FEEDBACK SUPPRESSION OF THE IMMUNE RESPONSE IN VITRO

II. IgV_H-restricted Antibody-dependent Suppression*

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In the preceding paper of this series (1), it was demonstrated that surface immunoglobulin (sIg)[1]- bearing, Lyt $1^-2^-3^-$ (B) lymphocytes, once triggered by antigen (sheep erythrocytes [SRBC]) in the presence of T cells, could exert potent specific suppressive effects on primary in vitro responses. A variety of B cell- and/or Ig-associated mechanisms might account for this effect. Recent studies by others have shown that auto-anti-idiotypic antibodies formed early during immune responses can suppress humoral immunity at the plaque-forming-cell (PFC) level (2, 3). Earlier studies have revealed that either anti-antigen antibodies, through interference with cellular recognition of antigenic determinants (classical antibody feedback) (4, 5), or heterologous anti-idiotypic antibodies, via receptor blockade (6) or suppressor T lymphocyte (T_s) induction (6–8), can reduce humoral immune responses. Finally, activated B cells have been suggested to cause the induction of T_s via idiotypic recognition of sIg in vivo (9) or in vitro.[2] Our series of experiments was undertaken to investigate if these or other mechanisms were involved in the B cell-determined suppression we have been studying.

This report documents that B cell-mediated suppression in vitro can occur via the production of a soluble product that bears Ig determinants and that binds to the eliciting antigen, SRBC. The activity of this antibody in suppressing anti-SRBC PFC responses is restricted in large measure to assay cultures containing B cells sharing V_H genes with the B cells producing the suppressive antibody. No suppression of anti-hapten (trinitrophenyl [TNP]) responses to derivatized SRBC (TNP-SRBC) is caused by this soluble product. These data are compatible with the theory that suppression is mediated by anti-antigen antibody, either (*a*) via blockade of different SRBC epitopes recognized by a limited set of B cell clones in each mouse strain, (*b*) via triggering of anti-idiotypic antibody or T_s responses restricted in activity to cultures

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[1] *Abbreviations used in this paper:* B6, C57BL/6; BDF, (C57BL/6 × DBA/2)F_1; $B_{BC\text{-}8}$, B_{B6}, BC-8 and B6 B cells; Co-SUP, supernate from control-educated cells; HRBC, horse erythrocyte(s); MEM, Eagle's minimum essential medium; NRG, normal rabbit globulin(s); PFC, plaque-forming cells; RAMIg, rabbit anti-mouse Ig antibody; sIg, surface Ig; sIg^+, sIg positive; sIg^-, sIg negative; SRBC, sheep erythrocyte(s); SRBC-SUP, supernate from SRBC-educated cells; TNP, trinitrophenyl; TNP-SRBC, heavily TNP-coupled SRBC; $T_{BC\text{-}8}$, T_{B6}, BC-8 and B6 T cells; T_H, T-helper cells; T_s, suppressor T lymphocyte(s).

[2] Horowitz, M., F. W. Shen, H. Cantor, and R. K. Gershon. Activated B cells stimulate antigen-specific Ly 1^-2^+ T suppression in vitro. Manuscript submitted for publication.

containing B cells sharing V_H structures with the original antibody, or (*c*) via antibody interference with idiotype-specific T-helper cell function.

Materials and Methods

Mice. (C57BL/6 × DBA/2)F_1 H-$2^{b/d}$, Ig-$1^{b/c}$) (BDF) mice, males or females, 2–6 mo old were used in most experiments and purchased from The Jackson Laboratory, Bar Harbor, Maine, or from the Health Research Inc., West Seneca, N. Y. C57BL/6 (H-2^b, Ig-1^b) (B6), B10.BR (H-2^k, Ig-1^b), DBA/1 (H-2^q, Ig-1^c), SJL (H-2^s, Ig-1^b) and A/J (H-2^a, Ig-1^c) mice were also obtained from The Jackson Laboratory. BALB/c (H-2^d, Ig-1^a) mice were purchased from the Charles River Breeding Laboratories, Wilmington, Mass. BAB14 (H-2^d, Ig-1^b) and CB-20 (H-2^d, Ig-1^b) mice were the gift of Dr. Martin E. Dorf, Harvard Medical School, Boston, Mass. BC-8 (H-2^b, Ig-1^a) mice were derived from breeding pairs generously supplied by Dr. Michael Potter, National Institutes of Health, Bethesda, Md. Either males or females were used at 2–6 mo of age. Mice were maintained in our animal facilities on standard laboratory chow and acidified, chlorinated water.

Antigens. The same SRBC and horse erythrocytes (HRBC) were used as in the previous report (1). Heavily TNP-coupled SRBC (TNP-SRBC) were prepared according to the method of Rittenberg and Pratt (10).

Culture Conditions for Cell Education and Test Cultures. These have been described (1). Briefly, the protocol developed by Eardley and Gershon (11) was used for the generation of SRBC-educated cells. Spleen cells (10^7/ml) were cultured for 4 d in the presence of SRBC (2 × 10^6/ml) under Mishell-Dutton conditions (12). Control-educated cells were cultured without SRBC. The cells were then titrated into test cultures set up with 7.5 × 10^6 viable spleen cells in 1 ml Mishell-Dutton medium. Cultures were stimulated with 2 × 10^6 SRBC, 3 × 10^6 HRBC, or 10^6 TNP-SRBC and assayed by a slide modification of the Jerne plaque assay on day 4 or 5 of culture, using SRBC, HRBC, or TNP-coupled HRBC (10).

Generation of SRBC Suppressor Cell Supernate (SRBC-SUP) from In Vitro Educated Cells. Spleen cells were educated with SRBC for 4 d as described above. At day 4, the cells were harvested, centrifuged, and then incubated for 4 min at 37°C in Tris-NH_4Cl buffer (0.83% NH_4Cl, nine volumes, buffered with 2% Tris-HCl, one volume; final pH: 7.0), to remove all erythrocytes. Cells were then washed twice in Eagle's minimum essential medium (MEM) which was buffered with 10 mM Hepes and which contained 5% fetal calf serum (1), and were filtered through a nylon mesh. At that stage, 5–10% of the initially harvested cells were lost. Viability of the remaining cells was 90–98% measured by trypan blue exclusion. The direct suppressive activity of SRBC-educated cells was not changed by this treatment. The washed cells were suspended at a concentration of 10^7/ml in complete Mishell-Dutton medium (12) and cultured for 24 (from 18 to 25) h in 60- × 15-mm tissue culture dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.; Falcon 3002) (2.5–3 ml/dish) on a rocking platform in a 10% CO_2, 83% N_2, and 7% O_2 atmosphere as used for cell education (1). After 24 h, the cells were resuspended, and the culture suspension harvested and centrifuged for 10 min at 1,500 rpm. The culture supernate was recovered and passed through a 0.45-μm-pore-size Millex filter (Millipore Corp., Bedford, Mass.) to remove cell fragments. Such supernate from SRBC-educated cells is termed SRBC-SUP. Control supernatant (Co-SUP) was obtained from control-educated cells that had been cultured for 4 d without SRBC.

These supernates were tested immediately or stored in aliquots at −20°C for up to 4 wks or at −80°C for up to 9 mo. Freezing and thawing once or twice did not alter the activity. The concentration of the undiluted supernate is defined as 10^7 cell equivalents/ml according to the initial cell concentration. We found that using 2 × 10^7 cells/ml for the 24-h culture period did not increase the absolute activity, and that a 48-h supernate had the same or less activity than a 24-h supernate. Addition of SRBC during the 24-h culture resulted in decreased activity with as few as 3 × 10^4 SRBC/ml.

Cell Separations. Normal spleen or educated spleen cells were separated into sIg-negative (sIg^-) and sIg-positive (sIg^+) cells by incubation on polystyrene Petri dishes (Falcon Labware, Div. of Becton, Dickinson & Co.) coated with rabbit anti-mouse Ig antibody (RAMIg) acording to the method of Mage et al. (13). The minor modifications and the reagents used for the preparation of the RAMIg and control separations which have been done have all been

described (1). The characterization of the separated SRBC-educated cell fractions was done by measuring the relative frequencies of anti-SRBC PFC, which was a more reliable method in our hands than immunofluorescence analysis of sIg^+ cells. These methods have been verified not to separate cells via Fc receptor binding, and to yield B (sIg^+) cell fractions devoid of Lyt^+ cells with detectable suppressive function (1).

Absorption of SRBC-SUP with SRBC or HRBC. One volume of factor was absorbed twice with one volume of packed SRBC or HRBC, each time for 45 min at 2°C.

Absorption on Anti-Mouse-Ig-coupled Sepharose Columns. A rabbit was immunized repeatedly with normal mouse Ig in complete Freund's adjuvant. The resulting rabbit serum was then passed over an immunoadsorbent prepared by coupling a 40% saturated ammonium sulfate precipitate of normal mouse serum to Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) and eluted as described for RAMIg used for cell separation (1). This anti-mouse Ig antibody and normal rabbit globulin (NRG) were coupled respectively to cyanogen bromide-activated Sepharose 4B. Aliquots of 0.7 ml of SRBC-SUP (10^7 cell equivalents/ml) were passed through 0.4-ml columns of Sepharose in 1-ml plastic syringes during a total time of 45 min at 2°C, yielding the column filtrates. Acid elution was performned with MEM adjusted to pH 2.8 with 1 M glycine-HCl. Such eluates were neutralized immediately with 1 M Tris-HCl, pH 8.0, and then dialyzed for 4½ h against MEM. All fractions were sterilized by filtration before adding them into test cultures. The anti-mouse Ig column reduced the direct and the RAMIg-facilitated, SRBC hemagglutination titers of 0.2 ml B6 anti-SRBC antiserum from 64 to 0 and from 2,048 to 16, respectively. The NRG-Sepharose column did not reduce these titers.

Results

Generation of SRBC-SUP. In the accompanying paper (1), it was shown that whole spleen cells cultured in the presence of SRBC (2×10^6/ml) according to the protocol for the education of feedback T_s developed by Eardley and Gershon (11) exerted optimal specific suppressive activity compared to control-educated cells cultured in absence of SRBC when transferred into test cultures after 4 d of education. The SRBC-educated cells generated IgM and IgG anti-SRBC PFC. The IgG PFC were just developing between days 4 and 5 of culture. In our experiments, essentially all the suppressive activity of the educated spleen cells could be accounted for by the activity of the B cell fraction (1).

The following experiment was done to test whether a soluble suppressor factor was generated by such SRBC-educated cells: on day 4, the SRBC-educated cells were harvested, treated with NH_4Cl buffer and then resuspended in fresh Mishell-Dutton medium (10^7/ml) and further cultured for 24 h. The 24-h supernate was then obtained after centrifugation of the cells and was filtered through a Millipore membrane (Millipore Corp.). Such supernate from SRBC-educated cells (SRBC-SUP) was added into test cultures of 7.5×10^6 spleen cells at the time of culture initiation. In Fig. 1 it is shown that SRBC-SUP exhibited a dose-dependent suppressive effect on the primary anti-SRBC IgM PFC response. This was compared to the effect of Co-SUP obtained from control-educated cells. For comparison, the direct suppressive activity of educated cells is also shown. A 50% suppression of the anti-SRBC IgM PFC response was observed with $1–2 \times 10^5$ cell equivalents of SRBC-SUP per culture; ~80% suppression was observed with 10^6 cell equivalents of SRBC-SUP. A similar suppression was found when $3–4 \times 10^4$ and $2–3 \times 10^5$ SRBC-educated cells, respectively, were added directly into the test cultures. Kinetic studies demonstrated that SRBC-SUP was active if added at culture initiation or after 24 h of culture, but lost suppressive activity if added on day 2 or 3 (data not shown). Co-SUP had some nonspecific enhancing activity. In all the following experiments, the suppression

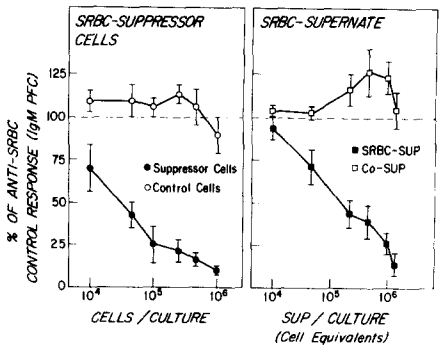

FIG. 1. Suppressive activity of the cell-free supernate from SRBC-educated BDF spleen cells compared to the direct suppressive effect of the educated cells. The educated cells were added into test cultures after they had been cultured for 4 d in presence of 2×10^6 SRBC/ml (suppressor cells) or in the absence of SRBC (control cells); at the same time they were used to generate 24-h supernates (SRBC-SUP and Co-SUP, respectively [Materials and Methods]). The results show the anti-SRBC IgM PFC response in test cultures of 7.5×10^6 spleen cells, expressed as the percent of the control response (8,040 ± 910 IgM PFC) (mean ± SE).

caused by SRBC-SUP will be expressed in comparison to the effect of the same concentration of Co-SUP, as a measure of SRBC-specific suppression.

Antigen Specificity of SRBC-SUP. The suppressive effect of SRBC-SUP was found to be antigen-specific in that it did not suppress the primary IgM PFC response against HRBC in test cultures stimulated with HRBC alone or (as shown in Table I) in cultures stimulated simultaneously with SRBC and HRBC. In this case the anti-SRBC PFC response was still suppressed. Furthermore, SRBC-SUP failed to decrease anti-TNP PFC responses to TNP-SRBC, whereas the anti-SRBC PFC responses of the same cultures were dramatically reduced (Table I). Antigen-binding studies revealed that activity of SRBC-SUP could be completely removed by absorption with SRBC, whereas control absorption with HRBC had no effect (Table II).

Characterization of the Cell Responsible for the SRBC-SUP Activity. In the previously reported experiments, education of sIg^- and sIg^+ cells together, but not of either cell fraction alone, led to the generation of SRBC-suppressor cells (1). The separately educated cell fractions were also tested for generation of suppressive supernate activity. Such activity was only obtained when unseparated cells or mixtures of sIg^- and sIg^+ cells were educated and paralleled the direct suppressive activity of the cells. Also, 5-d educated sIg^- cells did not generate suppressive supernate (data are not shown). When the educated spleen cells were separated into sIg^- and sIg^+ fractions using plastic dishes coated with RAMIg, it was found that SRBC-SUP from sIg^+ cells had 10 times the activity (in terms of cell equivalents needed for suppression) of supernate from unseparated educated cells. Supernate from sIg^- cells had almost no detectable activity (Fig. 2).

SRBC-SUP Activity is Absorbed on Anti-Mouse Ig Sepharose. Immunoabsorption experiments were performed to further characterize the nature of the suppressive material in SRBC-SUP. These were designed to test whether a reagent with anti-mouse Ig reactivity or reagents with reactivity towards H-2-encoded determinants could absorb the suppressive material in SRBC-SUP. Several T suppressor factors including L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} T-suppressor factor are known to carry I-J determinants and some T-helper factors carry I-A determinants (14). The SRBC-SUP

TABLE I
Antigen Specificity of SRBC-SUP

Experiment 1*	IgM PFC/test culture	
	Anti-SRBC	Anti-HRBC
Control response	16,050	2,320
10^6 cell equivalents of Co-SUP	16,200	2,800
10^6 cell equivalents of SRBC-SUP	3,030	2,960
Suppression	81%	−6%
Experiment 2‡	**IgM PFC/test culture**	
	Anti-SRBC	Anti-TNP
(A) Cells		
Control response	6,090	1,040
10^5 control-educated cells	6,845	995
10^5 SRBC-educated cells	725	1,150
Suppression	89%	−15%
(B) Supernates		
Control response	5,920	2,550
10^6 cell equivalents Co-SUP	6,610	2,980
10^6 cell equivalents SRBC-Sup	905	2,680
Suppression	86%	10%

* Cultures stimulated simultaneously with 2×10^6 SRBC and 3×10^6 HRBC.
‡ Cultures stimulated with 10^6 TNP-SRBC.

TABLE II
Specific Absorption of SRBC-SUP by SRBC

	Anti-SRBC IgM PFC/test culture		Suppression*
	Experiment 1	Experiment 2	
			%
Control response	5,040	10,600	
Co-SUP	6,045	12,080	
SRBC-SUP	1,785	1,520	78
SRBC-SUP, absorbed with SRBC	5,985	11,620	3
SRBC-SUP, absorbed with HRBC	2,235	1,540	75

10^6 cell equivalents of absorbed or unabsorbed supernates from educated BDF cells were added into the test cultures.
* Mean values from two experiments.

from SJL (H-2^s), B6 (H-2^b), or B10.BR (H-2^k) educated cells were passed over Sepharose columns coupled with mouse antisera of the following specificities: anti-whole I^k region, anti-$(K + IA)^k$, anti-I-J^s, anti-I-J^k, or anti-I-J^b. Significant absorption of suppressive activity was never observed (data are not shown).

It was then found that the suppressive activity of SRBC-SUP obtained from B6

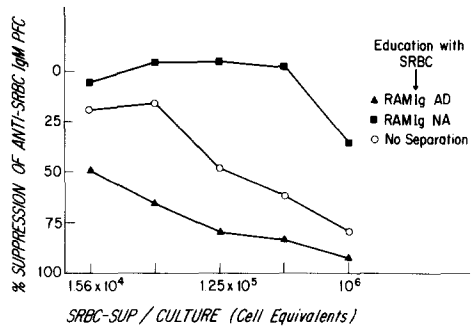

FIG. 2. Comparison of the suppressive activity in supernates of sIg$^+$ adherent (AD) and sIg$^-$ nonadherent (NA) cell fractions obtained by double cycle separation of educated BDF spleen cells on dishes coated with RAMIg. The recovery for AD cells was 12.5%, for NA, 37%. The anti-SRBC IgM and IgG PFC/10^6 recovered cells (measured 24 h after separation) were 1,755 IgM, 290 IgG for unseparated cells; 5,620 IgM, 1,880 IgG for AD cells; 205 IgM, 20 IgG for NA cells. The results show the percent specific suppression compared to the effects of supernate from control cells educated in the absence of SRBC. The results obtained with these controls were within a ± 15% range of the culture response in the absence of supernate (14,220 anti-SRBC IgM PFC).

cells could be completely removed by passage over a Sepharose column coupled with rabbit anti-mouse Ig (Table III). Control absorption with NRG-Sepharose did not significantly reduce the suppressive activity. Moreover, the suppressive activity could be largely recovered by acid elution of the material bound to the anti-Ig column, whereas similar elution of the NRG column yielded no suppressive activity.

IgV$_H$ Restriction of SRBC-SUP Activity. Some previous studies on suppression by soluble T cell products have demonstrated H-2 restriction of the activity of such materials (15). Eardley et al. (16) have shown that for T cell-mediated feedback suppression to SRBC, V$_H$ identity between inducer, Lyt 1^+ and target Lyt $1^+2^+3^+$ cells is required. Finally, auto-anti-idiotypic antibodies act in a V$_H$-restricted manner (3). Therefore, SRBC-SUP was tested for either H-2 or IgV$_H$ restrictions on its activity. Supernate from SRBC-educated or control-educated cells from several strains including H-2 congenics (B10 and B10.BR) were prepared and tested in a crisscross fashion in test cultures with spleen cells from these strains. A summary of eight experiments with seven different strains is shown in Table IV. The results obtained with B10 and B10.BR show a lack of H-2 restriction. However, differential susceptibility to the effect of SRBC-SUP was consistently observed in some strain combinations. Thus, SRBC-SUP from A/J or BALB/c cells was more active on the syngeneic cells than on all others. The same was found for DBA/1 supernate, except that it also exhibited suppressive activity with semi-syngeneic BDF cells. The cells from A/J BALB/c, and DBA/1 strains were, in turn, less susceptible to suppression by SRBC-SUP from SJL, B10, and B10.BR. One notes that the observed differences were relative rather than absolute but corresponded to ~5- to 10-fold differences in SRBC-SUP activity.

The Ig-1 allotypes of the various strains are also listed in Table IV as markers of the Ig heavy chain complex. Because it appears that a correlation between our results and these markers might exist, the hypothesis that Ig-complex-linked restriction and, in particular, IgV$_H$ restriction was involved in SRBC-SUP activity was tested using other inbred strains (Table V). The congenic strain CB-20 differs from BALB/c in

TABLE III

Absorption of SRBC-SUP Activity on Anti-Mouse Ig Immunoabsorbent Columns

	Anti-SRBC IgM PFC/test culture		Suppression*
	Experiment 1	Experiment 2	
			%
Control response	5,265	8,460	
Co-SUP	4,875	9,255	= Standard
SRBC-SUP	1,875	3,065	64
Column filtrates of SRBC-SUP			
NRG-Sepharose	2,265	3,225	60
Anti-mouse Ig-Sepharose	5,085	9,060	−1
Column eluates of SRBC-SUP			
Control response in elution medium	4,520	7,820	= Standard
NRG-Sepharose	4,530	7,715	1
Anti-mouse Ig-Sepharose	2,375	3,715	51

* In these experiments, 0.7-ml samples of SRBC-SUP from B6 cells (10^7 cell equivalents/ml) were passed through 0.4-ml Sepharose columns. The column filtrates were diluted five times compared to the initial sample and 0.5-ml aliquots were added to 0.5-ml test cultures of 7.5×10^6 B6 spleen cells. Unabsorbed samples of SRBC-SUP or Co-SUP were accordingly tested at a final concentration of 10^6 cell equivalents/ml. The acid buffer (pH 2.8) column eluates (Materials and Methods) were adjusted to the same volumes and tested as the filtrates.

* The mean values for the percent suppression are shown. The suppression observed with the column filtrates is expressed in comparison to the PFC response in presence of unabsorbed Co-SUP. The suppression observed with the acid buffer eluates is expressed in comparison to the PFC response in the presence of 0.5 ml of MEM that was acidified, reneutralized, and then dialyzed.

that it carries genes of the Ig heavy chain region of C57BL/Ka; the BAB-14 congenic recombinant strain carries the IgC_H genes (Ig allotypes) of C57BL/Ka, like the CB-20, but appears to have most of the IgV_H genes of BALB/c (17). The BC-8 strain is the reverse of CB-20 in that it possesses the Ig heavy chain of BALB/c on the C57BL/Ka ($H\text{-}2^b$) background (18). The experiments listed on Table V show that with regard to the type of SRBC-SUP produced as well as susceptibility to its effect, CB-20 resembles B6, whereas BC-8 and BAB14 resemble BALB/c, indicating the importance of IgV_H-linked genes in determining SRBC-SUP activity.

Genetic Restriction of B Cell-Feedback Suppression Correlates with the Phenotype of the sIg^+ Cells in Both the Educated Cell Population and in the Test Culture. The preceding experiments demonstrated that IgV_H-linked genes restricted suppression mediated by antibody derived from primary anti-SRBC cultures. Because it is well documented that both T and B cells may bear similar or identical V_H structures (19–21), and that both cell types can regulate immunity via V_H-linked networks (3, 6–9, 16), experiments were performed to determine if T cells, B cells, or both in the education and test cultures controlled the V_H restriction observed. This was accomplished by studying the specificity of suppression by cultures containing permutations of allotype congenic T and B cells assayed on similar T-B mixtures. Table VI demonstrates first, that cells from SRBC-educated cultures of B6 or BC-8 spleen show the same restrictions of

TABLE IV
Genetic Restriction of SRBC-SUP Activity

		Strains used for test cultures						
		SJL	B10.BR	B10	BDF	DBA/1	A/J	BALB/c
H-2		s	k	b	b × d	q	a	d
Ig-1 allotype*		b	b	b	b × c	c	e	a
Origin of SRBC-SUP	SRBC-SUP/culture (cell equivalents)	Suppression of anti-SRBC IgM PFC‡						
	× 10^{-5}	%						
SJL	5	68	74	63	70	20	32	26
	1	35	54	41				
B10.BR	5	66	67	65	57	21	27	25
	1	20	29	29				
B10	10	70	75	72	77	8	29	20
	3	36	42	46	39	0		
BDF	10	74	81	78	80	61	40	41
	3			70	60	50		
DBA/1	10	11	2	14	50	60	20	21
	3			4	39	39		
A/J	15	17	0	12	16	NT	66	25
	5						26	
BALB/c	15	21	19	20	28	12	34	65
	5							41

* The Ig-1 allotypes (Herzenberg classification) are listed according to the listing of Lieberman et al. (17). The following anti-SRBC IgM PFC responses were obtained in control cultures: 12,450 ± 1,230 for SJL; 9,160 ± 2,020 for B10.BR; 13,740 ± 1,310 for B10; 7,840 ± 1,380 for BDF; 5,210 ± 620 for DBA/1; 1,040 ± 290 for A/J; and 1,580 ± 180 for BALB/c (means ± SE).

‡ The values for percent suppression represent specific suppression of the anti-SRBC IgM PFC responses by SRBC-SUP as compared to the effects of the same amount of control supernates obtained from the same strains. These are mean values of two to four results with SE of ± 8% suppression. The data are from eight experiments and in one experiment all combinations shown were included. NT, not tested.

suppressive activity as SRBC-SUP derived from these strains (Table V); note that as for B6 SRBC-SUP, the genetic restriction was most noticeable at a reduced number of suppressor cells (5×10^4). Second, when reciprocal mixtures of BC-8 and B6 T cells ($T_{BC\text{-}8}$, T_{B6}) and BC-8 and B6 B cells ($B_{BC\text{-}8}$, B_{B6}) were educated with SRBC, the resulting suppressor cell activity was restricted to optimum activity on test cultures sharing Ig allotype with the B cells in the education culture. For example T_{B6} + $B_{BC\text{-}8}$ suppressed BC-8 responses to a much greater extent than B6 responses. In all combinations, the source of the T cell component of the educated cells had no detectable effect on the restrictions observed.

A similar analysis was carried out to answer the second question of whether the restriction of SRBC-SUP activity correlated with the phenotype of the B or T cells in the test cultures. SRBC-SUP obtained from educated B6 or BC-8 cells was added into assay cultures containing mixed populations of T and B cells from B6 or BC-8. The results show that the suppressive effect of SRBC-SUP was dependent upon the presence of B cells of the same genotype as the supernate-producing cells, irrespective of the type of T cells in the test cultures (Table VII).

TABLE V

Ig V_H Restriction of SRBC-SUP Activity

Origin of SRBC-SUP	Cell equivalents of SRBC-SUP added/test culture	Suppression of anti-SRBC IgM PFC*		
Experiment 1		B6	CB-20	BALB/c
	× 10^{-5}		%	
Control response	—	(16,980)‡	(5,080)	(2,550)
B6	3	63	67	23
	1	48	36	5
CB-20	15	53	54	10
	5	24	24	0
BALB/c	15	23	25	63
	5	0	14	45
Experiment 2		B6	BAB14	BALB/c
Control response	—	(9,330)	(870)	(1,160)
B6	3	71	28	28
	1	50	4	10
BALB/c	15	21	86	70
Experiment 3		B6	BC-8	BALB/c
Control response	—	(13,450)	(2,720)	(1,840)
B6	5	77	4	19
B10.BR	8	74	0	NT
BC-8	15	0	71	73
BALB/c	15	0	60	72

* The values for the percent suppression represent specific suppression of the anti-SRBC IgM PFC responses by SRBC-SUP as compared to the effects of the same amounts of control supernate obtained from the same strains. NT, not tested.

‡ Control responses are shown in parentheses.

TABLE VI

The Suppression of SRBC-educated Cells is Genetically Restricted According to the Genotype of the B Cells and Not of the T Cells in the Educated Population

Composition of the SRBC-educated cell population	Number of cells added per culture	IgM anti-SRBC PFC/test culture*	
		B6 cultures	BC-8 cultures
Control response	—	11,730	2,020
B6 spleen	5×10^4	4,800 (59)	1,525 (25)
$T_{B6} + B_{B6}$‡	5×10^4	5,640 (52)	1,735 (14)
$T_{BC\text{-}8} + B_{B6}$	5×10^4	5,570 (53)	1,760 (13)
$T_{B6} + B_{BC\text{-}8}$	3×10^5	11,850 (−1)	610 (70)
$T_{BC\text{-}8} + B_{BC\text{-}8}$	3×10^5	12,360 (−5)	845 (58)
BC-8 spleen	3×10^5	12,345 (−5)	645 (68)

* Suppression was compared to the control response observed in absence of educated cells. The percent suppression is in parentheses.

‡ T cell-enriched and B cell-enriched spleen cell fractions were obtained as sIg^- and sIg^+ cells, respectively, by double cycle separation on plastic dishes coated with RAMIg. The sIg^- and sIg^+ cells were recombined at a ratio of 2:3 and were educated with SRBC as described for whole spleen (Materials and Methods). The sIg^- and sIg^+ cells were also cultured separately for 4 d in presence of SRBC and were found to generate anti-SRBC IgM PFC levels of <4% compared to recombined cells.

TABLE VII

Suppression by SRBC-SUP Is Genetically Restricted According to the Genotype of the B Cells and Not of the T Cells in the Test Cultures

Composition of the test culture cell population	IgM anti-SRBC PFC/test culture*		
	Control supernate‡	SRBC-SUP from B6 cells‡	SRBC-SUP from BC-8 cells‡
B6 spleen	13,465	4,030 (70)	14,130 (−5)
T_{B6} + B_{B6}§	4,100	1,020 (75)	4,510 (−10)
T_{BC-8} + B_{B6}	4,985	1,100 (78)	5,980 (−20)
T_{B6} + B_{BC-8}	1,350	1,010 (25)	495 (63)
T_{BC-8} + B_{BC-8}	1,630	1,645 (0)	415 (75)
BC-8 spleen	2,725	2,630 (3)	790 (71)

* Suppression was compared to the responses observed in presence of control supernate from B6 cells. Co-SUP from BC-8 was found in other experiments to have the same effect as Co-SUP from B6. The percent suppression is in parentheses.

‡ All supernates were tested at a concentration of 10^6 cell equivalents per culture. Responses in the presence of control supernate did not differ greatly from responses of the same cells in the absence of supernate. The variations in total PFC between various cultures reflects the use of recombined T and B cells, and in particular, the predominant effect of the B cells on PFC levels.

§ T cell-enriched and B cell-enriched spleen cell fractions were obtained and recombined as described in legend of Table VI, and test cultures were set up with 7.5×10^6 cells. The sIg^- and sIg^+ cells were also cultured separately in presence of SRBC and were found to generate anti-SRBC IgM PFC levels of <4% compared to recombined cells.

Discussion

The data presented in this paper document that suppression of primary anti-SRBC humoral responses in vitro may be caused by either antigen-activated B lymphocytes or their Ig products. Such suppression is restricted by V_H-linked genes in the sense that optimal regulation is seen when the B cell source of the suppressive antibody and the B cells in the test culture share allotype-linked genes, irrespective of the genotype of the T cells involved in either stage. Finally, in accord with this B cell-determined restriction, suppression in this model does not affect a haptenic response to derivatized SRBC.

The most obvious question raised by these data concerns the mechanism of suppression. Several models, consistent with both the current data and earlier work, may be envisaged. One set of hypotheses invokes a direct action of antibody from the first culture on B cell activity in the assay culture. The most straightforward possibility is classical antibody feedback operating by interference with determinant presentation (4, 5). The SRBC-primed B cells release anti-SRBC antibody which could bind to the antigen in the test culture, block B cell recognition, and thus reduce responsiveness. The ability of SRBC to selectively absorb the activity of SRBC-SUP is consistent with this model, as is the finding that the greatest effect of the supernate occurs during the early culture period of initial antigen recognition. Similar observations were made with in vivo anti-SRBC antibodies (4, 5, 22–26). To explain the V_H restriction observed, one would have to postulate that the number of epitopes recognized by each allogroup is quite limited and differs for each V_H cluster. In this way, the antigenic determinants blocked by one set of antibodies would overlap minimally

with those seen by an allotype-mismatched B cell population, preventing effective suppression.

Alternatively, the active component of SRBC-SUP might in fact be anti-idiotypic antibodies, induced by the receptors of the activated B cells in the first culture and which block the receptors of the assay B cells. To account for the ability of SRBC to absorb the suppressive antibody, it would be necessary to assume that anti-SRBC antibody present in SRBC-SUP bound to the SRBC and formed an idiotypic absorbent for the anti-idiotypic antibodies. If this is the case, the activity of SRBC-SUP should be absorbed by SRBC-activated B cells or anti-SRBC early antibody from mice of the appropriate allotype, and such tests are underway. A variant of this proposal is that suppression by anti-SRBC antibody present in SRBC-SUP acts indirectly via the triggering of an anti-idiotypic antibody response in the test culture. These hypotheses are based on the recent demonstration that anti-idiotypic antibodies are triggered by early humoral responses in vivo, including anti-SRBC responses (27), and can suppress such responses in an antigen-specific manner (2, 3). In such cases, the suppression is V_H restricted (3) because the putative B cell target of this suppression must bear the (V_H linked) idiotype of the inducing antibody.

A second set of hypotheses postulates the induction of a T_s response by the addition of anti-SRBC B cells or antibody (SRBC-SUP) into the test culture, based on the demonstration of idiotype-specific T_s induction in vivo (6–8) or in vitro (28). Eardley et al. (16) have reported that T_s generation by stimulation with SRBC in vitro requires V_H-restricted interaction between Lyt 1^+:Qa 1^+ inducer and Lyt $1^+2^+3^+$, Qa 1^+ target cells. Recently it has been shown that Lyt 1^+ activation may occur via recognition of Ig structures (idiotype?) on activated B cells in vivo (9) or in vitro.[2] One might therefore consider the possibility that anti-SRBC antibody acts by triggering such a T cell feedback loop in the test culture. However, it should be noted that in the report by Eardley et al. (16) the V_H type of the B cells was not relevant to the V_H restrictions observed and that the T_s in the original feedback suppression model (11, 29, 30) decrease the haptenic responses to derivatized SRBC.

In contrast, if antibody- (idiotype) induced T_s are involved in the suppression described in this paper, it is the B, not T, cells in the test culture that play a crucial role in determining the genetic restrictions seen. The most straightforward explanation for this restriction and the lack of inhibition of the anti-TNP PFC response to TNP-SRBC is that the induced T_s act on the B cells themselves, as documented previously (31, 32). However, because the current experiments do not directly demonstrate that B cells are the actual target of the suppression, other mechanisms may be imagined by which the B cells in the assay culture may impose V_H restrictions on the suppression observed. It cannot be ruled out that other cells in the assay culture may acquire B cell-derived products and by this means become involved in a V_H-restricted suppressor network whose genetics are dictated by the B cells in the test system. Thus, the induced T_s could act on other T (helper?) cells (T-helper cells [T_H]) and not on the V_H-matched B cells. However, to account for the failure of these T_s to inhibit anti-TNP responses to TNP-SRBC one must postulate these target T_H to show idiotype and not only antigen-specific helper function (33, 34) and to provide the major proportion of the help required in primary anti-SRBC PFC responses in vitro (i.e., the suppressable help; [Table I]). In this regard, it might also be noted that simple competition between idiotype-bearing antibody and idiotype-bearing B cells for such T cell help could account for the observations reported here.

Whichever of these models is correct, our findings indicate that, contrary to expectation, the primary humoral response to what is thought of as a complex antigen must involve a very few (unique) B cell clones, to account for the ease with which V_H restrictions are seen in suppression. This apparent anomaly may be explained by the fact that SRBC are known to possess one (or a few) special determinants that lead to differential quantitative PFC responses in various mouse strains (35) with a pattern largely related to allotype-linked genes (36). Similarly, allotype-linked genes control the antibody levels to chicken erythrocytes in mice (37). It will be of interest to determine whether the crucial antigenic determinants on erythrocytes are protein or polysaccharide in nature. It has been well documented that anti-polysaccharide responses may show striking allotype specific (Ir gene-like) effects (18, 38–40).

Lastly, these results reemphasize the complexities involved in immune regulation. Future studies will hopefully identify the signals which cause one or another of the various regulatory pathways to become activated, and which determine the molecular mechanisms by which each pathway acts.

Summary

Feedback suppression of the primary humoral immune response to sheep erythrocytes (SRBC) in vitro was induced with cell-free supernate material derived from antigen- (SRBC) activated B (sIg$^+$) cells. This soluble product bears Ig determinants and binds to the eliciting antigen (SRBC). The activity of this antibody in suppressing anti-SRBC plaque-forming cell responses is restricted to spleen cell cultures containing B cells sharing V_H genes with the B cells producing the suppressive antibody. The anti-hapten (trinitrophenyl) response to derivatized SRBC is not affected by antigen-primed B cells or their products. These data are compatible with suppression being mediated by anti-antigen antibody, either (*a*) via blockade of different SRBC epitopes recognized by a limited set of B cell clones in each mouse strain, (*b*) via triggering of an anti-idiotypic response, either antibody or suppressor T cell in nature, restricted to activity in cultures containing B cells sharing V_H structures with the original antibody, or (*c*) via interference by preformed antibody with T cell help directed at idiotype bearing B cells.

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