RESTRICTED ADULT CLONAL PROFILES INDUCED BY NEONATAL IMMUNIZATION

Influence of Suppressor T Cells*

BY MARY ANN THOMPSON, SYAMAL RAYCHAUDHURI, AND MICHAEL P. CANCRO

From the Immunobiology Research Unit, Department of Pathology and Laboratory Medicine, University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania 19104

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Although the rearrangement strategies of immunoglobulin genes afford great antibody diversity (1–6), the ontogenetic mechanisms that dictate which members of this potential array exist among antigen-responsive B cells are poorly understood. Several laboratories have reported a staged developmental appearance of clonotypes among inbred individuals, suggesting that some steps involved in establishing the primary B cell repertoire are under intrinsic genetic control (7–17). In secondary situations, however, it is clear that prior contact with antigen can alter the repertoire as a result of selective clonal expansion, deletion, or suppression (17–22). Most previous studies have focused exclusively upon either the genetics of clonotype appearance (9–16, 23–26), or upon the effects of receptor-mediated changes in the repertoire (18–21). However, it is likely that these two parameters closely interact to shape the ultimate phenotype of an individual.

Useful information about these interactions has come from limiting dilution analysis of murine B cells responsive to the influenza A hemagglutinin molecule (HA).[1] Recent studies with this system have shown that the clonal composition of the primary HA-reactive B cell pool changes rapidly and regularly during early life (17), but that exposure to antigen perturbs this normal turnover by (*a*) preserving clones that are otherwise transiently expressed, and (*b*) preventing the appearance of certain other HA-specific clones.

These findings suggested that the B cell repertoire can be selectively fixed by antigen exposure, the particular clonotypes selected reflecting the composition of the primary B cell pool at the time of exposure. We have tested this notion by characterizing the HA-responsive repertoires of adults that had been immunized during neonatal life. The results show that varying the age at which immunization first occurs alters the adult clonal profiles obtained. Furthermore, the composition of the primary B cell pool at the time of initial antigen exposure

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[1] *Abbreviations used in this paper:* C′, complement; DME, Dulbecco's Minimum Essential Medium with high glucose; DNP, dinitrophenyl; HA, hemagglutinin molecule of influenza A virus; HAS, hemagglutination saline; HAU, hemagglutinating unit; HI, hemagglutination inhibition; i.p., intraperitoneal, i.v., intravenous; RIA, radioimmunoassay; RP-reactivity pattern; TRT, treatment; UV, ultraviolet.

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is predictive of the selected adult phenotype. The mechanisms responsible have also been investigated, and at least two events are instrumental in ligand-driven fixation of phenotype. First, responsive B cell clones present at the time of challenge are expanded and driven to a serologically distinguishable differentiation state that is resistant to induced T cell-mediated suppression. Second, suppressor T cells are concomitantly generated that ablate primary, but not secondary B cell responses in vivo.

Materials and Methods

Mice. Adult DBA/2 and BALB/cByJ mice were obtained from the Jackson Laboratory, Bar Harbor, ME. Neonatal BALB/c mice were produced in the breeding colony of M. P. Cancro.

Viruses and Immunizations. The following influenza A virus strains were used: PR8 (A/PR/8/34 [HON1]); WSE (A/WSE/33 [HON1]); MEL (A/Mel./45 [HON1]); BEL (A/Bel./42 [HON1]); Eq-PR8 (A/equine/Miami/1/63 [Heq 2]-A/PR/8/34 [N1]; BH (A/BH/35 [HON1]); WEISS (A/Weiss/43 [HON1]; CAM (A/Cam./46 [HIN1]; the influenza B virus, LEE; and E3376, the recombinant virus (A/PR/8/34-A/HK/8/68 [HON2]) originally obtained from P. Palese and J. Schulman, Mount Sinai School of Medicine, New York. All virus strains were initially provided by Dr. Walter Gerhard (Wistar Institute, Philadelphia, PA). Virus growth, purification, and quantitation by hemagglutination titration were accomplished as described previously (23–29). Recipient DBA/2 mice received 1,000 hemagglutinating units (HAU) of PR8 intraperitoneally (i.p.) 6 wk before use as recipients in splenic fragment cultures. Neonatal BALB/c mice were immunized i.p. at either 3 and 6 d of age or at 2 wk of age, with 1,000 HAU of UV-inactivated PR8 virus. These mice and their unimmunized control littermates, as well as the mother, were tested for virus-specific serum antibodies by radioimmunoassay. Virus was inactivated by irradiation with a General Electric G8T5 UV lamp for 15 s at a distance of 10 cm. The virus sample was in a small volume and constantly stirred during irradiation to ensure uniform inactivation.

Limiting Dilution Splenic Fragment Cultures. Monoclonal antiviral antibodies were obtained in vitro from splenic fragment cultures (23–29). Single-cell suspensions of donor spleen cells were transferred intravenously (i.v.) to PR8-immunized DBA/2 mice that had received 1,500 rads (15 grays) of whole body irradiation. Spleen cells were prepared from each donor and transferred in aliquots of 5×10^5–2×10^7 cells to individual recipients. Fragments were prepared from recipient spleens and stimulated with PR8 in vitro as described previously (23–29).

Radioimmunoassay (RIA) for Anti-influenza Antibody. Culture fluids were assayed for the presence of antiviral antibodies by solid-phase RIA (23–29). Briefly, 20 μl of either standard HA-specific hybridoma proteins, culture fluids, or serum dilutions, were added to wells of polyvinyl microtiter V-plates (Dynatech Laboratories, Arlington, VA), to which 20 HAU of purified virus had previously been adsorbed. Plates were developed with affinity-purified, radioiodinated goat antibody to mouse μ, γ, or α chains, or with rabbit antibody to mouse Fab.

Specificity and Reactivity Pattern Analysis of Monoclonal Antibodies. Monoclonal antiviral antibodies generated in limiting dilution culture were tested for HA specificity by RIA against three viruses: PR8, the stimulating virus; E3376, a recombinant virus that shares the hemagglutinin with PR8 and has the neuraminidase of (A/HK/8/68 [H3N2]); and B/LEE, an influenza B virus that shares with PR8 only chicken host component, a carbohydrate moiety acquired during viral growth in eggs. Only antiviral antibodies that reacted with PR8 and E3376 but failed to react with B/LEE were considerd HA-specific. Reactivity pattern (RP) analysis was performed on all HA-specific culture fluids by RIA using isotype-specific reagents. Each fluid was assessed for binding to PR8 and six heterologous viruses as previously described (23–29).

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Hybridoma Antibodies. Several hybridoma antibodies were used in conjunction with a mixture of rabbit and guinea pig complement (C′) in the preparation of cells for in vitro culture or in vivo adoptive transfer experiments.

The rat anti-mouse IgMk hybridoma, Jlld (30), was kindly provided by Dr. J. Sprent (Univ. of Pennsylvania School of Medicine, Philadelphia, PA). This antibody detects a cell surface antigen present on most mature B cells. The Jlld antibody was either used alone as a means of delineating primary from secondary splenic B cells, or was used in conjunction with an anti I-A^d monoclone for the removal of B cells from lymph node cell preparations.

The anti-I-A^d hybridoma, MKD6, originally described by Kappler et al. (31), was obtained from the American Type Culture Collection, Rockville, MD.

The anti-Thy-1 monoclone, JlJ, was also provided by Dr. J. Sprent (30), and was used to deplete T cells from spleen cell preparation.

The anti-Lyt-2.2 hybridoma antibody (32) 3.168.8, was used to deplete some lymph node T cell preparations of suppressor activity.

Preparation of Lymph Node T Cells. The cervical, axillary, inguinal, and mesenteric lymph nodes were removed from chronically immunized or normal BALB/c mice, placed in medium, and homogenized in a 15-ml ground glass tissue homogenizer. After passage through nylon mesh, the cell suspension was washed twice by centrifugation at 1,500 rpm for 10 min at 4°C. The final cell pellet was resuspended in 1 ml of medium and cell number and viability determined. Cells were then treated with a mixture of the monoclonal antibodies Jlld and MKD6. The lymph node cells were adjusted to 4×10^7 cells/ml in a final volume that was 1/5 Jlld (hybridoma supernatant) and 1/20 MDK6 (hybridoma ascites). After incubation for 45 min at 4°C, complement and DNAse (1 mg/ml) were added at 1/12 and 1/25 final volume, respectively. The resulting mixture was incubated for 40 min at 37°C. Subsequently an aliquot was taken to assess the cytotoxicity of the antibody-complement mixture and the suspension was diluted 1:1 cold medium. Aliquots of 3 ml were placed in siliconized 15-ml tubes (vacutainer, Becton-Dickinson), underlayered with 4 ml of Ficoll-Isopaque, and spun at 20°C at 3,000 rpm for 15 min. Cells were collected from the interface, diluted with cold medium, and washed twice at 4°C. Viability was >95%, and treatment with monoclonal anti-Thy-1 antibody routinely gave cytotoxicity indices of >95%. Cells were resuspended, counted, and diluted with DME for intravenous injection in 0.2-ml aliquots.

Preparation of Lyt-2-Depleted T Cells. Some lymph node T cell preparations were treated with monoclonal anti-Lyt-2.2 antibody plus complement for 45 min at 37°C. After treatment, cells were diluted with medium and spun over Ficoll-Isopaque, as above. The efficiency of Lyt-2^+ T cell depletion was assessed by treating the cell preparation with anti-Lyt-2 in a cytotoxicity assay. Residual Lyt-2^+ cells routinely accounted for <2% of the T cell preparation.

Treatment of Spleen Cells with Jlld. Spleen cell suspensions were prepared by perfusion of spleens and passage of the cells through nylon mesh. Cells were centrifuged at 1,500 rpm for 10 min at 4°C and then treated with ammonium chloride-Tris, 4 ml per spleen, at 37°C for 5 min. Volume was doubled with cold medium and the cells were washed twice at 1,500 rpm for 10 min at 4°C. Cells were suspended at 4×10^7 cells/ml in a mixture containing 0.5 ml Jlld per 1×10^8 cells, complement, and 40 μg/ml DNAse. Cells were incubated for 45 min at 37°C. An aliquot was taken to assess cytotoxicity, and the remaining diluted with an equal volume of medium, spun over Ficoll-Isopaque, and washed twice.

Hemagglutination Inhibition. HA-specific serum antibody was quantified by hemagglutination inhibition (HI). Serum (0.1 ml) was mixed with 0.1 ml of HAS and 0.1 ml of a 1/100 dilution of trypsin stock solution. The resulting mixture was incubated at 50°C for 30 min, then cooled to 26°C. Subsequently, 1.0 ml of 0.01 M $NaIO_4$ was added and the mixture allowed to stand for 30 min at 26°C. After this, 0.2 ml of glycerol in saline (60 g/l) was added. After 10 min, 0.5 ml of HAS was added to yield a 1:20 final dilution of the original serum.

The diluted, inactivated serum sample was titrated by doubling dilution. 15 μl of each

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dilution was mixed with 25 μl of HAS containing 4 HAU of either PR8 or CAM. Assays were carried out in 96-well round bottom plates (Linbro). After 1 h at 20°, 50 μl of a 1% suspension of washed chick erythrocytes in HAS was added.

After gentle agitation, the plates were allowed to stand for 40 min, and the number of inhibited wells were scored. Quantitation was accomplished with monoclonal HA-specific antibodies derived from hybridomas.

Results

In previous studies, (17, summarized in Fig. 1, panels *A*, *B*, and *C*), we showed that the PR8 HA-responsive primary repertoire changes considerably between 1 and 2 wk of age (panels *A* and *B*). Interestingly, this change is less marked if PR8 is administered at 1 wk of age and the immunized mice examined at 2 wk of age (panel *C*). Exposure to the antigen thus tends to "fix" the repertoire by preserving some existing specificities and deleting or concealing others. In addition, several clonotypes that had not been observed among normal neonates were repeatedly found among the immunized individuals (right side of Fig. 1, panel *C*).

Reactivity Patterns of Adult Mice Given PR8 as Neonates. In order to establish whether this was a transient or long-lived effect, BALB/c mice were initially immunized at both 3 and 6 d of age with 1,000 HAU of UV-inactivated PR8 virus, and then were either treated weekly with the same antigen dose (chronic regimen), or were untreated (acute regimen). Other mice received antigen chronically but beginning at 2 wk of age. At 7–8 wk of age, these mice were used as B cell donors for limiting dilution splenic fragment cultures. Previous studies (17) showed that such immunizations elicit virus-specific serum antibody, and that the procedures employed do not cross-immunize the nursing mothers.

Chronic exposure to PR8 increases the frequency of HA-responsive B cells 25-fold over that of unimmunized adults, and acute exposure results in a 6-fold increase in frequency (Table I).

The results of RP analysis are shown in Fig. 1, where the clonal composition of immunized animals is compared to that of normal individuals. The striking finding is that the RP seen among adult mice either chronically or acutely challenged with PR8 from 1 wk of age (Fig. 1, panels *D* and *E*) are very similar to the RP observed in 2-wk old mice primed at 1 wk (panel *C*). In each of these situations, the RP are more similar to those observed in normal 1-wk old mice than those seen in either unprimed 2-wk old mice (panel *B*) or adult mice (panel *G*). For example, after chronic immunization (panel *D*), six of the eight RP persist that are both characteristic of the 1-wk repertoire and only transiently expressed in unimmunized mice, while few RP characteristic of the normal 2-wk repertoire (panel *B*) are observed. Also, the repertoires of adults immunized at 1 wk of age contain the same novel clonotypes (RP) seen in our previous study of 2-wk old immunized animals (right side of panels *C*, *D*, and *E*). Likewise, the RP observed among adults given PR8 beginning at 2 wk of age (panel *F*) closely resembles the pattern characteristic of normal 2-wk old mice.

Statistical evaluation (34) upholds both the conclusion that immunized individuals display repertoires most similar to that characteristic of the age when immunization first occurred; as well as the conclusion that the repertoire is less diverse among neonatally immunized adults than among unimmunized adults. This is clear from inspection of Fig. 1, panel *G*, where (*a*) a similar size sample

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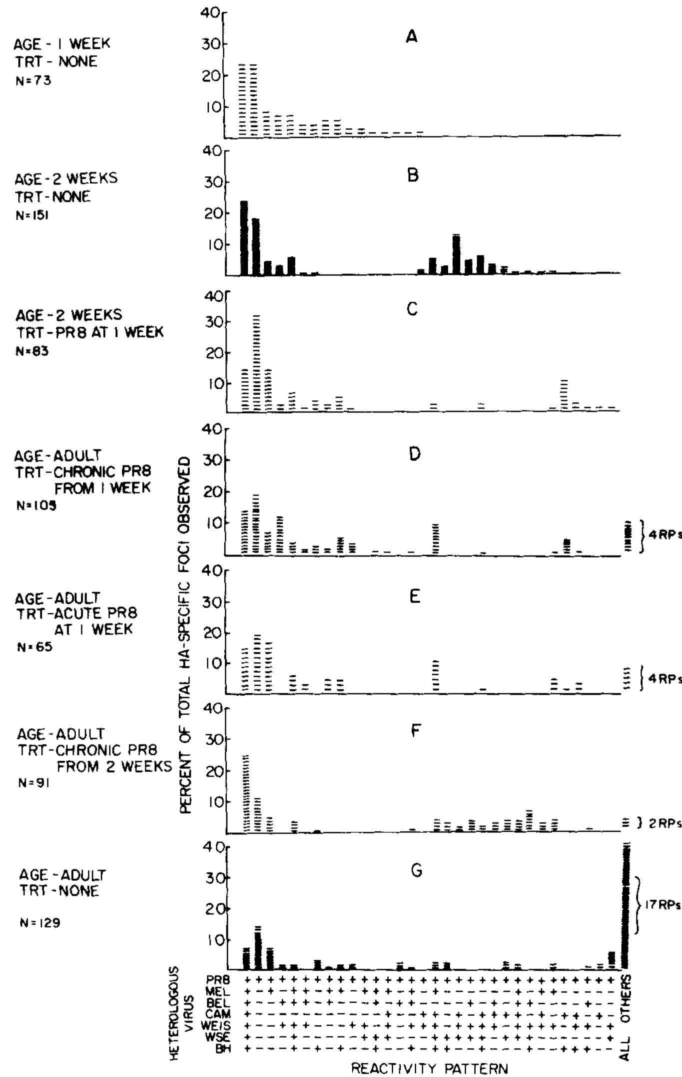

FIGURE 1. Reactivity pattern (RP) analysis of HA-specific antibodies from normal and neonatally immunized BALB/c mice. The HA-specific monoclonal antibodies derived from splenic fragment cultures were assayed for binding on six heterologous viruses by RIA. Because they are monoclonal, each antibody recognizes a single determinant on the immunizing HA molecule. Depending on the distribution of the recognized determinant among the heterologous viral HA, a characteristic array of positive and negative reactions is obtained for each antibody. Each potential RP defines a clonotype or small set of clonotypes that is necessarily distinct from clonotypes yielding any other RP. Each vertical bar shows the relative frequency of a given RP, and each horizontal line within a bar represents a single antibody. Each panel shows the results obtained from animals of a particular treatment (TRT) group. Panels *A* and *B* show the HA-responsive repertoires of unimmunized BALB/c mice at 3–6 and 12–14 d of age, respectively. Panel *C* shows the HA-responsive repertoire of 2-wk old mice immunized at 3 and 6 d of age. Panels *D* and *E* show the repertoires of adult mice that had been either chronically or acutely immunized beginning at 3–6 d of age. Panel *F* shows the repertoire of adult mice that had been chronically immunized beginning at 12–14 d of age. The repertoire observed among a sample of unimmunized adults is shown in panel *G*.

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TABLE I
*Frequency of HA-Specific B Cells Among Normal and Immunized BALB/c Mice**

Age	Age at initial PR8 immunizations‡	Immunization§ regimen	HA-Specific‖ cells per 10^6 splenic B cells
6 d	—	—	8
12–14 d	—	—	3
13–14 d	Day 3–6	Acute	19
Adult	—	—	13
Adult	Day 3–6	Chronic	321
Adult	Day 3–6	Acute	76
Adult	Day 12–15	Chronic	312

* The frequency of HA-responsive splenic B cells was determined by limiting dilution splenic fragment culture (23–28) as described in Materials and Methods.
‡ Mice were either unimmunized, or initially immunized with 1,000 HAU of UV-inactivated PR8 at the age shown.
§ Among immunized mice; either chronic or acute dose regimens were employed. Acutely immunized mice were given the initial dose of PR8 only. Chronically immunized mice received 1,000 HAU of UV-inactivated PR8 weekly following initial immunization; and received their final dose of virus 7 d before use as spleen cell donors in limiting dilution culture.
‖ The values given are after correction for the proportion of B cells in the inoculum (17), as well as homing and cloning efficiency (33).

from normal adults yields twice the number of different RP than within any group of neonates; and (*b*) a large proportion of the adult RP have never been observed among neonates (far right column of panel *G*).

These data imply that neonatal antigen exposure creates an oligoclonal adult repertoire, characterized by the presevation and dominance of clonotypes available at the time of initial exposure.

The persistence of particular clonotypes for prolonged periods after antigen priming is consistent with the well established view that most memory B cells have a long life span. However, it is less easy to explain the fact that priming restricts the *size* (heterogeneity) of the repertoire. Since the precursor frequencies of immunized mice are far higher than in unimmunized individuals (Table I), one could argue that the restricted repertoire of neonatally primed mice may be only quantitative, and reflect inadequate sample size. According to this idea, analysis of very large numbers of HA-specific B cells from immunized mice might eventually reveal all reactivity patterns found in normal adults. Alternatively, antigen priming might induce *suppression*, which prevents the emergence or stimulation of new anti-HA specificities. This explanation, however, forces one to argue that primed and unprimed B cells are qualitatively different, only unprimed B cells being susceptible to suppression.

To examine this possibility, we compared primed and unprimed HA-specific B cells for (*a*) the presence of the Jlld antigen, a putative marker for memory B cells, and (*b*) susceptibility to T cell-mediated suppression.

Expression of Jlld Antigen Among Primed vs. Unprimed HA-Specific B Cells. Bruce et al. (30) have reported that the rat anti-mouse monoclonal antibody, Jlld, lyses >90% of splenic B cells in the presence of complement; and greatly reduces the primary immune responses to antigens such as fowl gamma globulin. In marked contrast, *secondary* responses to these antigens are not detectably altered.

In the present experiments, the frequency of HA-specific B cells in the Jlld$^-$

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population of both normal and chronically immunized adults was determined by limiting dilution splenic fragment culture.

As shown in Table II, the few remaining splenic B cells that survive treatment with Jlld and complement have a similar frequency of HA-responsive cells as unseparated splenic B cells. Since this treatment lyses >90% of the Ig^+ cells, it follows that the vast majority of unprimed HA-specific B cells are $Jlld^+$. In contrast, treatment of primed B cells with Jlld and complement causes a marked (12-fold) enrichment in the frequency of HA-specific B cells. Thus HA-reactive cells from primed mice are predominantly $Jlld^-$. These data confirm that primary and secondary HA-reactive cells show qualitative differences and provide a means to assess functional changes associated with priming.

Suppressor T Cells that Differentially Affect Primary vs. Secondary B Cell Responses are Induced by Neonatal Antigen Challenge. T cells from either chronically immunized or normal BALB/c mice were transferred to both PR8-primed and normal BALB/c recipients. The recipients were immunized with PR8 2 d after T cell transfer, and bled 6, 8, and 10 d after immunization. The sera was tested for HA-specific antibody titer by hemagglutination inhibition.

T cells derived from chronically exposed mice routinely suppressed primary HA-specific antibody responses, whereas T cells from unimmunized mice had no effect (Table III). The suppressive effects were abolished by treating the T cell inoculum with anti-Lyt-2 and C′, as well as by irradiation of transferred cells with 1,500 rads. In contrast, secondary humoral responses were not susceptible to suppression even at doses of 10^7 transferred cells; which is 10-fold higher than the dose necessary to ablate primary responses. Finally, doses of 10^7 T cells had no effect upon primary DNP-specific responses induced by DNP-hemocyanin (data not shown). Neonatal immunization thus induces $Lyt\text{-}2^+$, radiation-sensitive, suppressors that act upon primary but not secondary B cell responses to the same antigen.

Studies with an adoptive transfer system showed that T cells from chronically PR8 primed mice failed to support responses by purified unprimed B cells. The

TABLE II

*Antigen Priming Changes the Distribution of HA-reactive B Cells Among Subsets Defined by the JIId Marker**

B cell donor status	HA-Specific foci‡ per 10^6 injected B lymphocytes	HA-Specific foci per 10^6 injected $JIId^-$ B lymphocytes
Normal	0.52 (0.35–0.62)	1.3 (0.6–2.2)
PR8 immune	12.70 (12.4–12.8)	161 (107–200)

* Splenic $Jlld^-$ B cells were prepared as described in Materials and Methods from either normal BALB/c adults, or age-matched animals that had been immunized with 1,000 HAu of PR8 at 3 and 6 d of age and weekly thereafter. Splenic $Jlld^-$ cells were pooled from several individuals, and either 2×10^7 cells from normal mice, or 1×10^5 from immunized mice were adoptively transferred to recipients for limiting dilution splenic fragment culture. Total B cell inoculums were prepared by treatment with anti-Thy-1 only.

‡ Values given are the arithmetic means of at least four experiments. The range of values between experiments are shown in parentheses.

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TABLE III

Neonatal Antigen Challenge Induces Suppressors Capable of Ablating Primary, but Not Secondary HA-Specific Humoral Responses In Vivo

Donor L.N.* T cells transferred	T cell donor‡ treatment	Recipient§	HA-Specific‖ antibody
			μg/ml
—	—	Normal	65; 130; 190
—	—	PR8 primed	350; 250; 360; 175
1×10^7	Chronic PR8	Normal	<10; <10; <10; 30
1×10^6	Chronic PR8	Normal	<10; 50; <10; 30
5×10^5	Chronic PR8	Normal	75; 175; 80; 80
1×10^7	Chronic PR8	PR8 primed	350; 375
1×10^6	Chronic PR8	PR8 primed	350; 750; 750; 750
1×10^7	None	Normal	90; 180; 165; 90; 260
1×10^7	None	PR8 primed	350; 700; 750; 750
1×10^7 after anti-Lyt-2 and C′	Chronic PR8	Normal	150; 175
1×10^7 T after irradiation	Chronic PR8	Normal	180; 260

* All lymph node cells were treated with anti-I-A^d (MKD6) and Jlld plus complement to yield a 97% Thy-1^+ cell population. In the last two treatment groups, these cells were also either treated with a monoclonal anti-Lyt-2 reagent (3.168.8) and C′, which kills 22% of L.N.T-cells; or were irradiated (1,500 rads).

‡ BALB/c donor mice were either untreated, or had received UV-inactivated PR8 chronically (1,000 HAU/wk) beginning 3–6 d after birth.

§ Recipients were either normal BALB/c mice or age-matched, PR8 immunized BALB/c mice. All recipients received 1,000 HAU of PR8 i.p. 2 d after T cell transfer, and were bled at days 6, 8, 10 after immunization.

‖ HA-specific antibody was determined by hemagglutination inhibition. This allows the detection of HA-specific antibody, but not antibodies directed to other viral antigens. Each number represents mean of triplicate determinations on an individual animal.

same T cells, however, provided excellent help for primed B cells, including the purified J11d⁻ subset. Table IV shows the results obtained from such an experiment. Neither whole splenic B cells nor J11d⁻ B cells from unimmunized mice respond to PR8 in the absence of cotransferred T cells. Both such pools from immunized mice give rise to some PR8-specific serum antibody after challenge; suggesting that secondary, J11d⁻, B cells may have less stringent requirements for T help than do primary cells. The key elements of the experiment, however, are the groups that show that J11d⁻ B cells from chronically immunized mice respond even in the presence of T cells that abolish the response of splenic B cells from nonimmune individuals.

The data in Tables III and IV thus imply that: (*a*) PR8 primed T cells comprise a mixture of suppressor and helper cells; (*b*) unprimed B cells are very sensitive to the suppressors; and (*c*) primed, J11d⁻ B cells are resistant to suppression but remain responsive to help.

PR8-Induced Suppressors Ablate B Cell Responses Directed Towards Heterologous Viral HA. To establish whether the suppressors induced by neonatal challenge at 3–6 d of age exhibit specificity via epitopes recognized by responding B cells; or instead exhibit broadly specific effects; the following experiment was performed. T cells from mice chronically immunized with PR8 were tested for their ability to ablate primary responses to the heterologous influenza virus, CAM. This virus

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TABLE IV

HA-Reactive, J11d⁻ B Cells from Immunized Mice are Resistant to Antigen-induced Suppression *

Spleen cell donor treatment	Spleen cell treatment	Lymph node T cell donor treatment	Recipient serum HA-specific antibody after PR8 challenge‡
			μg/ml
—	—	Normal	<10 <10
—	—	Chronic PR8	<10 <10
Normal	Thy-1	—	<10 <10
Chronic PR8	Thy-1	—	130; 65
Normal	Thy-1	Normal	130; 130
Normal	Thy-1	Chronic PR8	<10; <10; <10
Chronic PR8	Thy-1	Normal	735; 735; 735
Chronic PR8	Thy-1	Chronic PR8	520; 735; 735
Chronic PR8	Thy-1/J11d	Normal	520; 1040; 735; 735
Chronic PR8	Thy-1/J11d	Chronic PR8	370; 735; 1040; 370; 735
Normal	Thy-1/J11d	Normal	30; <10; 15; 30;
Normal	Thy-1/J11d	Chronic PR8	<10; <10; 30; 15; <10

* Spleen and lymph node cells were prepared from either normal BALB/c mice; or BALB/c mice that had been immunized chronically with PR8 beginning at 3 to 6 d of age. All donors were 7–8 wk of age. Spleens and nodes were taken from the same animals. Lymph node T cells were prepared by treatment with monoclonal anti-I-A and J11d as described in Materials and Methods. Spleen cells were treated with either anti-Thy-1 only, or with both with anti-Thy-1 and J11d. These cells were transferred intravenously to irradiated (950 r) BALB/c mice. In all cases, doses of 2.5×10^7 B cells and 1×10^7 T cells were used.

‡ 2 d after transfer, recipients were immunized with 1,000 HAU of UV-inactivated PR8 intraperitoneally. Serum was collected on days 6, 8, and 10 after immunization, and assayed for PR8-specific antibody by both RIA and hemagglutination inhibition. The figures given are based on the day-6 sera.

was chosen because it shares very few epitopes recognized by the 3–6-d PR8-responsive B cell repertoire. This is evident from Fig. 1, where few of the PR8-specific antibodies from normal 6-d old mice react with CAM.

The results (Table V) indicate that PR8-induced suppressors are effective in preventing primary responses to CAM, as well.

Discussion

This work demonstrates a strong relationship between the normal turnover of primary B cell repertoire composition and the effects of antigen-driven events. Precedence for receptor-mediated repertoire changes exists in several experimental systems that involve neonatal exposure to either antigen or antiidiotypic antibodies. These treatments have led either to the loss of dominant clonotypes, or to the enhanced representation of minor clonotypes (18–22, 35–37). The present study extends these findings in several ways: First, the behavior of the entire HA-responsive repertoire has been monitored, providing a more complete understanding of the processes exemplified by individual clonotypes in previous studies. Second, in conjunction with our previous work, which defined the dynamics of the neonatal HA-specific repertoire (17), the present findings help explain the time-dependent nature of these effects. Third, these experiments show that induction of characteristic clonal profiles involves changes in not only the frequency but the functional properties of stimulated B cell clones. Finally, active suppression seems involved in the continued maintenance of a particular

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TABLE V

Suppressors Generated by PR8 Immunization Ablate Primary B Cell Responses to Heterologous Influenza A Virus Strains

Donor L.N. T cells transferred*	Donor treatment‡	Immunizing antigen§	HA-Specific antibody reactive with:	
			PR8	CAM
			µg/ml	
—	—	PR8	130; 200; 160; 160	<10; 30; <10; <10
—	—	CAM	40; 20; <10; <10	200; 110; 80; 160
1×10^7	Chronic PR8	PR8	20; <10; 20; <10	<10; <10; <10; <10
1×10^6	Chronic PR8	PR8	20; 20; 40; 20	<10; <10; <10; <10
1×10^7	Chronic PR8	CAM	<10; <10; <10; <10	<10; <10; <10; <20
1×10^6	Chronic PR8	CAM	<10; <10; <10; <10	<10; <10; <10; <10
1×10^7	None	PR8	100; 80; 40; 160	40; <10; <10; 20
1×10^7	None	CAM	40; 20; <10; <10	200; 110; 80; 160

* Lymph node cells were prepared as in Table III.

‡ Donor mice were treated as those in Table III.

§ Recipients were normal (unimmunized) BALB/c mice at 6–8 wk of age. Each recipient received 1,000 HAU of either PR8 or CAM 2 d after T cell transfer. Sera was taken at days 6, 8, and 10 after immunization.

‖ HA-specific antibody was determined by hemagglutination inhibition. For each individual recipient, titration was performed for both PR8- and CAM-reactive antibody, by using the appropriate virus in the HI assay.

clonal profile.

The resistance of primed B cells to T cell-mediated suppression clearly confers a selective advantage in subsequent responses, since primary cells arising in the intervening period will presumably be susceptible to suppression. Although relative resistance of secondary B cells to T cell-mediated suppression has been described in several other systems (38, 39), the present experiments indicate that the clonotypes within the suppression-resistant population can depend strongly upon the time of initial antigenic exposure.

The distribution of HA-reactive cells among J11d$^+$ and J11d$^-$ B cells also changes markedly following immunization. Immunization may either preferentially expand pre-existing J11d$^-$ B cells, or may instead induce the differentiation of J11d$^+$ B cells, which results in loss of the marker. Although we presently favor the latter possibility because of our inability to reconstitute HA-specific responses with J11d$^-$ cells from unprimed donors (Table IV), additional experiments will be required to assess these possibilities.

Interpretive caution must be exercised regarding the J11d marker's apparent association with suppression susceptibility. For example, the J11d$^+$ to J11d$^-$ transition may occur only after certain types of priming (e.g., thymus dependent vs. thymus independent), whereas functional changes may occur in all cases.

Lengthened cellular half-life may also accompany stimulation and clonal expansion. Several studies have suggested a rapid turnover for primary B cells (40, 41), but more protracted decay rates for secondary B cells (42). Because PR8-primed cells are serologically distinguishable from their unprimed counterparts by the J11d marker, the possibility of induced changes in half-life properties should be amenable to definitive analysis.

The apparent absence of clonotypes known to exist among unimmunized

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adults may also involve several mechanisms. First, B cell clones that arise in the presence of antigen might be tolerized (43–45). The similar skewing of adult phenotype after acute as well as chronic doses of antigen, however, makes it unlikely that this is the sole mechanism responsible. The results suggest instead that active mechanisms play a role, since suppressor populations are produced by neonatal immunization. The in vivo T cell transfer experiments that demonstrate complete suppression of the primary HA-specific responses to both CAM and PR8 suggest that suppression is at least in part specific for conserved regions of the virus that are not necessarily those recognized by the B cells. This provides a plausible explanation for our inability to detect the broad array of clonotypes known to emerge at later ages. The properties of these suppressors closely parallel those of Ts populations described by Herzenberg et al. (46). The antigen-specific nature of suppression raises several issues. First, although primary clones that subsequently emerge will not participate in HA-specific responses, they may remain available for primary responses to other antigens. Second, antigen challenge that routes particular B cell clonotypes to a suppression resistant state, while at the same time inducing suppressors, could lead to *apparent* clonotype-specific suppression; but actually reflect the distribution of clonotypes within suppression-resistant vs. suppression-sensitive subsets.

A particularly strong association can be made between the present observations and those of Fazekas de St. Groth and Webster (47), who showed that children previously immunized to one strain of influenza tended, upon secondary challenge with a heterologous strain, to produce antibodies of greatest avidity for the HA of the virus used in the primary challenge. The findings presented herein closely parallel this observation, and they may provide a mechanism to account for this phenomenon, termed "original antigenic sin." Although the classical explanation also invokes stimulation of previously expanded, cross-reactive B cell clones (48), our results indicate that active suppression may also be a major contributing factor.

Summary

The effects of neonatal antigen exposure on the adult B cell repertoire have been examined by characterizing the influenza hemagglutinin (HA)-specific response of adult BALB/c mice given antigen soon after birth. Ligand exposure during early life exerts a profound and lasting effect upon the B cell repertoire, characterized by the expansion and preservation of particular antigen-reactive clones and the apparent loss of others. The precise subset of clonotypes selectively preserved depends upon the age at which antigen is first encountered; and is predictable given a knowledge of the emerging primary pool's dynamics and composition.

The preserved (secondary) B cells differ from their unprimed precursors with respect to (*a*) expression of the surface marker detected by the monoclonal antibody J11d, and (*b*) susceptibility to T cell-mediated suppression.

These studies thus demonstrate a strong relationship between the heritable dynamics of the emerging primary B cell repertoire and the effect of ligand-driven events upon repertoire phenotype. In addition, they provide a mechanistic model for certain forms of antigen-induced oligoclonal dominance, especially

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the phenomenon of original antigenic sin.

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