CELLULAR BASIS FOR NEONATALLY INDUCED T-SUPPRESSOR ACTIVITY

Primary B Cell Maturation Is Blocked by Suppressor-Helper Interactions Restricted by Loci on Chromosome 12

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The relative frequency of different B cell clonotypes is governed by several mechanisms. Genetic control over the initial appearance of primary clonotypes has been demonstrated (1–15), but antigen-driven events also play a large role in shaping an individual's ultimate clonal profile (16–21). We recently demonstrated regular clonal turnover patterns during early life within the influenza hemagglutinin (HA)[1]-specific repertoire, which are altered by antigen challenge (22, 23). Specifically, the expression of clonotypes extant at the time of challenge is maintained, while the expression of clonotypes that normally arise subsequently is prevented. This induced oligoclonal dominance was shown to be due to suppressor T lymphocytes (Ts), which prevent primary, but not secondary B cell responses in vivo (23). In order to better understand the role played by these Ts cells in maintaining adult clonal profiles, we have herein examined their mechanism of action.

Using an adoptive transfer system described previously (23), the events in primary B cell activation that are blocked by Ts cells in vivo have been assessed by measuring B cell proliferation, surface differentiation antigen changes, antibody production, and the generation of long-lived memory B cell populations. In addition, the effect of neonatally induced Ts cells on HA-specific helper T cell (Th) priming was determined by limiting-dilution analysis of Th cell activity.

Our results indicate that Ts cells prevent activation of Th cells necessary for primary B cell maturation to either secretory or long-lived secondary B cells. The initial proliferative response of HA-specific B cells, however, proceeds normally in the presence of Ts cells, but the lifespan of these clones is short, unlike B cells stimulated in the absence of Ts cells.

In addition, there is an interaction between Ts and Th cells that is restricted by genetic elements on mouse chromosome 12. This has been demonstrated by

[1] *Abbreviations used in this paper:* C', complement; HA, hemagglutinin; HAU, hemagglutination unit; HAS, hemagglutination saline; HI, hemagglutination inhibition; LN, lymph node; mAb, monoclonal antibody RIA, radioimmunoassay; Th, helper T cell; Ts, suppressor T cell.

in vivo reconstitution experiments, in which adoptively transferred Ts, Th precursors, and primary B cells were derived from BALB/c, C.B20, or BALB/c × C.B20 F_1 individuals.

Materials and Methods

Mice. Adult BALB/c ByJ and DBA/2 mice (6–8 wk old) were purchased from The Jackson Laboratory, Bar Harbor, ME. Neonatal BALB/c ByJ mice were produced in the breeding colony of M. P. Cancro. C.B20 and BAB14 mice were obtained from the colony of Dr. M. Potter (National Institutes of Health, Bethesda, MD) maintained by contract NCI-CB-94326.

Viruses and Immunizations. The following influenza A virus strains were used: PR8 (A/PR/8/34 [H1N1]); Eq-PR8 (A/equine/Miami/1/63 [Heq 2]-A/PR/8/34 [N1]); the influenza B virus, LEE; and E3376, the recombinant virus (A/PR/8/34-A/HK/8/68 [H1N2]) 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 (22–27). Recipient DBA/2 mice received 1,000 hemagglutinating units (HAU) of PR8 intraperitoneally, 6 wk before use as recipients in splenic fragment cultures for limiting-dilution B cell analyses. Neonatal BALB/c mice were initially immunized intraperitoneally at 3 and 6 d, and weekly thereafter with 1,000 HAU of UV-inactivated PR8 virus (27), to generate Ts populations.

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 IgM κ rat anti-mouse hybridoma, J11d (28), was kindly provided by Dr. J. Sprent (Scripps Clinic and Research Foundation, La Jolla, CA). This antibody detects a cell surface antigen present on most mature primary B cells. The J11d antibody was either used alone, or was used in conjunction with an anti-I-A^d monoclonal antibody (mAb) for the removal of B cells from lymph node (LN) T cell preparations.

The anti-I-A^d hybridoma, MKD6, originally described by Kappler et al. (29), was obtained from the American Type Culture Collection, Rockville, MD.

The anti-Thy-1 mAb, J1J, was also provided by Dr. J. Sprent (28), and was used to deplete T cells from splenic B cell preparations.

The anti-Lyt-2.2 hybridoma antibody 3.168.8 (30), was used to deplete some LN T cell preparations of suppressor activity.

The anti-Lyt-1.2–producing hybridoma line C3PO, originally described by Dr. J. Klein (31); was kindly provided by Dr. Jack Bennik (Wistar Institute).

Preparation of LN T cells. LN were removed from either immunized or normal mice, and T cells were prepared as described previously (23), by treatment with a cocktail of J11d and anti-I-A^d plus C′. Viability was >95%, and treatment with anti-Thy-1 mAb routinely gave cytotoxicity indices of >95%. Cells were resuspended, counted, and diluted appropriately for intravenous injection in 0.2 ml-aliquots.

Preparation of Lyt-2-depleted T Cells. Some LN T cell preparations were treated with anti-Lyt-2.2 mAb plus C′ for 45 min at 37°C. After treatment, cells were diluted with medium and spun over Ficoll-Isopaque. 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 J11d. Spleen cell suspensions were prepared by perfusion, treated with ammonium chloride–Tris for 5 min at 37°C to lyse red blood cells, and 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 J11d per 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.

Limiting-dilution Analysis of HA-specific B Cells. The frequency of HA-responsive splenic B cells was assessed by limiting-dilution splenic fragment culture (32) as previously described (11–13, 22–27). Briefly, single-cell suspensions of donor splenic B cells were transferred intravenously to PR8-immunized DBA/2 mice that had received 1,500 rad of whole body irradiation. Fragments were prepared from recipient spleens 18 h later, stimulated with PR8 in vitro, and the culture supernates tested for HA-specific antibody production by radioimmunoassay (RIA).

Limiting-dilution Analysis of Virus-specific Th Cells. The relative frequencies of Th cells capable of enabling HA-specific responses were determined by a modification of the technique originally described by Pierce et al. (33). Briefly, various numbers of Lyt-2-depleted LN T cells, prepared as described above, were mixed with splenic B cells such that the desired numbers of T and B cells could be transferred intravenously to irradiated (1,500 rad) recipients in a volume of 0.2 ml. ~18 h after transfer, the recipient spleens were removed and diced with a McIlwain tissue chopper (Brinkman Instruments, Westbury, NY) to yield 1 mm^3 fragments. The fragments were distributed individually to 96-well tissue culture plates, and stimulated with 20 HAU of PR8 in 0.2 ml of medium. The medium was changed at 3 d intervals and the culture supernatants collected beginning at day 9 of culture. Supernatants were assayed for anti-viral antibody and HA-specificity by RIA.

RIA for Anti-influenza Antibody. Culture fluids were assayed for the presence of antiviral antibody by solid-phase RIA (22–27). Briefly, 20 μl of either standard HA-specific hybridoma proteins or culture fluids 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 against mouse μ, α, or γ chains, or with rabbit antibody against mouse $F(ab)_2$.

Specificity of mAb. Antiviral mAb generated in limiting-dilution culture were tested for HA-specificity by RIA using three viruses: PR8, the stimulating virus; E3376, a recombinant virus that shares the HA molecule with PR8 but 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 considered HA-specific.

Hemagglutination Inhibition (HI). HA-specific serum antibody was quantified by HI. Serum was inactivated as described previously (23), and titrated by doubling dilution. 25 μl of each dilution was mixed with 25 μl of HA-saline (HAS) containing 4 HAU of PR8. Assays were carried out in 96-well round-bottom plates (Linbro Chemical Co., Hamden, CT). After 1 h at 20°C, 50 μl of a 1% suspension of washed chicken red blood cells in HAS was added.

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

Results

Previous experiments (23) showed that adoptive recipients of PR8-induced Ts cells fail to produce HA-specific serum antibody in response to PR8 challenge. To determine which aspects of B cell stimulation these Ts cell populations blocked, we assessed their effect on B cell proliferation and differentiation.

Ts Cells Prevent Neither B Cell Proliferation Nor Differentiation to J11d⁻ Subset. We immunized BALB/c mice with PR8 after transferring Lyt-1⁻,2⁺ lymph node T cells that had been derived from either normal or neonatally immunized mice. As previously reported, individuals that received T cells from immunized mice produced little HA-specific serum antibody (Table I, *left*). 8–10 d following such pretreatments, we used these mice as splenic B cell donors in limiting-dilution culture, and determined the frequency of HA-responsive B cells. The

TABLE I

Neonatally Induced Ts Prevent Antibody Secretion But Not Proliferation by Primary HA-specific B Cells In Vivo

Pretreatment of B cell donor				HA-specific foci per 10^6 injected donor cells among:*	
T cell source[‡]	T cells transferred	Immunizing antigen	HA-specific serum antibody	Whole splenic B cells	J11d⁻ splenic B cells
			µg/ml		
None	None	None	<12	0.31	0.08
None	None	PR8	100	5.0	25.0
Normal	10^7	PR8	115 (87–187)	5.7	40.0
Chronic	10^7	PR8	19 (13–25)	3.8	50.0

The effects of neonatally induced Ts on antibody secretion in vivo, and on HA-specific B cell proliferation and differentiation to the J11d⁻ subset were assessed by immunizing BALB/c mice that had received Lyt-1⁻,2⁺ T cells (from either normal or neonatally immunized individuals) with PR8. 8–10 d later, these mice were used as splenic B cell donors in limiting-dilution culture, and the relative frequency of HA-specific B cells was determined. Donor B cells were prepared by treatment with anti-Thy-1.2 and C′ as described in Methods.

* Serum HA-specific antibody from B cell donors was assessed by HA inhibition 6–8 d following T cell transfer and PR8 challenge.

‡ T cells were derived from either normal BALB/c mice or from BALB/c mice that had been immunized weekly with 1,000 HAU of PR8 intraperitoneally, beginning at 3–6 d. The transferred cells were treated, as described in Methods, with both J11d and anti-Lyt-1 plus C′ prior to transfer to remove B cells and Th.

results (Table I, *right*) show that, although adoptively transferred Ts prevent HA-specific serum antibody production, the frequency of HA-responsive B cells increases substantially after immunization.

Because the adoptively transferred Ts failed to prevent a proliferative response by HA-specific primary B cells, we wanted to establish whether other changes associated with B cell priming occurred. Previous studies (23) showed that the majority of HA-specific B cells in unprimed mice bear a surface marker detected by mAb J11d, whereas HA-specific B cells in chronically primed mice are predominantly J11d⁻. Accordingly, we examined the relative representation of HA-specific B cells within J11d⁺ and J11d⁻ subsets after priming in the presence of Ts cells. A portion of the splenic B cells derived from adoptive Ts cell recipients was treated with J11d and C′ before the in vitro limiting-dilution analysis of HA-specific B cell frequency.

These results (Table I) indicate that, even in the presence of Ts cells, a shift to J11d⁻ subset occurs, similar to when Ts cells are absent.

In conjunction, the findings in Table I show that although antibody secretion by primary B cells is prevented by Ts cells, neither initial proliferation nor loss of the J11d marker is blocked. In addition, these results suggest that the shift in surface phenotype from J11d⁺ to J11d⁻ occurs early in the activation of B cells, prior to antibody secretion.

Fate of B Cell Clones Stimulated in Presence of Suppressors. Because secondary B cells are generally believed to be long-lived, these results appeared contrary to the notion that Ts cells play a role in maintaining oligoclonal adult B cell profiles.

Alternatively, it remained possible that, although proliferation and transition from the J11d$^+$ to J11d$^-$ subset occurred, the antigen-responsive lifespan of B cell clones expanded in the presence of Ts cells is short. To test this possibility, we determined the frequency of HA-specific B cells at various times after immunization.

The results (Fig. 1, A and B) show that although the initial B cell proliferative response occurs whether or not Ts cells are present, the persistence of responsive B cell clones expanded in the presence of Ts is relatively short, compared to individuals that received normal T cells before priming.

Two explanations could be invoked to explain the rapid decay of HA-responsive cells following priming in the presence of Ts. The clonal lifespan of these cells might remain short, rather than increasing, as is characteristic for secondary cell populations. Alternatively, it could also be argued that the homing properties of these cells was altered such that they became sequestered in the LN and did not return to the spleen. To test this latter possibility, we performed similar experiments in which we determined the frequency of HA-reactive B cells in the LN of mice primed in the presence or absence of Ts. The results (Fig. 1, C and D) indicate that the same pattern of expansion and decay of the HA-responsive cell population is observed within the LN as in the spleen, although with delayed kinetics. Moreover, in separate experiments using ^{51}Cr-labelled cells, we have
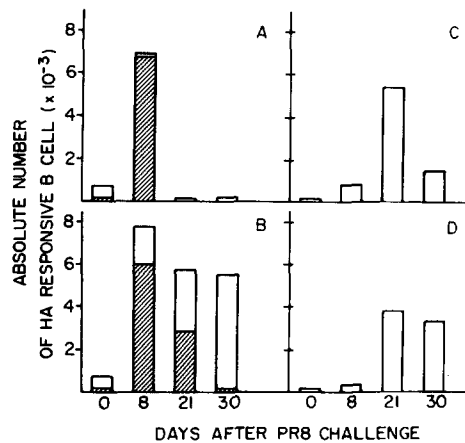

FIGURE 1. Kinetics of splenic and LN HA-specific B cell frequencies and J11d phenotype following PR8 challenge in the presence of Ts cells. BALB/c mice were immunized with 1,000 HAU of PR8 virus 24 h after receiving 10^7 Lyt-1$^-$,2$^+$ T cells from either normal or neonatally primed individuals. At various times after immunization, these mice were used as splenic and LN B cell donors in limiting dilution splenic fragment cultures as described in Methods. Both whole splenic B cells, prepared by treatment with anti-Thy-1.2 plus C', as well as J11d$^-$ B cells, prepared by treatment with both anti-Thy-1.2 and J11d plus C' were analyzed. Only whole LN B cells were analyzed. The absolute number of B cells was calculated based upon previous estimates of total adult splenic or LN B cells (22), and after correction for the homing and cloning efficiency of the limiting-dilution culture system (32). A shows results obtained with splenic B cells from mice that received T cells from neonatally immunized mice. B shows results obtained with splenic B cells from mice that received T cells from normal mice. The cross hatched areas indicate the proportion of B cells that lack the J11d surface marker. C shows the results obtained with LN B cells from mice that received T cells from neonatally immunized mice. D shows results obtained with LN B cells from mice that received T cells from unimmunized mice.

shown that LN B cells home to adoptive recipient spleens with the same efficiency as do splenic B cells (data not shown), which makes it unlikely that we have overlooked a population of HA-reactive cells sequestered in the LN.

Based upon these findings, we favor the notion that the clonal lifespan of B cells stimulated in the presence of Ts is relatively short. Further, because these clones initially expand to roughly the same extent as cells primed in the absence of suppression, this would have to be an actual difference in cellular half-life, rather than a reflection of smaller burst size.

The J11d Marker Is Associated with Resting B Cells. In the same experiments, we assessed the relative number of HA-responsive B cells within the J11d$^-$ and J11d$^+$ populations by treating a portion of the donor splenic B cells with J11d and C$'$ before limiting-dilution analysis. The results (Fig. 1, A and B) indicate that the transition from J11d$^+$ to J11d$^-$ is transient, and that most B cells regain this marker once the primary response has subsided. These experiments were not performed on LN B cells because of the paucity of J11d$^-$ cells within the nodes (28).

Antigen-induced Ts Prevent Specific Th Priming. Because the proliferative response of primary B cells was unaffected by Ts cells, but maturation to Ig secretion was blocked, it seemed likely that suppression might act via Th. As a test of this notion, we titrated Lyt-1$^+$,2$^-$ T cells from mice pretreated as in the above experiments at limiting dilution into irradiated BALB/c recipients, along with an excess of normal adult splenic B cells (33). This allowed comparison of the relative number of PR8-specific Th cells generated by priming in the presence or absence of Ts cells.

The frequency of HA-specific Th in unprimed mice is quite low, resulting in less than one responding culture per 10^6 injected T cells (Table II, *top*). In control mice, which were primed after the transfer of Lyt-2$^+$ cells from normal mice, the frequency of HA-specific Th increases about 10-fold, yielding values of 4–5 responding cultures per 10^6 injected cells. In contrast, among the mice that were primed after the transfer of Ts populations, the increase in Th frequency usually seen after priming is completely ablated. These results are consistent with the idea that Ts cells prevent the proliferation and activation of the Th cells required for maturation of primary B cells.

Ts Activity Is Restricted by Loci Linked to the Igh-C Complex. Several laboratories have reported (34–38) that Ts activity is restricted by genetic elements linked to the Igh-C complex. We therefore tested the ability of neonatally induced Ts cells to act across differences on chromosome 12. In preliminary experiments, BALB/c Ts cells were adoptively transferred to normal C.B20 mice. In these experiments, the HA-specific responses of the C.B20 mice were unaffected, although the Ts compromised BALB/c responses in the same experiment (Table III). In reciprocal experiments, C.B20 Ts suppressed C.B20 HA-specific responses, but not BALB/c responses (Table III).

Since the Ts cells appeared to operate by preventing Th cell activation and proliferation in BALB/c individuals (Table II), we expected that HA-specific Th cell priming might proceed normally in the C.B20 individuals receiving BALB/c Ts cells. We tested this parameter in the C.B20 individuals by limiting-dilution Th analysis. The results (Table IV) show that this is indeed the case. In

TABLE II

Neonatally Induced Ts Inhibit Priming of HA-specific Th

Pretreatment of Th donor[‡]	Limiting dilution analysis of HA-specific Th[*]			
	B cells per recipient ($\times 10^{-6}$)	Th per recipient ($\times 10^{-6}$)	Total HA-specific foci per total recipients	HA-specific foci per 10^6 injected Th[§]
None	40	—	0/3	—
	—	1.0	0/2	<0.5
	40	1.0	1/3	0.3
	40	0.5	1/2	0.5
	40	0.1	0/2	0
BALB/c LN T cells	—	1.0	0/3	<0.3
	40	1.0	23/5	4.6
	40	0.5	12/6	4.0
	40	0.1	3/6	5.0
LN T cells from neonatally immunized BALB/c	—	1.0	0/3	<0.3
	40	1.0	2/5	0.4
	40	0.5	1/6	0.2
	40	0.1	0/6	0

The effect of neonatally induced Ts on HA-specific Th priming in vivo was established by limiting-dilution analysis of Th activity following antigen challenge in the presence of Ts (33). Controls were either untreated (top group), or received T cells from normal, rather than neonatally primed individuals (middle group).

[*] 8 d after Ts transfer and PR8 challenge, Lyt-1^{+},2[−] LN T cells were prepared from Ts recipients and control groups, as described in Methods. These cells were transferred to irradiated (1,500 rad) BALB/c recipients, along with an excess of normal splenic B cells. 18 h later, the Th recipient spleens were removed and diced into 1 mm³ fragments. The fragments were then stimulated individually in vitro, as described in Methods. Supernatants were assayed by RIA for HA-specific antibody production.

[‡] Before use as Th donors in limiting-dilution cultures, BALB/c mice received 5 × 10⁶ Lyt-1^{−},2[+] LN T cells, derived from either normal BALB/c mice or BALB/c mice that had been immunized with PR8 at 3–6 d, and immunized weekly thereafter, to generate Ts. Except for the untreated controls, Th donor mice were immunized with 1,000 HAU of PR8 1 d after these transfers.

[§] Values shown were calculated by dividing the total cells injected at a given Th dose by the total HA-specific foci observed. This value represents a relative estimate of the Th frequency in a given donor population. In addition, the relative constancy of this value over the dose range shown demonstrates a linear relationship between responding cultures and number of input Th, indicating that a single cell type is being titrated.

reciprocal experiments, we have established that Ts generated by neonatally immunizing C.B20 individuals also fail to affect BALB/c HA-specific responses.

Ts Activity Requires Syngeny Between Ts and Th Cells at Loci on Chromosome 12. The foregoing experiments suggested that the primary effect of induced Ts cells is at the level of Th cell activation, and that this activity requires syngeny at loci on chromosome 12. In order to establish which cell types must share alleles at these loci, we performed an adoptive reconstitution experiment. Lethally irradiated (900 rad) BALB/c mice were reconstituted with different combinations of primed Ts cells, Th cell precursors, and primary splenic B cells derived from either C.B20 or BALB/c individuals. 1 d later, recipients were challenged with PR8 and their HA-specific serum antibody titres were determined. Table V shows the results of such an experiment. Mice that received no

TABLE III

Ts Activity Is Restricted by Genetic Elements Linked to the Allotype Locus

T cell donor strain	Donor treatment	T cells trans-ferred	Recipient strain	HA-specific serum anti-body after PR8 chal-lenge
				(µg/ml)
—	—	none	BALB/c	100; 87
—	—	none	C.B20	100; 87
BALB/c	none	5 × 10⁶	BALB/c	187; 87; 100; 100
BALB/c	none	5 × 10⁶	C.B20	87; 100; 180; 87
BALB/c	chronic PR8	5 × 10⁶	BALB/c	13; 13; 25; 25
BALB/c	chronic PR8	5 × 10⁶	C.B20	87; 87; 87; 100
C.B20	none	5 × 10⁶	BALB/c	200; 200; 200
C.B20	none	5 × 10⁶	C.B20	200; 200; 200
C.B20	chronic PR8	5 × 10⁶	BALB/c	200; 200; 200
C.B20	chronic PR8	5 × 10⁶	C.B20	25; 50; 50

T cells were prepared from either normal BALB/c donors, or BALB/c mice that had been immunized weekly with 1,000 HAU of PR8 beginning at 3–6 d of age. To perform the reciprocal transfer experiment, T cells were also prepared from C.B20 mice that were either normal, or had been immunized weekly. 2 d after the transfer of Ts cells, recipient mice were immunized with 1,000 HAU of PR8. 8 d following the immunization, the recipient mice were bled, and their serum assayed for HA-specific antibody by HI. The figures given are values obtained from individual mice.

cells (group I), Ts cells alone (group II), primary B cells alone (group III), or Th cells alone (group IV) failed to respond to PR8. Groups that received both B and Th cells responded with HA-specific antibody (group V). The donor origin of the Th and B cells, however, had little effect. In cases where C.B20 Th cells were cotransferred with BALB/c B cells, HA-specific titres were similar to those measured when the transferred Th and B cells were syngeneic. Taken together, the results of these controls (groups I–V) show that the responses elicited were derived from the transferred B and Th cells, and that the interactions of Th and primary B cells are largely unaffected by donor genotype at chromosome 12. Control group VI indicates that the Ts cell populations alone did not support humoral responses in the adoptive recipients.

Experimental transfers are shown in Table V, group VII. When BALB/c Ts cells were transferred with BALB/c Th and B cell populations, the HA-specific serum responses were reduced, indicating that the Ts populations could act effectively in the adoptive transfer system. Ts were equally effective when the Ts and B cell donors were mismatched at chromosome 12. In contrast, when the transferred Ts and Th cells were disparate at chromosome 12, the Ts failed to prevent HA-specific serum antibody responses. Thus, Ts activity requires syngeny between the Th precursor populations and the environment in which Ts priming occurred, whereas the genotype of the primary B cells at loci on chromosome 12 does not influence Ts effector activity.

In order to further substantiate this conclusion and rule out the possibility that a rejection phenomenon was selectively acting upon the transferred Ts cell

TABLE IV

Neonatally Induced Ts Do Not Inhibit Priming of HA-specific Th from Allotypically Dissimilar Mice

Pretreatment of Th cell donor	Th donor strain	Limiting-dilution analysis of HA-specific Th cells			
		B cells per recipient ($\times 10^{-6}$)	Th cells per recipient ($\times 10^{-6}$)	Total HA-specific foci per total recipients	HA-specific foci per 10^6 injected Th cells
None	—	40	0	0/2	—
	BALB/c	0	1.0	0/2	—
	BALB/c	40	1.0	1/2	0.5
	BALB/c	40	0.5	0/2	—
	C.B20	0	1.0	0/2	—
	C.B20	40	1.0	2/4	0.5
	C.B20	40	0.5	1/3	0.7
BALB/c LN T cells	BALB/c	0	1.0	0/2	—
	BALB/c	40	1.0	17/3	5.6
	BALB/c	40	0.5	16/6	5.3
	C.B20	0	1.0	0/2	—
	C.B20	40	1.0	20/3	6.7
	C.B20	40	0.5	9/3	6.0
	C.B20	40	0.1	2/3	6.7
LN T cells from neo-natally immunized BALB/c	.BALB/c	0	1.0	0/2	—
	BALB/c	40	1.0	1/3	0.3
	BALB/c	40	0.5	1/4	0.5
	C.B20	0	1.0	0/2	—
	C.B20	40	1.0	15/2	7.5
	C.B20	40	0.5	7/2	7.0
	C.B20	40	0.25	3/2	6.0

The effect of neonatally induced BALB/c Ts cells on HA-specific Th priming in C.B20 individuals. The experimental approach was identical to that described in Table II, except that C.B20 mice were used as recipients of Ts cells, and subsequently as Th cell donors in limiting-dilution analyses.

populations, we performed a similar experiment in which fully reciprocal groups of Ts and Th populations were cotransferred, and BALB/c × C.B20 F_1 mice were used both as B cell donors and as recipients.

In this experiment, the controls again indicate that the HA-specific serum response may be reconstituted with the appropriate transfer of Th cells and B cells (Table V, groups I-VI). Further, this experiment confirms the observation that the inducing environment of the Th and Ts cells must be matched with respect to a locus or loci chromosome 12 in order for effective suppression to occur (group VII).

Discussion

Our previous experiments had shown that oligoclonal adult profiles are induced by neonatal immunization, and are maintained in part by Ts cells. Here,

TABLE V

Ts Activity Requires Syngeny With Th Cells, Not B Cells

Group	BALB/c Ts cells transferred*	Th cell source[‡]	Primary B cell source[§]	Mean HA-specific antibody after PR8 challenge[l]
				(µg/ml)
I	—	—	—	<13
II	5 × 10⁶	—	—	<13
III	—	BALB/c	—	<13
	—	C.B20	—	<13
IV	—	—	BALB/c	<13
	—	—	C.B20	<13
V	—	BALB/c	BALB/c	125
	—	BALB/c	C.B20	125
	—	C.B20	BALB/c	125
	—	C.B20	C.B20	100
VI	5 × 10⁶	—	BALB/c	<13
	5 × 10⁶	—	C.B20	<13
VII	5 × 10⁶	BALB/c	BALB/c	25
	5 × 10⁶	C.B20	BALB/c	110
	5 × 10⁶	BALB/c	C.B20	30
	5 × 10⁶	C.B20	C.B20	125

Irradiated (900 rad) recipients were given mixtures of primed Ts cells, Th cell precursors, and normal splenic B cells derived from either C.B20 or BALB/c mice. 24 h later, the recipients were immunized with 1,000 HAU of PR8. Serum HA-specific antibody was measured 8 d after immunization.

* Lyt-1⁻,2⁺ LN T cells were prepared as described in Methods from BALB/c mice that had been immunized with PR8 at 3–6 d of age, and weekly thereafter until 7 wk of age.

‡ Lyt-1⁺,2⁻ Ln T cells were prepared from normal mice of the strains shown by treatment with a mixture of J11d, anti-I-A^d, anti-Lyt-2 plus C'.

§ Splenic B cells from the strains shown were prepared as described in Methods by treatment with anti-Thy-1.2 plus C'.

l HA-specific antibody, 8 d after challenge, was determined by HI. Values given are the means of four mice in each group.

we probe the events and cellular interactions involved in the action of these Ts cells, and show that (*a*) the early events in primary B cell triggering are not blocked by Ts, but maturation to antibody secretion and memory cell generation are prevented; (*b*) the surface marker defined by J11d is associated with resting B cells, since its loss is associated with the early steps of B cell activation, and it reappears once an active B cell response has subsided; (*c*) Ts generated by neonatal immunization prevent Th cell proliferation, and probably act by preventing Th signals necessary for the maturation of primary B cells to secretory and memory cells; and (*d*) Ts interact with Th cells in a fashion that is restricted by loci on murine chromosome 12.

Several questions are raised by our finding that primary B cells proliferate in response to antigen but fail to secrete antibody in the presence of Ts cells. Since

TABLE VI

Reciprocal Transfers of C.B20 and BALB/c Th and Ts Populations in F_1 Recipients

Group	Source of Ts cells*	Ts cells transferred	Th cell source[†]	Primary B cell source[§]	Mean HA-specific antibody after PR8 challenge
					(μg/ml)
I	—	—	—	—	<13
II	BALB/c	5 × 10⁶	—	—	<13
	C.B.20	5 × 10⁶	—	—	<13
III	—	—	BALB/c	—	<13
	—	—	C.B20	—	<13
IV	—	—	—	BALB/c × C.B20 F_1	<13
V	—	—	BALB/c	BALB/c × C.B20 F_1	125
	—	—	C.B20	BALB/c × C.B20 F_1	100
VI	BALB/c	5 × 10⁶	—	BALB/c × C.B20 F_1	<13
	C.B20	5 × 10⁶	—	BALB/c × C.B20 F_1	<13
VII	BALB/c	5 × 10⁶	BALB/c	BALB/c × C.B20 F_1	<13
	BALB/c	5 × 10⁶	C.B20	BALB/c × C.B20 F_1	150
	C.B20	5 × 10⁶	BALB/c	BALB/c × C.B20 F_1	150
	C.B20	5 × 10⁶	C.B20	BALB/c × C.B20 F_1	13

Irradiated (900 rad) BALB/c × C.B20 F_1 recipients were given a mixture of primed Lyt-2⁺ cells, Lyt-1⁺ cell precursors, and normal F_1 B cells. Immunization protocol and serum response measurements are described in Table V.
* Lyt-1⁻,2⁺ cells from BALB/c and C.B20 mice were prepared as described in Table V.
[†] Lyt-1⁺,2⁻ LN T cells were prepared from BALB/c and C.B20 mice, as described in Materials and Methods.
[§] Splenic B cells from BALB/c × C.B20 F_1 donors were prepared as described in Materials and Methods.
[∥] HA-specific antibody was determined as described in Materials and Methods. Experimental values given are the means of six mice in each group.

the direct action of Ts cells appears to be at the level of Th cell activation, the initial proliferative response of B cells to influenza HA may be a relatively T-independent process.

Although the response to influenza HA is generally thought to be a T-dependent process, previous findings from both this as well as other laboratories are consistent with the possibility that certain aspects of this response are T-independent. Virilizier, et al. (39) performed studies showing that thymectomized mice, when primed with PR8, fail to mount a serum antibody response. However, when these mice were chronically primed and then provided a source of T cell help, the resulting response resembled a secondary response in terms of isotype, specificity, and kinetics. These authors concluded that the initial priming events for HA-specific B cells were T-independent. Second, in studies with *nu/nu* BALB/c mice, we have established that, although serum antibody titres are not elevated upon priming, the frequency of HA-reactive B cells, measured in

limiting-dilution cultures 2 wk after priming, is similar to that observed in normal individuals. These findings are consistent with the observations presented herein, and support the idea that early events in the activation and proliferation of HA-reactive B cells are T-independent.

Alternatively, proliferative responses may simply require helper signals that are constitutively present and distinct from those necessary for differentiation to antibody secretion and memory cell formation. Several recent studies of soluble Th factors suggest distinct mediators for the induction of B cell proliferation vs. maturation and secretion (37, 41), lending support to this possibility. If this is the case, then Ts cells might selectively effect either different signals generated by the same Th cell, or separate subpopulations of Th cells, only one of which induces B cell maturation and is sensitive to Ts activity. The results of previous studies (40, 41) using cloned Th cell lines, as well as those of others (42–46), suggest that Th subpopulations with distinct functional activities exist, and would be consistent with this possibility. However, the effects of Ts cells have not yet been assessed in these systems.

This model of Ts action might help explain why, in this and other experimental systems, secondary B cells seem resistant to suppression (23, 47, 48). Secondary B cells may have less stringent Th requirements, either qualitatively or quantitatively, than do primary B cells. This idea has been supported by results from several studies, both with HA-specific B cells (23, and Gerhard and Cancro, unpublished observations) as well as in hapten-carrier systems (49). If this is indeed the case, then Ts cells may be unable to effectively block either the Th population, or the signals required for memory B cell activation.

These studies also confirm and extend previous work with the B cell surface marker, Jl1d. Although the original description of this marker (28), as well as our previous studies (23), had indicated that antigen-primed B cells are predominantly Jl1d$^-$, the findings presented herein extend this observation in two ways. First, although the Jl1d marker is lost shortly after challenge, it is regained once the active humoral response subsides. Second, the loss of this marker is an early event in B cell stimulation that occurs during initial antigen-driven proliferation, and before differentiation to antibody secretion or generation of memory populations.

The requirement for syngeny between the Ts effector and Th precursor populations at loci on chromosome 12 raises several questions of the interactions and recognition elements involved in Ts activity, as it is unclear how these Igh-C-linked loci exert their effects. It is possible that, during their antigen-driven induction, the Ts receptor repertoire is shaped by interactions that are, either directly or indirectly, dependent on the responding B cell receptor repertoire. This would result in a restriction of Ts effector function that would "map" with the Igh-V haplotype of the environment in which Ts are generated. We must be cautious regarding speculation of the actual loci involved in this apparent restriction of Ts–Th interaction. Although C.B20 is frequently used to infer the participation of Ig constant or variable region loci, we must bear in mind that a considerable portion of chromosome 12 in the C.B20 is derived from the C57BL parent. In fact, all of the presently typed loci are of C57BL origin, and these span a map distance of >20 centimorgans. Thus, it is equally possible that loci

distinct from those that encode Ig may be responsible for our observations. For example, cell surface molecules that nonspecifically enhance or stabilize cellular interactions might be encoded in this region. Several polymorphic cell surface molecules that are present on large subsets of T cells have been mapped to the right of the Igh-C complex (16), and might have such functional activity.

Clearly, these possibilities are neither mutually exclusive nor exhaustive, and additional experiments will be required to determine the exact nature of this restriction, as well as to more precisely map the loci responsible.

Summary

The cellular mechanism and genetic restriction of neonatally induced HA-specific suppressor T (Ts) cells have been examined. The in vivo effect of these Ts cells on antibody production, primary B cell proliferation, B cell surface marker changes, and helper T (Th) cell priming during primary responses to HA have been determined. The results indicate that, although antigen-induced B cell proliferative responses and surface marker changes occur in the presence of Ts cells, differentiation to Ig secretion, and long-lived memory B cell production are prevented. Further, antigen-specific Th cell priming is completely ablated by Ts cells, suggesting that Ts act by preventing the delivery of Th signals required for both the later stages of primary B cell maturation, and the formation of memory B cell populations. Finally, in vivo cell mixing experiments using congenic mice indicate that this Ts–Th interaction is restricted by loci on mouse chromosome 12.

References

1. Silverstein, A. M., J. W. Uhr, K. L. Kraner, and R. J. Lukes. 1963. Fetal response to antigenic stimulus II. Antibody production by the fetal lamb. *J. Exp. Med.* 117:799.
2. Rowlands, D. T., D. Blakeslee, and E. Angala. 1974. Acquired immunity in opossum (*Didelphis virginiana*) embryos. *J. Immunol.* 112:2148.
3. Klinman, N. R., and J. L. Press. 1975. The characterization of the B-cell repertoire specific for 2,4-dinitrophenyl and 2,4,6-trinitrophenyl determinants in neonatal BALB/c mice. *J. Exp. Med.* 141:1133.
4. Sigal, N. H., P. J. Press, and N. R. Klinman. 1976. Late acquisition of a germline specificity. *Nature (Lond.)* 259:51.
5. Sigal, N. H., A. R. Pickard, E. S. Metcalf, P. J. Gearhart, and N. R. Klinman. 1977. Expression of phosphorylcholine-specific B cells during murine development. *J. Exp. Med.* 146:933.
6. Fernandez, C., and G. Möller. 1978. Immunological unresponsiveness to native dextran B512 in young animals of dextran high responder strains is due to lack of Ig receptor expression: evidence for a non-random expression of V-genes. *J. Exp. Med.* 147:645.
7. Bona, C., J. J. Mond, K. E. Stein, S. House, R. Lieberman, and W. E. Paul. 1979. Immune responses to levan. III. The capacity to produce anti-insulin antibodies and cross-reactive idiotype appears late in ontogeny. *J. Immunol.* 123:1484.

8. Nutt, N. B., A. N. Wiesel, and A. Nisonoff. 1979. Neonatal expression of a cross-reactive idiotype associated with anti-phenylarsonate antibodies in strain A mice. *Eur. J. Immunol.* 9:864.

9. Huang, H. V., and W. J. Dreyer. 1978. Bursectomy *in ovo* blocks the generation of immunoglobulin diversity. *J. Immunol.* 121:1738.

10. DuPasquier, L., B. Blomberg, and C. C. A. Bernard. 1979. Ontogeny of immunity in amphibians: changes in antibody repertoire and appearance of adult major histocompatibility antigens in Xenopus. *Eur. J. Immunol.* 9:900.

11. Cancro, M. P., D. E. Wylie, W. Gerhard, and N. R. Klinman. 1979. Patterned acquisition of the antibody repertoire: diversity of the hemagglutinin specific B-cell repertoire in neonatal BALB/c mice. *Proc. Natl. Acad. Sci. USA.* 76:6577.

12. Cancro, M. P., and N. R. Klinman. 1981. B-cell repertoire ontogeny: heritable but dissimilar development of parental and F_1 repertoires. *J. Immunol.* 126:1160.

13. Cancro, M. P. 1982. Multiple loci mediate B-cell repertoire ontogeny: analysis of the hemagglutinin-specific repertoires in neonatal C.B20 and (C.B20 × B10.D2)F_1 mice. *J. Immunol.* 128:1307.

14. Sigal, N. H. 1982. Regulation of azophenylarsonate-specific repertoire expression. I. Frequency of cross-reactive idiotype-positive B cells in A/J and BALB/c mice. *J. Exp. Med.* 156:1352.

15. Denis, K. A., and N. R. Klinman. 1983. Genetic and temporal control of neonatal antibody expression. *J. Exp. Med.* 157:1170.

16. Augustin, A., and J. Cosenza. 1976. Expression of new idiotypes following neonatal idiotypic suppression of a dominant clone. *Eur. J. Immunol.* 6:497.

17. Acolla, R. S., P. J. Gearhart, N. H. Sigal, M. P. Cancro, and N. R. Klinman. 1977. Idiotype-specific neonatal suppression of phosphorylcholine-responsive B-cells. *Eur. J. Immunol.* 7:876.

18. Eig, B. M., S.-T. Ju, and A. Nisonoff. 1977. Complete inhibition of the expression of an idiotype by a mechanism of B-cell dominance. *J. Exp. Med.* 146:1574.

19. Hirneaux, J., C. Bona, and P. J. Baker. 1981. Neonatal treatment with low doses of anti-idiotypic antibody leads to the expression of a silent clone. *J. Exp. Med.* 153:1004.

20. Etlinger, H. M., and C. H. Heusser. 1982. Expression of a distinct B-cell clonotype profile after antigen-induced unresponsiveness. *Eur. J. Immunol.* 12:533.

21. Etlinger, H. M., and C. H. Heusser. 1983. Alteration of immunologic function through early ontogenetic experiences. Selective inactivation of T15-positive B cells in neonatal mice is related to receptor avidity and is independent of thymus function. *Eur. J. Immunol.* 13:180.

22. Thompson, M. A., and M. P. Cancro. 1982. Dynamics of B-cell repertoire formation: normal patterns of clonal turnover are altered by ligand interaction. *J. Immunol.* 129:2372.

23. Thompson, M. A., S. Raychaudhuri, and M. P. Cancro. 1983. Restricted adult clonal profiles induced by neonatal immunization: influence of suppressor T cells. *J. Exp. Med.* 158:112.

24. Cancro, M. P., M. A. Thompson, and D. M. Hilbert. 1983. Developmental aspects of B-cell repertoire phenotype. *Surv. Immunol. Res.* 2:62.

25. Gerhard, W., T. J. Bracialle, and N. R. Klinman. 1975. The analysis of the monoclonal immune response to influenza virus I. Production of monoclonal anti-viral antibodies *in vitro. Eur. J. Immunol.* 5:720.

26. Cancro, M. P., W. Gerhard, and N. R. Klinman. 1978. The diversity of the influenza-specific primary B-cell repertoire in BALB/c mice. *J. Exp. Med.* 147:776.

27. Gerhard, W. 1976. The analysis of the monoclonal immune response to influenza virus. II. The antigenicity of the viral hemagglutinin. *J. Exp. Med.* 144:985.

28. Bruce, J., F. W. Symington, T. J. McKearn, and J. Sprent. 1981. A monoclonal antibody discriminating between subsets of T and B cells. *J. Immunol.* 127:2496.

29. Kappler, J. W., B. Skidmore, J. White, and P. Marrack. 1981. Antigen inducible, H-2-restricted, interleukin 2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition. *J. Exp. Med.* 153:1198.

30. Sarmiento, M., A. L. Glasebrook, and F. W. Fitch. 1980. IgG or IgM monoclonal antibodies reactive with different determinants on the molecular complex bearing Lyt-2 antigen block T-cell mediated cytolysis in the absence of complement. *J. Immunol.* 125:2665.

31. Mark, C., F. Figueroa, Z. Nagg, and J. Klein. 1982. Cytotoxic monoclonal antibody for the Lyt-1.2 antigen. *Immunogenetics.* 16:95.

32. Klinman, N. R., A. Pickard, N. H. Sigal, P. J. Gearhart, E. S. Metcalf, and S. K. Pierce. 1976. Assessing B-cell diversification by antigen receptor and precursor cell analysis. *Ann. Immunol. (Paris).* 127c:489.

33. Pierce, S. K., Cancro, M. P., and Klinman, N. R. 1978. The helper function of individual antigen-specific T-lymphocytes in enabling the expression of multiple antibody isotypes. *J. Exp. Med.* 148:759.

34. Owen, F. L., R. Riblet, and P. D. Gottlieb. 1982. T suppressor cells activated by anti-TSU^d serum are Igh-V restricted. *Eur. J. Immunol.* 12:94.

35. Owen, F. L. 1983. T cell alloantigens encoded by the IgT-C region of chromosome 12 in the mouse. *Adv. Immunol.* 34:1.

36. Herzenberg, L. A., T. Tokuhisa, and L. A. Herzenberg. 1980. Carrier priming leads to hapten-specific suppression. *Nature (Lond.).* 285:664.

37. Sy, M.-S., M. H. Dietz, A. Nisonoff, R. N. Germain, B. Benacerraf, and M. I. Greene. 1980. Antigen- and receptor-driven regulatory mechanisms. V. The failure of idiotype-coupled spleen cells to induce unresponsiveness in animals lacking the appropriate Vh genes is caused by the lack of idiotype-matched targets. *J. Exp. Med.* 152:1226.

38. Weinberger, J. Z., B. Benacerraf, and M. E. Dorf. 1979. Hapten-specific T cell responses to 4-hydroxy-3-nitrophenylacetyl. III. Interaction of effector suppressor T cells is restricted by H-2 and Igh-V genes. *J. Exp. Med.* 151:1413.

39. Virelizier, J. L., R. Postlethwaite, G. C. Schild, and A. C. Allison. 1974. Antibody responses to antigenic determinants of influenza virus hemagglutinin. I. Thymus dependence of antibody formation and thymus independence of immunological memory. *J. Exp. Med.* 140:1559.

40. Petterson, S., G. Pabor, A. Bandeira, and A. Coutinho. 1983. Distinct helper activities control growth or maturation of B-lymphocytes. *Eur. J. Immunol.* 13:249.

41. Julius, M. H., H. von Boehmer, and C. L. Sidman. 1982. Dissociation of two signals required for activation of resting B cells. *Proc. Natl. Acad. Sci. USA.* 79:1989.

42. Zauderer, M., H. Campbell, D. R. Johnson, and M. Seman. 1984. Helper function of antigen-induced specific and autoreactive T-cell colonies. *J. Mol. Cell. Immunol.* 1:65.

43. Imperiale, M. J., D. A. Faherty, J. F. Sproviero, and M. Zauderer. 1982. Functionally distinct helper T cells enriched under different culture conditions cooperate with different B cells. *J. Immunol.* 129:1943.

44. Marrack (Hunter), P. C., and J. W. Kappler. 1975. Antigen-specific and nonspecific mediators of T cell/B cell cooperation. I. Evidence for their production by different T cells. *J. Immunol.* 114:1116.

45. Keller, D. M., J. E. Swierkosz, P. Marrack, and J. W. Kappler. 1980. Two types of functionally distinct, synergizing helper T cells. *J. Immunol.* 124:1350.

46. Parker, D. C. 1982. Separable helper factors support B cell proliferation and maturation to Ig secretion. *J. Immunol.* 129:469.
47. Owen, F. L., and A. Nisonoff. 1978. Effect of idiotype-specific suppressor T cells on primary and secondary responses. *J. Exp. Med.* 146:182.
48. Pierce, S. K., and N. R. Klinman. 1977. Antibody-specific immunoregulation. *J. Exp. Med.* 146:509.
49. Yefenof, E., E. C. Snow, J. W. Uhr, and E. S. Vitetta. 1984. Activation of antigen-specific B-cells isolated from unprimed and TNP-primed mice. *J. Cell. Biochem.* (Suppl. 8A):213. (Abstr.)