however, somatically mutated C4 B cells are represented in secondary PR8 HA–specific B cell responses (24). By comparing HA104 mice with non-Tg mice for their HA-specific B cell responses, we have found that the HA-specific, IgG-secreting B cells (including C12 B cells), which arise rapidly after virus immunization of non-Tg mice, are substantially reduced in HA104 mice. By contrast, HA-specific, IgM-secreting B cells (including C4 B cells) are generated with similar frequencies in HA104 and non-Tg mice. The findings demonstrate that autoreactive B cells directed to a ubiquitously expressed membrane-bound self antigen can be heterogeneous with respect to their regulation; a subset of HA-specific B cells that undergoes rapid differentiation to produce IgG ASC during the primary response to PR8 virus in non-Tg mice is also substantially eliminated from the primary B cell repertoire of HA104 mice. By contrast, a subset of HA-specific B cells that gives rise to IgM ASC persists in HA104 mice and can be activated by virus immunization.

Materials and Methods

Mice. A previously described DNA construct containing a cDNA copy of the PR8 HA linked to the SV40 early region promoter/enhancer and polyadenylation signals (30) was modified to remove a single base deletion that introduces a premature stop codon in the HA-coding sequence. The COOH-terminal portion of the HA-coding sequences was replaced with corresponding sequences from a PR8 HA plasmid kindly provided by Dr. Peter Palese (Mount Sinai School of Medicine, New York). This produced a plasmid encoding a full-length copy of the HA inserted between the SV40 early region enhancer and the T antigen coding region, in the BglII site of the expression vector pKSV10 (Pharmacia Fine Chemicals, Piscataway, NJ). Plasmid sequences were removed and purified construct DNA was injected into fertilized BALB/cxC57Bl/6 zygotes. The HA104 lineage was developed from one of four founder mice by successive back-crossing to BALB/c mice (Charles River Breeding Laboratories, Inc., Wilmington, MA, or The Jackson Laboratory, Bar Harbor, ME). The mice used here had been back-crossed three to five generations to BALB/c mice. Mice were genotyped by PCR (31) of excised tail DNA (32) using the primers HA2 and HAamp3′ (30). Non-Tg mice were in most cases transgene-negative HA104 mice; in a few instances, BALB/c mice that had been maintained in our colony were used as non-Tg mice. Mice were housed in microisolator cages and were between 2 and 5 mo old before immunization.

Transgene mRNA Expression. RNA was isolated from the kidney, spleen, thymus, heart, lung, intestine, and bone marrow of a 6-wk-old HA104 mouse by lysis of tissues in guanidinium isothiocyanate followed by passage over a CsCl gradient (33). cDNA was produced by reverse transcription (RT) of 20 μg of total RNA primed with the previously described primers SV40 poly(A), which contains oligo dT plus a four-nucleotide extension corresponding to the SV40 early region poly(A) site that forms the 3′ end of the transgene mRNA, as well as the MHC class I–specific primer $H2D^d$ 3′ (30). The resulting cDNA was amplified in the PCR for 35 cycles under standard conditions (31) with either SV40 poly(A) and SV40-PCR5′, or $H2D^d$ 3′ and $H2D^d$ 5′ (30). To identify transgene-specific amplification products, gels were Southern blotted and probed with a ^{32}P-labeled oligonucleotide (SV40 probe) (30).

Immunohistochemistry. A three-step immunoalkaline phosphatase method was used to localize expression of the HA protein in frozen sections of spleen and thymus or cytocentrifuge preparations of mononuclear bone marrow cells, which were obtained from a 5-wk-old HA104 mouse. All samples were fixed in acetone before incubation with mouse Ig (50 μg/ml; Sigma Chemical Co., St. Louis, MO) in Tris-buffered saline (TBS, 10 mM Tris, 0.15 M NaCl, pH 7.3) to prevent nonspecific binding of primary antibodies. Duplicate samples were incubated for 30 min at 20°C with either the rat anti–PR8 HA IgG mAb 10-1 or a control rat IgG antibody of irrelevant specificity diluted in TBS + 1% BSA. Slides were washed in TBS, incubated with a secondary biotinylated mouse anti–rat antibody (mouse adsorbed; Vector Laboratories, Burlingame, CA) for 30 min at 20°C, washed, and incubated with an alkaline phosphatase–conjugated avidin–biotin complex (Vector Laboratories). After washing, the samples were incubated with a tertiary alkaline phosphatase–conjugated rat anti–alkaline phosphatase antibody (Harlan Bioproducts, Indianapolis, IN), washed again, and then enzyme activity was developed using the Vector Red substrate (Vector Labortories). Samples were washed, air dried, and mounted for photomicroscopy.

Viruses and Immunizations. Influenza viruses PR8 [A/Puerto Rico/8/34 (H1N1)], T3 (a mutant PR8 virus containing an aspartic acid to glycine interchange at amino acid 225) (21), and J1 [a H3N1 reassortant between PR8 (H1N1) and A/Hong Kong/1/68 (H3N2), in which the PR8 HA has been replaced by the serologically non–cross-reactive H3 subtype HA] (34, 35) were grown in the allantoic cavity of 10-d-old embryonated hen's eggs

and purified by sucrose gradient centrifugation. Virus titers were determined by agglutination of chicken erythrocytes and expressed as hemagglutinating units (HAU) as described (36). Mice were immunized by injecting 1,000 HAU of purified virus intravenously in 0.2 ml PBS.

ELISPOT Analysis. Purified PR8, T3, and J1 viruses (1,000 HAU/ml in PBS) were applied in 0.1-ml vol to nitrocellulose-based 0.45-μM filters in 96-well microtiter plates (Multiscreen-HA; Millipore Corp., Bedford, MA). After overnight adsorption at 4°C, plates were washed and blocked by incubation at 37°C for at least 1 h with IMDM (GIBCO BRL, Gaithersburg, MD) supplemented with 50 μM 2-ME, 5 μg/ml iron-saturated transferrin (Sigma), 50 μg/ml gentamycin (ISC), plus 5% FBS (Hyclone Laboratories, Logan, UT) (ISC + 5%). Splenocytes from immunized mice were erythrocyte depleted and washed three times in ISC + 5%. Cells were added in quadruplicate in 0.2 ml vol at 10^6, 2.5×10^5, 6.3×10^4, and 1.6×10^4 cells/ml in ISC + 5% to virus-coated wells, and were incubated for 4 h at 37°C in a humidified incubator containing 7% CO_2. Filters were then washed, blocked for 30 min with PBS + 1% BSA, and incubated with alkaline phosphatase–conjugated goat antisera specific for mouse IgM or IgG H chains (Fisher Scientific Co., Pittsburgh, PA) (100 μl vol of a 1:1,000 dilution in PBS + 1% BSA) overnight at 4°C. Wells were washed and developed with 0.33 mg/ml nitro blue tetrazolium and 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M $NaHCO_3$ and 0.001 M $MgCl_2$, pH 9.8. Spots were counted with the aid of a dissecting microscope, and mean counts derived from cell dilutions that yielded discrete spots were expressed as numbers of ASC per 10^5 splenocytes. In control experiments, splenocytes from unimmunized mice were generally found to yield spots at cell concentrations $>10^6$/ml; no difference was apparent in unimmunized HA104 and non-Tg mice. Accordingly, the lower detection limit for this assay is roughly one ASC per 10^5 splenocytes.

Hybridoma Generation. Hybridomas were generated from T3-immunized mice 5 d after immunization by fusion with Sp2/0-Ag14 (37) as previously described (28), except that hypoxanthine-azaserine (Sigma) was used to select for hybridomas. After fusion, cells were plated at limiting dilution and generally gave rise to <30 hybridomas per 96-well plate. HA-specific antibodies were initially selected by screening fivefold diluted culture supernatants for reactivity with T3 but not J1 viruses; those displaying more than threefold higher reactivity with T3 than J1 were expanded, and their culture supernatants were analyzed in serial dilution for reactivity with T3, PR8, and J1 viruses, and for antibody isotype. Roughly 70% of the hybridomas that were initially selected from either non-Tg or HA104 mice yielded stable hybridoma cell lines secreting HA-specific antibodies. The hybridomas were generated and screened in three experiments, each containing one transgene-positive and one transgene-negative mouse. In the hybridoma designation, the prefix indicates the individual donor mouse, and the suffix indicates the individual hybridoma number. Mice 920 (Tg^+), 921 (Tg^+), and 922 (Tg^-) were littermates, as were 1343 (Tg^+) and 1344 (Tg^-). Mouse 963 was a Tg^- mouse.

Virus-Binding ELISA. Purified T3, PR8, or J1 viruses (20 HAU in 25 μl PBS) were adsorbed to the wells of polyvinyl 96-well plates by incubation at 4°C overnight, after which they were washed and blocked by incubation with PBS + 1% BSA. Hybridoma culture supernatants or serum antibody samples were diluted in PBS + 1% BSA, added in duplicate (25 μl/well), and incubated at room temperature for 2 h. The wells were then washed and incubated with either a biotinylated goat anti–mouse total Ig polyclonal antiserum (diluted 1:1,000; Fisher), which was used in preliminary screening of hybridomas, or biotinylated isotype-specific antisera (diluted 1:1,000; Sigma), which were used to determine antibody isotype. After incubation for 2 h at room temperature, wells were washed and incubated with extravidin-alkaline phosphatase (diluted 1:2,000; Sigma) and incubated for 30 min at room temperature. Plates were again washed and incubated with *p*-nitrophenyl phosphate (1 mg/ml in 0.1 M $NaHCO_3$, 0.001 M $MgCl_2$, pH 9.8) for 30 min after which the reaction was terminated by addition of 0.3 M NaOH. Absorbance at 405 nm was determined using a microplate reader.

Sequence Analysis of Antibody Variable Regions. Roughly 10^6 hybridoma cells were washed with 1 ml of NTE (0.1 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) at 4°C, and then lysed for 5 min on ice in 200 μl of cold NTE + 0.5% NP-40. Nuclei were pelleted, and supernatants were added to 200 μl of 0.3 M NaCl, 0.2 M Tris-HCl, pH 8.0, 25 mM EDTA, 2% SDS, 400 μg/ml proteinase K, and were incubated at 37°C for 1 h. RNA samples were extracted with phenol/chloroform, ethanol precipitated, and redissolved in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA. The L chain constant region–specific primer Cκ (5′-dAGATGGATACAGTTGGT-3′) and the appropriate H chain constant region–specific primer [Cγ cross (5′-dGGGGCCAGTGGATAGAC-3′), specific for IgG2a and IgG2b constant regions; Cγ3 (5′-dATGGGGCTGTTGTTTGTA-3′), specific for IgG3 constant regions; Cμ (5′-dAGACATTTGGGAAGGAC-3′), specific for IgM constant regions] were used to prime cDNA synthesis from aliquots (~1/10 of the total RNA sample) using RT in 100 μl vol under standard conditions (33). After incubation, reaction mixtures were boiled and aliquots (2 μl) were subjected to 35 cycles of amplification in the PCR in 50 μl vol under standard conditions (31) using the appropriate constant region–specific primers and the following V region primers. For H chains, separate reactions containing either VH5′1 (5′-dGAGGTGAAGCTGGTGGAG(A/T)C(A/T)GG-3′) or VH5′2 (5′-dCAGGTCCAGTTGCAGCAG(A/T)C(A/T)GG-3′) were carried out (27). For L chains, one reaction was carried out with the degenerate Vκ-specific primer L5 (38) that can amplify VL regions derived from a wide variety of Vκ gene groups, but does not amplify an out-of-frame mRNA that is present in hybridomas generated using the Sp2/0-Ag14 fusion partner (27, 39). A second PCR reaction was carried out using a mixture of the primers VκC12 (5′-dGTCATATTGTCCAGTGGA-3′) and VκC4 (5′-dCAGTCTCCATCCTCCCTGAC-3′), which can hybridize to the VκC12 and VκC4 gene segments. Amplified reaction products were fractionated by electrophoresis on a 1.5% agarose gel and purified using GeneClean II (Bio101, La Jolla, CA). PCR reaction products were directly sequenced as double-stranded DNA using Sequenase (U.S. Biochemical Corp., Cleveland, OH) under standard conditions using the appropriate constant region–specific primer. For the hybridomas 922-22, 922-23, and 922-42, additional short-range sequencing reactions were carried out using ^{32}P-end–labeled constant region primers to confirm the presence of IgG2a-, IgG2b-, and IgG3-specific nucleotide substitutions. The L chain sequence of hybridoma 922-177 (which contains a putative somatic mutation) was established by sequencing PCR products generated from two independent cDNA reactions.

Results

To generate transgenic mice that express the PR8 HA glycoprotein as a neo-self antigen, a full-length, double-stranded DNA copy of the PR8 virus HA gene segment was linked

to sequences containing the SV40 early region promoter/enhancer and polyadenylation signals. Transgenic mice were generated by injecting the HA/SV40 DNA construct into fertilized BALB/cxC57BL/6 zygotes, followed by subsequent back-crossing to BALB/c mice. Offspring from the HA104 lineage were found by Northern hybridization analysis to express low levels of transgene mRNA in several tissues, including the intestine, thymus, and spleen (data not shown). A more sensitive analysis of the tissues in which the transgene mRNA is expressed was obtained using RT-PCR to amplify transgene mRNA sequences from various tissues (Fig. 1). Because the HA/SV40 construct uses SV40-derived splicing signals (which do not contain conventional introns), it was not possible to use intron-spanning PCR primers to ensure that transgene-specific PCR reaction products were derived from mRNA and not from contaminating DNA that might be present in the mRNA preparations. Accordingly, one of the primers used in the RT-PCR reactions [comprised of oligo(dT) plus a four-nucleotide extension corresponding to the SV40 early region poly(A) site (40)] was designed to hybridize to the poly(A) tail present at the 3′ end of the transgene mRNA. The low sequence complexity of this primer allows it to hybridize to other cellular mRNAs, yielding multiple PCR reaction products. Among these reaction products, transgene mRNA-specific sequences were detected by Southern hybridization analysis in all tissues that were examined (Fig. 1). Importantly, this primer was unable to direct amplification of transgene-specific sequences from genomic DNA (Fig. 1, lane *D*). We also performed immunohistochemical analysis of tissue sections from the spleen and thymus, as well as of cytocentrifuge preparations of mononuclear bone marrow cells, and we observed staining of individual cells using a PR8 HA–specific monoclonal antibody (Fig. 2). Together, these data indicate that the HA is an immunoreactive membrane-bound neo-self antigen that is expressed at low levels in many tissues in HA104 mice.

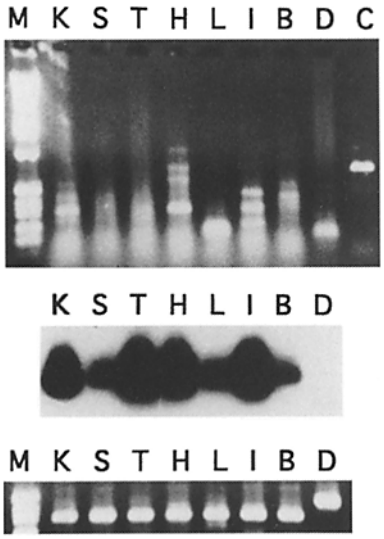

Figure 1. HA mRNA is expressed in many tissues in HA104 Mice. Total RNA was isolated from multiple organs (*K*, kidney; *S*, spleen; *T*, thymus; *H*, heart; *L*, lung; *I*, intestine; and *B*, bone marrow) of a 6-wk-old HA104 mouse, and used as a template for reverse transcription primed by a poly-T oligonucleotide anchored by four bases of homology to the known SV-40 polyadenylation signal, which forms the 3′ end of the HA transgene mRNA (40). The resulting cDNA was amplified by PCR using the same anchored primer and a transgene-specific 5′ primer. PCR products were separated on a 1.2% agarose gel and stained with ethidium bromide (*top panel*). This primer pair yields a 294-bp fragment upon amplification of transgene mRNA, which is not apparent by ethidium bromide staining, but is detected by hybridization with a transgene-specific probe (*middle panel*). To establish that the anchored primer pair only amplifies mRNA and not DNA, 1 μg of genomic DNA from the same mouse was used in a parallel PCR reaction (lane *D*). Although PCR products were generated from genomic DNA, transgene-specific hybridization was not observed (*middle panel*, lane *D*). The presence of the transgene in the genomic DNA was confirmed by amplification using the set of HA-specific primers used to genotype the HA Tg mice, which yield a 740-bp fragment (*top panel*, lane *C*). The integrity of the RNA and DNA samples was confirmed by reverse transcriptase PCR using MHC class I–specific primers. The amplified products were fractionated on a 1.2% agarose gel and stained with ethidium bromide (*bottom panel*). The MHC class I–specific primers span an intron, resulting in a large product from DNA (*bottom panel*, lane *D*) than from RNA (*bottom panel*, lanes *K–B*).

Induction of PR8 HA-specific Antibody Secreting Cells in HA104 and Non-Tg Mice. To examine how the neo-self PR8 HA affects the peripheral B cell repertoire, HA104 mice were compared with non-Tg mice for their ability to generate HA-specific B cell responses. Since the HA is expressed in the thymus of HA104 mice, the induction of $CD4^+$ T cell tolerance to the HA could limit their ability to generate HA-specific, T-dependent B cell responses (16, 41, 42). We therefore immunized HA104 and non-Tg mice with intact influenza virus so that T cells directed to other proteins in the virus particle could provide a source of intermolecular cognate help to HA-specific B cells (43). A similar approach has been applied in other transgenic systems to analyze B cell responses to neo-self antigens under conditions in which T cell tolerance was also established to the neo-self antigen (16, 41, 42). HA104 and non-Tg mice were immunized intravenously with purified PR8 virus; 5 d later, the mice were killed and the frequency of HA-specific ASC in the spleen was determined by ELISPOT assays (44). Previous studies have demonstrated a peak of HA-specific ASC formation in splenocytes 5 d after intravenous immunization with influenza virus (45). To determine the frequency of HA-specific ASC, the number of ASC that reacted with PR8 virus was compared to the number that reacted with the control virus J1. J1 is a reassortant virus that contains a serologically non–cross-reactive H3 subtype HA, but that is otherwise identical to PR8 (34, 35). The frequency of J1-specific ASC quantitates the ASC that are directed to non-HA viral components (i.e., other virus proteins and components such as carbohydrates that derive from propagating the virus in hen's eggs); the frequency of PR8 HA–specific ASC is therefore indicated by the excess frequency of PR8-specific ASC over J1-specific ASC.

In non-Tg mice, the mean frequency of PR8 HA–specific ASC was 58.2 ± 22.5 per 10^5 splenocytes and comprised both IgM and IgG ASC (mean frequencies = 34.2 ± 16.0 and 24.0 ± 17.2 ASC per 10^5 splenocytes, respectively) (Fig. 3). These values are in good agreement with previous estimates of the frequencies of HA-specific IgM and IgG ASC in splenocytes 5 d after intravenous immunization with

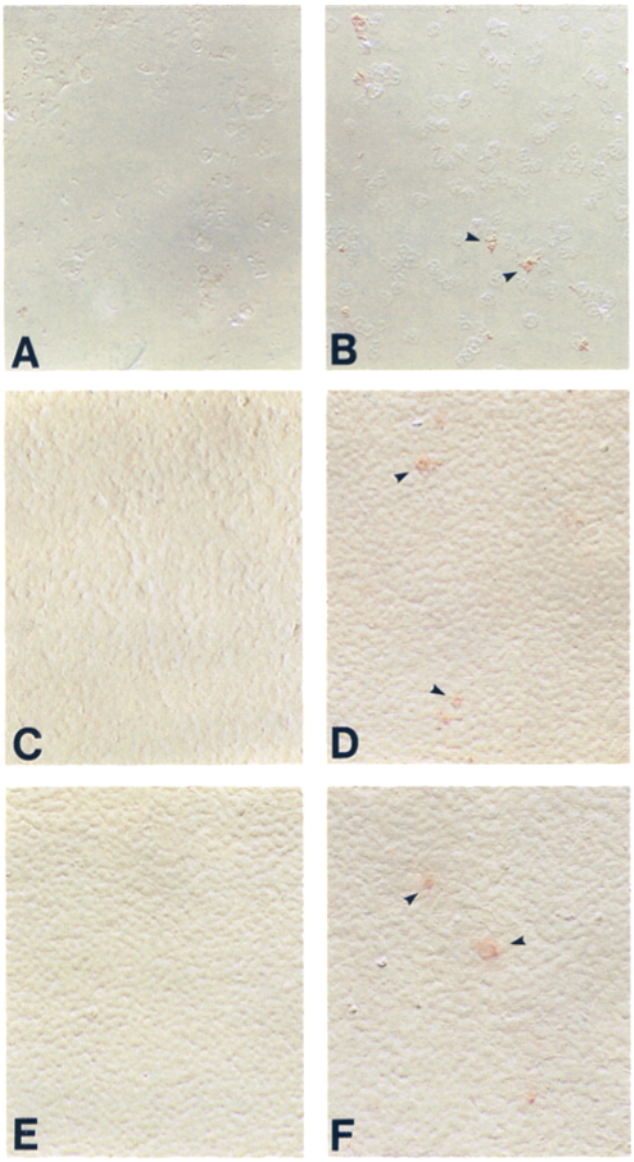

Figure 2. Immunohistochemical analysis of HA protein expression in HA104 mice. Cytocentrifuge preparations of bone marrow mononuclear cells (*A* and *B*), spleen sections (*C* and *D*), and thymic sections (*E* and *F*) from a HA104 mouse were incubated with a control rat IgG antibody (*A*, *C*, and *E*), or with a rat anti-PR8 HA IgG antibody (*B*, *D*, and *F*), followed by a three-step immunoalkaline phosphatase staining to detect bound rat IgG. Arrows indicate some of the individual cells that display PR8 HA-specific staining in *B*, *D*, and *F*. ×320.

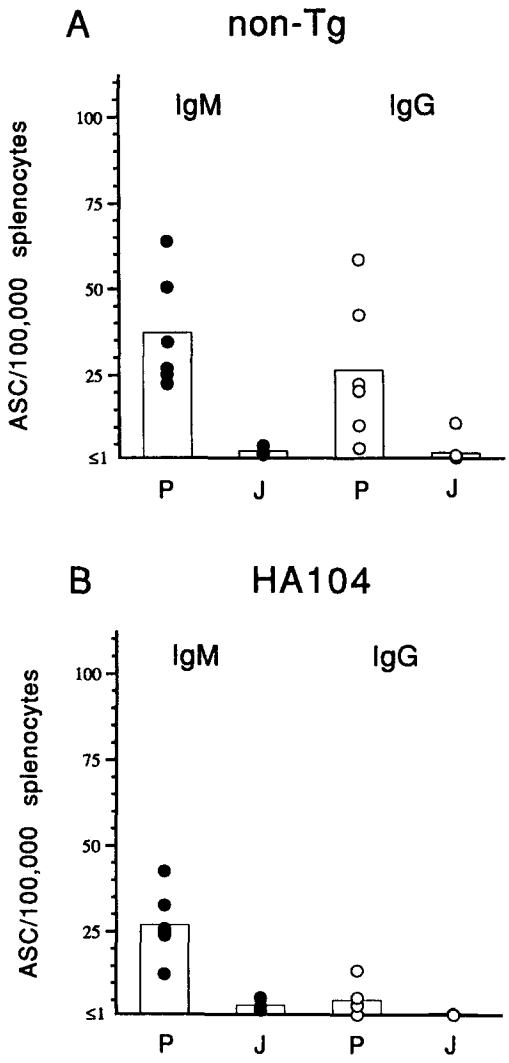

Figure 3. HA-specific ASC formation in PR8-immunized non-Tg and HA104 mice. Six non-Tg (*A*) and six HA104 (*B*) mice were each immunized with 1,000 HAU PR8 virus intravenously, and 5 d later the frequencies of PR8 (P)- and J1 (J)-specific ASC in splenocytes were determined by ELISPOT. *Closed symbols*, the frequencies of IgM ASC; *open symbols*, the frequencies of IgG ASC from individual mice; *columns*, the mean frequencies in each set.

influenza virus (45). In HA104 mice, the mean frequency of PR8 HA–specific ASC was half of that observed in non-Tg mice (27.7 ± 10.2 ASC per 10^5 splenocytes). This decrease was caused by a substantial reduction in the frequency of PR8 HA–specific IgG ASC: although the frequency of IgM ASC was reduced slightly relative to non-Tg mice (23.4 ± 8.9 in HA104 mice versus 34.2 ± 16.0 in non-Tg mice), the frequency of IgG ASC was more than fivefold lower in HA104 mice than in non-Tg mice (4.3 ± 4.5 ASC per 10^5 splenocytes in HA104 mice versus 24.0 ± 17.2 in non-Tg mice).

HA-specific Antibody Responses of HA104 Mice to Mutant Virus T3. To examine the basis for the reduced frequency of PR8 HA–specific IgG ASC in HA104 mice, we analyzed the specificity and kinetics of their B cell responses to a mutant virus (T3) that differs from the PR8 HA by a single aspartic acid to glycine interchange in one of the four major B cell antigenic sites (antigenic site Ca) (21). We have previously shown that the majority of HA-specific antibodies from BALB/c mice react equally well with the T3 and PR8 HA molecules; a fraction (~15%) of T3 HA–specific antibodies from BALB/c mice react with T3 but do not cross-react with PR8 because they recognize epitopes that include the mutated glycine at residue 225 (28). We therefore examined whether negative selection of PR8 HA–specific B cells skewed the specificity of the B cell re-

sponse to the mutant virus T3 away from reactivity with the neo-self PR8 HA in HA104 mice, as occurs in xeno- or alloantibody responses to variants of self proteins (46–48).

Accordingly, HA104 and non-Tg mice were immunized intravenously with purified T3 virus; at various intervals, individual mice were killed, and the frequency of HA-specific ASC in splenocytes was determined. To determine the frequencies of T3 and PR8 HA–specific ASC, the frequencies of ASC that reacted in ELISPOT with T3 virus, PR8 virus, and the control virus J1 were determined. In non-Tg mice, HA-specific ASC were induced by day 3 after T3 immunization and were exclusively IgM (Fig. 4). The frequency of HA-specific ASC reached a peak on day 5 and comprised IgM and IgG ASC in roughly equal proportions. HA-specific ASC declined in frequency through day 7, and by day 9, they were ~15% of their frequency on day 5. In some instances, a small excess of T3 over PR8 HA–specific ASC was evident (particularly on the fifth day after injection), corresponding to ASC that were directed to the mutated glycine at residue 225. Overall, however, the frequencies of T3- and PR8 HA–specific ASC in T3-immunized, non-Tg mice were similar, consistent with our previous studies indicating that the majority of T3-specific antibodies in BALB/c mice are directed to shared epitopes on the T3 and PR8 HA molecules (28).

In HA104 mice, T3- and PR8 HA–specific IgM ASC were present 3 d after immunization at similar frequencies to non-Tg mice (Fig. 4). On day 5, the mean frequencies of T3 and PR8 HA–specific IgM ASC were similar to non-Tg mice. The frequency of PR8 HA–specific IgG ASC 5 d after immunization in HA104 mice, however, was substantially reduced compared to non-Tg mice. Nonetheless, T3 HA–specific IgG ASC were present on day 5 in HA104 mice. On days 7 and 9, the frequencies of T3- and PR8 HA–specific IgM and IgG ASC were similar to those of non-Tg mice. ELISPOT analysis therefore indicated that HA-specific IgM ASC were generated with similar kinetics and frequencies in HA104 and non-Tg mice. By contrast, the PR8 HA–specific IgG ASC that arise 5 d after immunization in non-Tg mice were substantially reduced in HA104 mice. Moreover, the mean frequency of day 5 IgG ASC that reacted with T3 only (indicated by the excess frequency of T3- over PR8-specific IgG ASC) was similar in HA Tg mice and non-Tg mice. Thus, the reduction of PR8 HA–specific IgG ASC

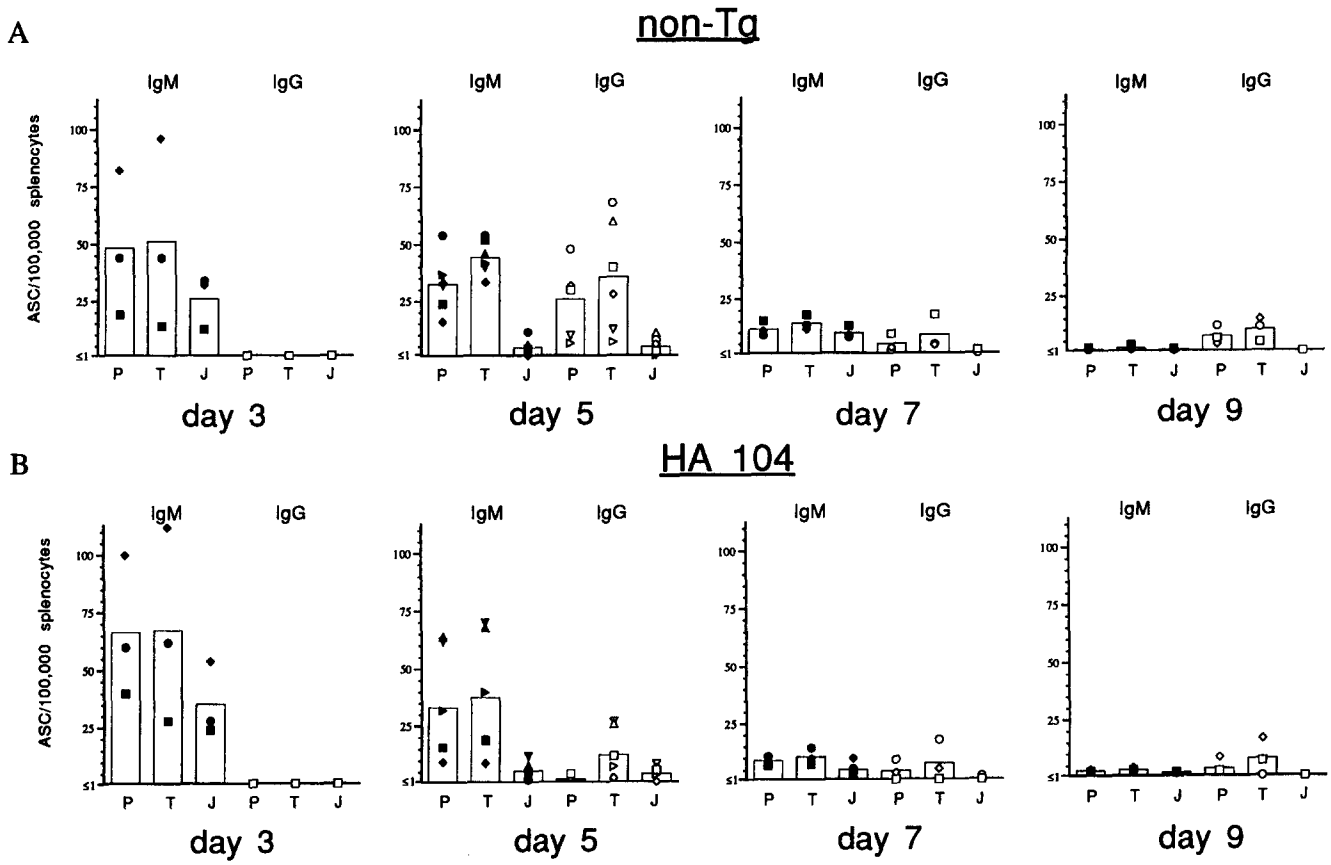

Figure 4. Kinetics and specificity of HA-specific ASC formation in T3-immunized non-Tg and HA104 mice. Non-Tg (*A*) and HA104 (*B*) mice were immunized with 1,000 HAU T3 virus; at various time points, animals were killed and splenocytes were analyzed for the frequencies of PR8 (P)-, T3 (T)-, and J1 (J)-specific ASC by ELISPOT. Closed symbols indicate the frequencies of IgM ASC, and open symbols indicate the frequencies of IgG ASC. Three pairs of non-Tg and HA104 mice were analyzed on days 3, 7, and 9, and six pairs of mice were analyzed on day 5. At each time point, shared symbols indicate the frequencies of ASC derived from individual mice, and columns indicate the mean frequencies in each set.

was specific to those ASC that can react with the neo-self PR8 HA.

Reduced Titers of Anti-Viral IgG Serum Antibody in HA104 Mice. To examine how the frequencies of HA-specific ASC affect virus-specific antibody titers in HA104 and non-Tg mice, sera from the T3-immunized mice analyzed by ELISPOT were compared for their levels of PR8 virus–specific serum antibody by ELISA (Fig. 5). PR8 virus–specific IgM antibody was induced to equivalent levels and with similar kinetics in non-Tg and HA104 mice, reaching a maximum 5 d after immunization. PR8 virus–specific IgG titers reached maximum levels 7 d after immunization in non-Tg mice. The titers of PR8 virus–specific IgG in HA104 mice were ~10% of the titers in non-Tg mice, correlating with the reduced frequency of PR8 HA–specific IgG ASC that were detected by ELISPOT analysis.

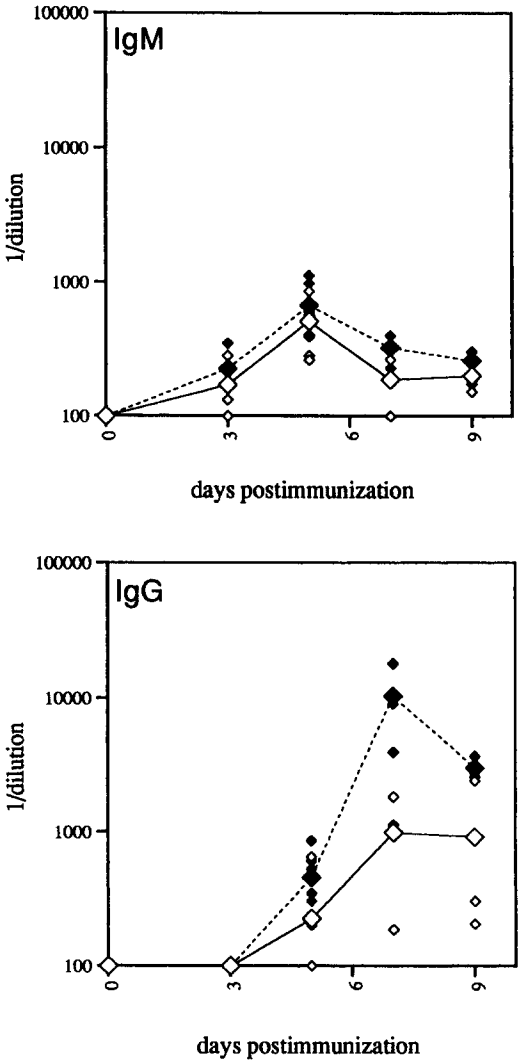

Figure 5. Kinetics of PR8 virus–specific serum antibody responses in T3-immunized non-Tg and HA104 mice. Serum from the T3-immunized non-Tg (*closed symbols*) and HA104 (*open symbols*) mice described in Fig. 4 were analyzed for their level of PR8 virus–specific serum antibody by ELISA. Data are expressed as the reciprocal of the dilution necessary to yield an A_{405} of 0.4. *Small symbols* indicate data points from individual mice; *large symbols* indicate mean values at each time point, and they are linked by lines.

Specificity Analysis of T3-Specific Hybridomas from HA104 Mice. To analyze the HA-specific B cell responses of HA104 mice in greater detail, B cell hybridomas were generated from the splenocytes of T3-immunized HA104 and non-Tg mice at the peak of HA-specific ASC formation (5 d after immunization). Hybridoma supernatants were initially screened for their reactivity with T3 or J1 viruses. T3 HA–specific hybridomas were isolated, and their supernatants were screened in serial dilution for their reactivity with T3 and PR8 viruses. Hybridomas were designated as being T3-specific if they displayed >10-fold higher reactivity with T3 than with PR8 (28). In most cases, however, no detectable reactivity with PR8 was observed among the hybridomas that were designated as T3 HA-specific. Hybridomas that displayed equivalent reactivity with T3 and PR8 were designated as being PR8 HA–specific since they were directed to epitopes that are shared between the T3 and PR8 HA molecules. The numbers of T3- and PR8 HA–specific hybridomas that were obtained from individual HA104 and non-Tg mice is shown in Table 1, along with their antibody heavy chain isotype (all of the hybridomas from HA104 and non-Tg mice used κ isotype L chains). The overall numbers and H chain isotype of T3 HA–specific hybridomas from HA104 and non-Tg mice were similar. The number of PR8 HA–specific hybridomas isolated from HA104 mice (28 hybridomas), however, was reduced by nearly half relative to non-Tg mice (55 hybridomas). As a result, the relative frequency of T3-specific hybridomas doubled from 15% (10/65) in non-Tg mice to 33% (14/43) in HA104 mice. Among IgG hybridomas, the difference in relative frequency of T3-specific hybridomas was even greater: 4/43 (9%) of the T3 HA–specific IgG hybridomas from non-Tg mice reacted with T3, but did not cross-react with the PR8 HA. By contrast, 5/10 (50%) of the T3 HA–specific IgG hybridomas from HA104 mice could not cross-react with the PR8 HA. Specificity analysis of hybridomas from T3-immunized mice therefore paralleled the results obtained by ELISPOT analysis: T3 HA–specific IgM and IgG hybridomas, and PR8 HA-specific IgM hybridomas were isolated with similar frequencies from HA104 and non-Tg mice. PR8 HA–specific, IgG-secreting hybridomas, however, were isolated at greatly reduced frequencies from HA104 versus non-Tg mice.

Differential Expression of PR8 HA-specific B Cell Clonotypes among Hybridomas from HA104 and Non-Tg Mice. We next examined the hybridomas from HA Tg and non-Tg mice for their usage of antibody V region sequences that define B cell clonotypes that are reproducibly expressed at different stages of the immune response to the PR8 HA in BALB/c mice. We focused on two major clonotypes: the first (designated C12) uses a characteristic Vκ gene segment (VκC12) from the Vκ4/5 group in combination with one of two closely related VHJ558 gene segments (VHC12.1 and VHC12.2) (26). The second clonotype (designated C4) uses a characteristic Vκ gene segment (VκC4) from the Vκ8 group in asso-

Table 1. *Specificity and H Chain Isotype of T3 HA-specific Hybridomas from Non-Tg and HA104 Mice*

Non-Tg

Donor mouse*	Number of hybridomas‡	T3 only§				T3 and PR8‖			
		M	G2a	G2b	G3	M	G2a	G2b	G3
922	244	1	3	0	0	2	9	3	1
963	248	0	1	0	0	5	10	0	0
1344	353	5	0	0	0	9	14	1	1
Total	845	6	4	0	0	16	33	4	2

HA104

Donor mouse*	Number of hybridomas‡	T3 only§				T3 and PR8‖			
		M	G2a	G2b	G3	M	G2a	G2b	G3
921	217	4	0	0	0	11	1	0	0
920	330	4	4	0	0	6	2	0	1
1343	329	1	1	0	0	6	0	1	0
Total	876	9	5	0	0	23	3	1	1

Hybridomas generated 5 d after immunization with T3 virus were analyzed for their reactivity with T3 and PR8 viruses and for their H chain isotype.

*Donor mouse number.

‡Number of hybridomas screened from each mouse.

§Number and H chain isotype of hybridomas that react with T3 only (>10-fold higher binding to T3 virus than to PR8 virus; binding to PR8 in most cases was undetectable).

‖Number and H chain isotype of hybridomas that react with both T3 and PR8.

ciation with the Jκ5 gene segment, and can combine with VH gene segments derived from several gene families (including VHS107, VHQ52, VH7183, and VH36-30) to generate specificity for the PR8 HA (24). Although the C12 and C4 clonotypes were defined in immune responses of BALB/c mice to PR8 virus, they are also induced in response to the mutant T3; this is expected since the T3 mutation is located in an antigenic site (Ca) that does not form part of the epitopes that are recognized by C4 and C12 antibodies (which recognize epitopes in the Sb and Cb antigenic sites, respectively) (21). In addition, although the HA104 mice used here are not fully back-crossed to BALB/c mice and will contain genes derived from the C57BL/6 background, we have found that the C12 and C4 clonotypes are present among day 5 hybridomas from T3-immunized BALB/c × C57Bl/6 F1 mice (data not shown).

The representation of the C12 and C4 clonotypes was examined among the PR8 HA–specific hybridomas that were isolated from HA104 mice. We also analyzed the PR8 HA–specific hybridomas from one of the non-Tg mice (mouse 922) to establish the frequencies with which the C12 and C4 clonotypes are expressed among hybridomas generated from a non-Tg mouse under the experimental conditions we adopted here. To examine C12 and C4 clonotype expression, hybridoma mRNA samples were amplified in the PCR with primers that are specific for the VκC12 and the VκC4 gene segments, and with degenerate Vκ- and VH-specific primers, and the sequences of PCR products were determined. In some cases, hybridoma sequences could not be determined because the amplification products yielded mixed sequences, presumably because of amplification of V region sequences derived from productive and nonproductive rearrangements. Nonetheless, hybridomas whose sequences were not determined could usually be assigned as not using the VκC4 or VκC12 gene segments (designated "not C4, C12") if they yielded mixed PCR reaction products with the degenerate Vκ primer, but did not amplify with the C12 or C4 primers. The deduced amino acid sequences of the hybridoma VH and Vκ regions are shown in Figs. 6 and 7. V gene sequences that are designated as using the VκC12, VHC12, and VκC4 gene segments are shown relative to these previously described gene segments; the remaining VH and Vκ sequences are assembled according to VH family or Vκ group (51–53). In most cases, the VH and Vκ sequences from individual hybridomas were either identical (reflecting the use of the same germline gene segment) or they displayed multiple substitutions that suggest the use of different germline gene segments (27). One likely somatic mutation was apparent in hybridoma 922-163, which contains a substitution relative to VκC12 in L chain CDR 1. The Vκ sequences of 921-25, and of 920-289 and 921-1415 also display a few differences relative to other hybridomas that could be caused by somatic mutation or by the use of closely related germline gene segments. Overall, however, somatic mutation appears to be limited among these hybridomas, consistent with other studies indicating that somatic mutation is rare among B cells 5 d after immunization (54). Several sets of hybridomas were also found to contain identical VH/D/JH and Vκ/Jκ rearrangements, including identical H chain CDR3 sequences. These sets of hybridomas are most likely to have been derived from the clonal progeny of single B cells (55). In some instances, the clonal progeny expressed different H chain constant region isotypes, confirming that they had been derived independently of each other. The VH and Vκ gene segment usage of the hybridoma panels is summarized in Table 2.

Among the 17 hybridomas from non-Tg mouse 922, five hybridomas (all IgG isotype) used the VHC12.1/VκC12 combination of the C12 clonotype, and two hybridomas (both IgM isotype) used the VκC4/Jκ5 combination that characterizes the C4 clonotype. The C12 hybridomas included one pair of clonally-related hybridomas; two additional hybridomas that express the VκC12 L chain were not included in this set because they used VH regions that are not known to be associated with C12 antibodies. In contrast to non-Tg mouse 922, the C12 clonotype was rare

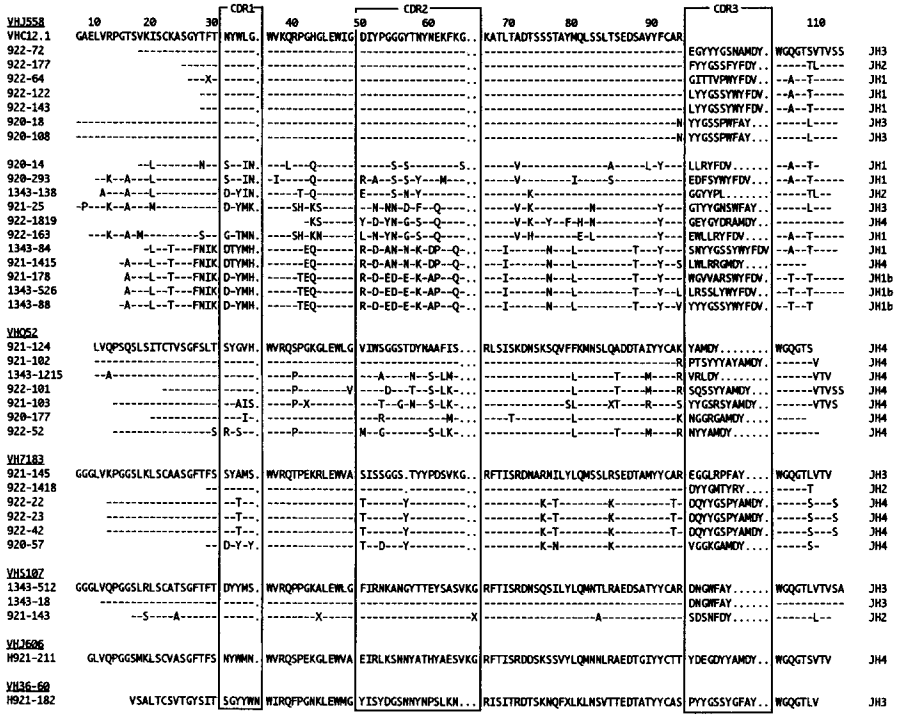

Figure 6. Deduced amino acid sequences of VH regions of PR8 HA-specific hybridomas from non-Tg and HA104 mice. Hybridomas with the prefix 922 were derived from the non-Tg mouse 922; those bearing the prefixes 920, 921, or 1343 were derived from HA104 mice 920, 921, or 1343, respectively. Sequences have been assembled according to the VH gene family (which is indicated), and they are displayed in reference to a member of each group; hybridomas that use the VHC12.1 gene are shown relative to the previously described VHC12.1 sequence (26). Identity with the representative sequence is indicated by dashes, differences are indicated by the single-letter amino acid code, and ambiguities are indicated by X. The sequences are numbered according to Kabat et al. (49), and the locations of the CDRs are indicated. Gaps that have been introduced to maintain alignment are indicated by dots. The JH gene segment that is used is also indicated; JH1b refers to an allele of the JH1 gene segment that derives from the C57Bl/6 background (50). The nucleotide sequences of these V regions are available from EMBL/Genbank/DDJB under accession numbers U37842–U37877.

among hybridomas from HA104 mice. No C12 hybridomas were identified among hybridomas from two of the three HA104 mice. The only C12 antibodies that were identified from HA104 mice were a pair of clonally-related hybridomas derived from mouse 920 (hybridomas 920-18 and 920-108) that expressed the VHC12.1/Vκ12 combination in conjunction with either IgG2a or IgM constant regions. By contrast, nearly half of the hybridomas from HA104 mice expressed the VκC4/Jκ5 combination that characterizes C4 B cells. The C4 hybridomas were all IgM

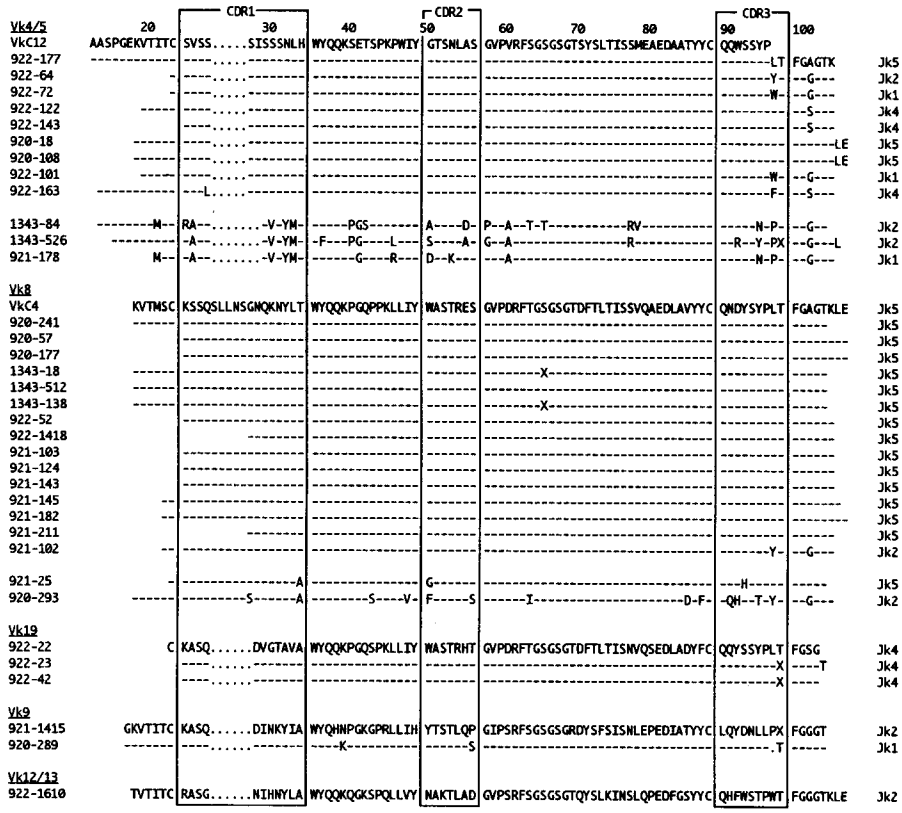

Figure 7. Deduced amino acid sequences of VL regions of PR8 HA–specific hybridomas from non-Tg and HA104 mice. Hybridoma designations are as described in Fig. 5. Sequences have been assembled according to Vκ gene group (which is indicated), and they are displayed in reference to a member of each group; hybridomas that use the VκC12 and VκC4 genes are shown relative to these previously described sequences (24, 26). Identity with the representative sequence is indicated by dashes, differences are indicated by the single-letter amino acid code, and ambiguities are indicated by X. The sequences are numbered according to Kabat et al. (49), and the locations of CDR are indicated. Gaps that have been introduced to maintain alignment are indicated by dots. The Jκ gene segment that is utilized is also indicated. The nucleotide sequences of these V regions are available from EMBL/Genbank/DDJB under accession numbers U37878–U37912.

Table 2. *Differential Expression of C12 and C4 Clonotypes in HA-specific Hybridomas from Non-Tg and HA104 Mice*

Non-Tg				HA104			
Hybridoma	H isotype	VH/JH	Vκ/Jκ	Hybridoma	H isotype	VH/JH	Vκ/Jκ
922-64	G2a	**VHC12.1/JH1**	**VκC12/Jκ2**	921-124	M	VHQ52/JH4	**VκC4/Jκ5**
922-72	G2a	**VHC12.1/JH3**	**VκC12/Jκ1**	921-103	M	VHQ52/JH4	**VκC4/Jκ5**
922-122*	G2a	**VHC12.1/JH1**	**VκC12/Jκ4**	921-143	M	VHS107/JH2	**VκC4/Jκ5**
922-143*	G2a	**VHC12.1/JH1**	**VκC12/Jκ4**	921-145	M	VH7183/JH3	**VκC4/Jκ5**
922-177	G2a	**VHC12.1/JH2**	**VκC12/Jκ5**	921-182	M	VH36-60/JH3	**VκC4/Jκ5**
				921-211	M	VHJ606/JH4	**VκC4/Jκ5**
922-101	G2a	VHQ52/JH4	VκC12/Jκ1	921-102	M	VHQ52/JH4	VκC4/Jκ2
922-163	G2a	VHJ558/JH1	VκC12/Jκ4				
				921-25	M	VHJ558/JH3	Vκ8/Jκ5
992-52	M	VHQ52/JH4	**VκC4/Jκ5**	921-148	M	ND	(not C4, C12)
922-1418	M	VH7183/JH2	**VκC4/Jκ5**	921-151	M	ND	(not C4, C12)
				921-178	G2a	VHJ558/JH1b	Vκ4,5/Jκ1
922-22*	G3	VH7183/JH4	Vκ19/Jκ4	921-1415	M	VHJ558/JH4	Vκ9/Jκ2
922-23*	G2b	VH7183/JH4	Vκ19/Jκ4				
922-42*	G2a	VH7183/JH4	Vκ19/Jκ4	920-18*	G2a	**VHC12.1/JH3**	**VκC12/Jκ5**
				920-108*	M	**VHC12.1/JH3**	**VκC12/Jκ5**
922-207	M	ND	(not C4, C12)				
922-1311	G2b	ND	(not C4, C12)	920-14	M	VHJ558/JH1	**VκC4/Jκ5**
922-1610	G2b	ND	Vκ12,13/Jκ1	920-57	M	VH7183/JH4	**VκC4/Jκ5**
922-1819	G2a	VHJ558/JH4	(not C4, C12)	920-177	M	VHQ52/JH4	**VκC4/Jκ5**
				920-241	M	ND	**VκC4/Jκ5**
				920-257	G3	ND	(not C4, C12)
				920-289	M	ND	Vκ9/Jκ1
				920-293	G2a	VHJ558/JH1	Vκ8/Jκ2
				1343-18*	M	VHS107/JH3	**VκC4/Jκ5**
				1343-512*	M	VHS107/JH3	**VκC4/Jκ5**
				1343-138	M	VHJ558/JH2	**VκC4/Jκ5**
				1343-84	M	VHJ558/JH1	Vκ4,5/Jκ2
				1343-88	M	VHJ558/JH1b	(not C4, C12)
				1343-1215	M	VHQ52/JH4	ND
				1343-526	G2b	VHJ558/JH1b	Vκ4,5/Jκ2

The VH and Vκ gene families, as well as the JH and Jκ gene segments used by PR8 HA-specific hybridomas from non-Tg mouse 922, and by HA104 mice 921, 920, and 1343 are shown. Gene segment combinations that are characteristic of the C12 and C4 clonotypes are shown in bold. The hybridoma H chain isotype is also indicated. For each mouse, hybridomas that use the same or related V gene sequences have been grouped together. These include sets of clonally related hybridomas, which are indicated by asterisks (*). Hybridomas that yielded mixed L chain sequences when analyzed using a degenerate Vκ-specific primer, but that did not amplify in the PCR with VκC4- or VκC12-specific primers, are indicated as (not C4, C12). ND, not determined.

isotype, and they used a similar variety of VH regions as have previously been identified among C4 B cells from BALB/c mice. Three of the remaining hybridomas from HA104 mice (including two of the five IgG-secreting B cell hybridomas that were isolated) used a particular VHJ558/JH1 gene segment combination that was derived from the C57/BL6 background. Since we have not extensively characterized how this gene segment participates in the anti-HA responses of non-Tg mice, the significance of this finding is not clear. It is, however, apparent that hybridomas expressing the C12 clonotype were substantially less frequent among day 5 hybridomas from HA104 mice. C4 hybridomas, on the other hand, were readily isolated from HA104 mice.

Discussion

To analyze how reactivity with a defined self antigen shapes the primary B cell repertoire, we generated transgenic mice that express the influenza virus PR8 HA as a membrane-

bound neo-self antigen (HA104 mice). HA104 mice were compared with non-Tg mice for the specificity of their HA-specific B cell responses after primary virus immunization. Overall, our analyses indicate that functionally distinct subsets of PR8 HA–specific B cells can be activated, or are negatively selected from the primary B cell repertoire in HA104 mice. In particular, a subset of HA-specific B cells that give rise to IgM ASC was induced to similar levels in virus-immunized non-Tg and HA104 mice. By contrast, a subset of IgG-secreting ASC that arise rapidly and disappear abruptly after primary virus immunization in non-Tg mice was substantially reduced in HA104 mice.

Several previous reports have described the induction of autoantibody responses to transgene-encoded neo-self antigens. For example, autoantibodies could be induced in transgenic mice expressing low levels of soluble lysozyme, membrane-bound vesicular stomatitis virus glycoprotein in a variety of tissues, and the influenza virus HA expressed on pancreatic β islet cells (16, 41, 56, 57). In each case, autoantibody responses were induced when mice were immunized with the neo-self antigen linked to a carrier protein as a source of cognate T cell help. Since these transgenic mice mounted reduced T cell responses to the neo-self antigen and failed to generate autoantibody responses without a source of cognate help, these findings indicated that the induction of T cell tolerance can play an important role in controlling autoantibody responses to self-antigens (16, 41, 42). We have recently demonstrated that transgenic mice expressing the HA in the thymus mediate negative selection of $CD4^+$ T cells directed to the major I-E^d–restricted T cell determinant from the HA (30). To rule out the possibility that changes in the B cell responses in HA104 mice might be caused by T cell tolerance to the HA, we challenged HA104 mice with intact influenza virus so that $CD4^+$ T cells directed to other viral proteins could provide a source of intermolecular cognate help to HA-specific B cells (16, 41–43). Under these conditions, we observed a clear reduction in the frequency of PR8 HA–specific, IgG-secreting B cells that were induced 5 d after virus immunization. One explanation for this could be that despite the provision of help to HA-specific B cells by T cells directed to other virus proteins, the induction of IgG B cells in the primary HA-specific response requires direct help from HA-specific T cells. In adoptive transfer experiments, however, T cell clones directed to the virus matrix protein and the HA were previously found to be equally efficient in promoting class-switching of the PR8 HA–specific antibody responses of nude mice (43). A lack of appropriate help from $CD4^+$ T cells in HA104 mice appears unlikely to explain the failure to induce the subset of PR8 HA–specific IgG B cells that arises 5 d after immunization in non-Tg mice.

The specificity of B cell responses to the mutant virus T3 provided direct evidence that PR8 HA–specific IgG B cells are negatively selected in HA104 mice. Several previous studies have demonstrated that xeno- or alloantisera raised against variants of self proteins such as MHC molecules or hemoglobin are focused toward epitopes that contain amino acid differences from the self protein (46–48). This skewing of the antibody response toward foreign determinants reflects the regulation of autoreactive B cells that can cross-react with shared epitopes on the self protein, and provided some of the earliest evidence for B cell tolerance to self proteins (58). The frequency of T3 virus–induced hybridomas that reacted with the T3 HA but did not cross-react with the PR8 HA doubled from 15% of the hybridomas from non-Tg mice to 33% of the hybridomas from HA104 mice. The difference in the relative frequency of T3 HA–specific hybridomas was even greater among IgG hybridomas, increasing from 9% of the hybridomas from non-Tg mice, to 50% of the hybridomas isolated from HA104 mice. Moreover, the increase in the relative frequency of IgG hybridomas that could only react with the mutant T3 epitope was caused by a decrease in the absolute frequency of PR8 HA–specific IgG hybridomas in HA104 versus non-Tg mice. A similar skewing in reactivity toward the T3 epitope, and away from reactivity with the PR8 HA, was apparent among the IgG ASC present in splenocytes 5 d after immunization with T3 virus. By contrast, no skewing in reactivity toward the T3 epitope was observed among the IgM B cells in HA104 mice. Indeed, no substantial differences were evident in the kinetics or frequencies of HA-specific IgM ASC that were induced in HA104 and non-Tg mice. Together, these data strongly suggest that the PR8 HA–specific IgG-secreting B cells that arise 5 d after immunization of non-Tg mice are negatively selected from the primary B cell repertoire of HA104 mice because of their specificity for the PR8 HA.

The conclusion that a subset of HA-specific B cells is negatively selected in HA104 mice is further supported by the relative representation of hybridomas expressing V region sequences that characterize the C12 and C4 clonotypes. The C12 and C4 clonotypes have previously been defined from analyses of the anti-PR8 HA B cell responses of BALB/c mice. C12 antibodies recognize epitopes in the antigenic site Cb, and they dominate the early primary response to the PR8 HA in BALB/c mice; roughly a quarter of the PR8 HA–specific B cell hybridomas generated 5 d after immunization of BALB/c mice express C12 antibodies (26). C4 antibodies recognize epitopes in the antigenic site Sb, and they are also characteristic of day 5 hybridomas from BALB/c mice (24). Consistent with these previous studies, C12 antibodies comprised roughly a quarter of the hybridomas that were generated here from a non-Tg mouse. C4 antibodies were also represented in this panel, at a lower frequency than C12. In hybridomas from HA104 mice, however, the representation of C12 and C4 antibodies was reversed: no C12 hybridomas were identified among hybridomas from two of the three HA104 mice. The only C12 antibodies isolated from HA104 mice were a pair of clonally-related C12 hybridomas (expressing either IgM or IgG constant regions) from mouse 920. By contrast, roughly half of the hybridomas from each of the three HA104 mice expressed C4 antibodies. Moreover, the C12 antibodies from non-Tg mice were all IgG isotype, and the C4 antibodies from either non-Tg or HA104 mice were all IgM isotype. Accordingly, the differential expression of the C12 and C4 clonotypes correlates well with the other data here indicating that the PR8

HA–specific IgG-secreting B cells that arise 5 d after immunization in non-Tg mice are reduced in HA104 mice. Indeed, C12 B cells appear to be substantially eliminated from the primary B cell repertoire of HA104 mice.

The finding that C12 and C4 B cells differ in their susceptibility to negative selection from the primary repertoire of HA104 mice is noteworthy in light of the different phenotypic potentials that B cells expressing these clonotypes display in virus-immunized BALB/c mice. Although C12 B cells dominate day 5 hybridoma panels, they are absent from hybridoma panels generated 10 d after immunization; moreover, C12 antibodies make little or no contribution to secondary responses to the HA (29). In the case of C12 B cells, therefore, virus immunization appears to induce terminal differentiation primarily into IgG ASC, and it results in the elimination of C12 B cells from the HA-specific repertoire of BALB/c mice. Clonal elimination as a consequence of a strong immune response has been observed in mature T cells responding to Mls (59) and to certain viral antigens (60), and it is consistent with early findings of hyporesponsiveness among animals previously injected with large antigen doses (61, 62). By contrast, C4 B cells participate in both primary and secondary responses to the HA; C4 hybridomas can be isolated from hybridoma panels generated 10 d after primary virus immunization (63), and somatically mutated C4 antibodies can be obtained from secondary anti-HA responses of BALB/c mice (24). Since C12 cells but not C4 B cells are eliminated from the primary repertoire of HA104 mice, the findings of this report provide evidence that similar processes may induce a subset of HA-specific B cells to undergo terminal differentiation to produce IgG ASC during the primary response to the HA in BALB/c mice, and also signal their negative selection during primary repertoire formation in HA104 mice.

Why do C12 and C4 B cells differ in their susceptibility to negative selection in HA104 mice, and display distinct phenotypic potentials in non-Tg mice? It is possible that C12 B cells are generally more sensitive to activation than C4 B cells, perhaps because they have higher affinities for the HA. By this model, the higher sensitivity of C12 B cells could promote their terminal differentiation into IgG-secreting ASC in virus immunized non-Tg mice. Likewise, a greater sensitivity to activation could make C12 B cells more susceptible to negative selection in HA104 mice. C4 B cells, on the other hand, might persist in HA104 mice because their reactivity with the HA falls below a threshold that signals negative selection of C12 B cells. C12 B cells could be negatively selected in HA104 mice by reacting with the neo-self HA as a membrane-bound self antigen during their development in the bone marrow (13, 64), or because they react with the HA in the periphery in the absence of T cell help (65, 66). Our data also do not exclude the possibility that C12 B cells could be particularly sensitive to T cell–mediated and/or idiotype-specific suppression in HA104 mice (and in non-Tg mice undergoing primary responses to the HA) (67), although it is unclear how such suppression might be mediated.

Alternatively, the C4 and C12 clonotypes might represent antigen-specific B cells that differentiate along different pathways in response to stimulation by the HA. The notion that B cells can undergo different developmental programs in response to antigenic stimulation is consistent with current models that ASC and memory B cells develop as phenotypically distinct populations in discrete anatomical locations (periarteriolar lymphoid sheaths and germinal centers, respectively) (3, 6, 9). Moreover, ASC and germinal center B cells appear to have quite different potentials for postactivation events, such as antibody secretion and somatic mutation (4–10). The factors that drive B cell differentiation along these different pathways are not yet understood. Germinal center B cells and primary response ASC may derive from common precursor pools and undergo differentiation along different pathways in response to receptor-mediated (or perhaps stochastic) events (8), or may derive from B cell populations that are precommitted to develop along these different pathways (4). The results here suggest that receptor mediated and/or developmentally programmed events may likewise induce HA-specific B cells to develop into functionally distinct subsets of HA-specific ASC, affecting their activation potential in non-Tg mice and influencing their negative selection from the primary repertoire of HA104 mice. In this regard, it will also be interesting to examine the development of C4 B cells at later stages of the primary response and after secondary virus immunization of HA104 mice since this may provide insights into the regulation of self-reactive B cells in the germinal center pathway.

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