CELLULAR INDUCTION OF CHRONIC ALLOTYPE SUPPRESSION OF IgG2a IN $Igh^{b/b}$ HOMOZYGOUS MICE AND ITS ABROGATION BY IN VIVO TREATMENT WITH ANTI-CD8 MONOCLONAL ANTIBODY

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Immunoglobulin (Ig) allotype suppression was first described in heterozygous rabbits by Dray (1), who showed in 1962 that perinatal exposure to antibodies directed against paternally inherited Ig allotypes (2-5) would suppress their expression. One of the interesting developments in the field made possible by this pioneer work were successful attempts to induce allotype suppression in homozygous rabbits. However, for this purpose, new methods had to be used, because large amounts of maternal Ig pass through the placenta and are present in the fetus, thereby blocking the effects of eventual anti-allotypic antibody treatment commonly used to induce suppression. This problem was originally circumvented in 1967 by Dubiski (6), who mated a heterozygous female rabbit subjected to suppression for a paternal allotype with a male rabbit homozygous for this allotype. Other methods were then elaborated, such as homozygous embryo transfer to surrogate mothers of appropriate genotype (7, 8). The obtention of allotype-suppressed homozygous rabbits allowed in particular the discovery of new Ig allotypic specificities and some insights in the regulation of Ig expression (9). For instance, the compensatory effect (1) observed in allotype-suppressed rabbits was due to an increased expression either of allelic genes when considering heterozygous rabbits (1) or of genes at different loci in the case of homozygous rabbits (6).

Studies in mice on the effect of isoantibodies on paternal Ig allotype started soon after Dray's discovery of rabbit allotypic suppression (10). They were mainly achieved in (BALB/c × C57BL/6)F_1 mice and allowed the obtention of short term allotypic suppression (11). Then, chronic allotypic suppression was induced (12) in BALB/c (Igh^a) × SJL (Igh^b)F_1 mice, where the BALB/c mothers were immune to IgG2a of Igh^b haplotype (Igh-1b). At 6 mo of age, a chronic Igh-1b suppression was observed in 50% of the offsprings. It should be noted that antibody-induced Igh-1b chronic suppression occurred only in SJL-related hybrids. This suppression exhibited several differences with the phenomenon described in rabbits, but it provided a powerful approach in understanding its cellular bases (reviewed in reference 13).

We have recently succeeded in inducing chronic allotype suppression of Igh-1b expression in $Igh^{a/b}$ hybrid mice issued from matings between Igh congenic partners

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that were not SJL-related. This was achieved by injecting the heterozygous mice at birth with T splenocytes either from Igh^a normal parental donors (14) or, with a much higher efficiency, from Igh^a parental donors sensitized against either Igh-1b-coated autologous splenocytes or Igh^b congenic B splenocytes (15). This sensitization procedure was particularly powerful, as it allowed the induction of this Igh-1b suppression in $Igh^{b/a}$ mice having maternally inherited the Igh^b haplotype (15). This, together with the fact that anti-allotypic antibodies were shown to be absent from this induction procedure (15), led us to attempt to induce Igh-1b suppression in homozygous mice without resorting either to embryo transfer, as in experiments with rabbits, or to irradiation of adult recipients, as in the mouse experimental model of Bosma and Bosma (16). Homozygous mice ($Igh^{b/b}$) received at birth a single injection of T splenocytes from Igh^a congenic mice sensitized against parental Igh^b B splenocytes. We report here that in two different systems of Igh congenic mice, all the treated animals developed a chronic suppression of the Igh-Ib expression without affecting the expression of IgM, IgD, and IgA of the Igh^b haplotype. Further, we show that the suppression can be experimentally abrogated and that $CD8^+$ lymphocytes are essential for its maintenance.

Materials and Methods

Mice. 9–13-wk-old female or male BALB/c, CB20, C57BL/6, and BC8 mice were from our animal facilities. CB20 is a BALB/c congenic strain having the Igh genes (Igh^b) of the C57BL/6 strain, while BC8 is a C57BL/6 congenic strain having the Igh genes (Igh^a) of the BALB/c strain. To designate the F_1 mice, we used the usual convention, namely, the mother's name indicated first, followed by the father's name.

Cell Preparation. Splenocytes were separated on nylon wool columns as previously described (17). Using a rat anti–mouse Thy-1.2 mAb (a gift of Dr. Coutinho, Institut Pasteur, Paris) followed by goat anti–rat antibodies adsorbed on mouse Ig (Cappel Laboratories, Cochranville, PA) and labeled with FITC, we found 75% positive cells within the nylon wool–nonadherent splenocyte population (Tj),[1] while the use of FITC-labeled Fab fragments of goat anti–mouse Ig (Nordic Immunological Laboratories, Tilburg, The Netherlands) revealed, in the same population, <5% positive cells. To prepare large amounts of B-enriched splenocytes, total splenocyte populations were incubated with the rat anti-Thy-1.2 mAb plus guinea pig complement. To prepare peripheral blood lymphocytes, ~1 ml of blood per mouse was collected on heparin by retro-orbital sinus puncture. This blood was diluted with 1 ml of balanced salt solution and carefully layered on 2 ml of Lympholyte M (Cedarlane Laboratories, Hornby, Ontario, Canada). After centrifugation at room temperature for 17 min at 420 *g*, white cells were harvested at the interface and washed twice before use.

Mouse Sensitizations and Newborn Treatments. Treatments were carried out as previously described (15). For sensitizations, female BALB/c or BC8 mice were injected, by the intravenous route, with 0.1–0.2 ml of balanced salt solution containing 5×10^7 B-enriched splenocytes from either female CB20 or C57BL/6 mice, respectively. Mice were sensitized, either once or twice, in which case the second injection was given 2 wk later. Between 24 and 72 h after birth, appropriate homozygous mice were injected intraperitoneally with Tj splenocytes collected from such sensitized mice 1 wk after the unique or the second sensitization.

Preparation of Anti-allotypic Antibodies and of Allotypes and Their Detection. The details were given elsewhere (15), except for one mAb obtained from Dr. Coutinho: RS31 (18), which is a mouse mAb anti-Igh-6a (IgM of Igh^a haplotype). This mAb was used to quantify the corresponding allotype by RIA, as described (19). In this study, the mAb 9-8 anti-Igh-1a (20)

[1] *Abbreviations used in this paper:* Tj, nylon wool–nonadherent splenocytes; Tj1s, Tj2s, Tj splenocytes from mice sensitized either once or twice, respectively.

was linked to β-galactosidase (21) and used in an ELISA, allowing a better sensitivity than in RIA.

In Vivo Treatments by Anti-T Cell Subset mAbs. The GK1.5 clone producing a rat anti-L3T4 (CD4) IgG2b mAb (22) and the H.35-17.2 clone producing a rat anti-Lyt2 (CD8) IgG2b mAb (23) were kindly supplied by Dr. Coutinho. These clones were cultured in RPMI 1640 medium supplemented with 100 IU penicillin per ml, 100 μg of streptomycin per ml, 2 mM L-glutamine, 5×10^{-5} M 2-ME, 1 mM sodium pyruvate, and 10% FCS. mAb treatments were carried out on allotypically suppressed CB20 or C57BL/6 mice.

In one experiment, we used ascitic fluid obtained from irradiated (300 rad) BALB/c host mice for these clones. The crude ascitic fluid was partially purified by 18% Na_2SO_4 precipitation. The immuno-fluorescence titers of these fractions were determined by thymocyte suspension staining with FITC-labeled goat anti-rat Ig antibodies as the second step (the stained cells were analyzed in a FACScan system; Becton Dickinson Immunocytometry Systems, Mountain View, CA). The GK1.5 and the H.35-17.2 fractions had a final titer of 1:32,000 and 1:8,000, respectively. Mice received 300 μl of saline intravenously containing either 50 μl of the GK1.5 fraction or 200 μl of the H.35-17.2 fraction (fourfold less of the anti-CD4 reagent compared with the anti-CD8 fraction, taking into account their different immunofluorescence titers). 4 d later, these mice received, intravenously, half of the first dose of the same mAb fraction. They were bled 2 wk after this second injection and then every fortnight. The eventual presence of Igh-1b in their sera was estimated by RIA.

In a second experiment, culture supernatants were used as source of mAb (the immunofluorescence titers were of 1:50 and 1:240 for the GK1.5 and H.35-17.2 supernatants, respectively). Mice were separated in two groups and received daily, during 7 d, 250 μl intravenously and 500 μl intraperitoneally injections of supernatants of either H.35-17.2 or GK1.5 clone. These mice were bled 1 d after the last injection and then weekly. Here, also, serum Igh-1b concentrations were estimated.

Fluorescence Staining and Cytofluorographic Analysis. Before cell staining, red cells were removed from splenocyte suspensions by treatment with Gey's solution (24). The two-step staining procedure followed to determine the percentages of splenocytes positive for surface Igh-5a and Igh-5b (Igh-5 is the allotypic locus for IgD) has been previously described (15). It was also used to quantitate the percentages of $CD4^+$ and $CD8^+$ cells. For this purpose, anti-CD4 mAb was purified from GK1.5 ascitic fluid (see above) and biotin labeled (25) while the biotin-labeled anti-CD8 mAb (53.6.72, reference 26) was purchased from Becton Dickinson & Co. 10^6 splenocytes were incubated with either 0.5 μg of the anti-CD4 or 1 μg of the anti-CD8 biotin-labeled mAb. We verified that under these conditions, addition of various amounts of H35-17.2 culture supernatant (containing the anti-CD8 mAb) to normal splenocytes before incubation (or concomitantly) with biotin-labeled anti-CD8 mAb then revealed by FITC-streptavidin, did not modify the $CD8^+$ cell percentage as compared with the value obtained in the absence of culture supernatant. As control for cell saturation by unlabeled mAb, incubations of normal splenocytes with the various amounts of culture supernatant used then revealed by FITC-anti-rat Ig antibodies gave the same $CD8^+$ cell percentage value than above. The same held true for the estimation of $CD4^+$ cell percentages. Therefore, cell-bound mAb was effectively displaced in our cell-staining conditions and thus, eventual reduction of cell percentages will reflect disappearance of corresponding cells. Percentages of cells positive for surface Ig were estimated by staining with FITC-labeled Fab fragments of goat anti-mouse Ig used at a final dilution of 1:80. The stained cells were analyzed in a FACScan system, as fully described (15).

Southern Blot Hybridization. High molecular weight genomic DNA was prepared (27) from liver and spleen of four different mouse strains: BALB/c (Igh^a); BC8 (Igh^a); C57BL/6 (Igh^b); and CB20 (Igh^b). Each DNA sample was digested to completion by either Bgl II or Pvu II restriction endonucleases. 10–15 μg of each digest were run on a 0.8% agarose gel and transferred to Genescreen membranes (New England Nuclear, Boston, MA). These were hybridized with a probe labeled with P^{32} by the random priming technique (28). This probe is a 380-bp Xho I-Pst I fragment essentially covering the first intron of the γ 2a gene of BALB/c strain, isolated from the pBG2a-4 fragment (29). Final post-hybridization washes were performed in 0.1× SSC at 65°C, under which conditions, hybridization to alleles of γ 2a gene only is detected.

Results

Induction of Allotype Suppression in CB20 Mice. The procedure followed was essentially that previously established with heterozygous mice (15). We also used the Igh congenic partners: CB20 (Igh^b) and BALB/c (Igh^a) mice. CB20 homozygous newborns received either 4×10^7 Tj or 2×10^7 Tj splenocytes from BALB/c mice twice sensitized against CB20 B splenocytes. The sera of the untreated controls and of the treated CB20 mice were collected for >9 mo. They were studied for the expression of the Igh^b allotypic forms of the IgG2a (Igh-1b), IgA (Igh-2b), and IgM (Igh-6b) according to Herzenberg's nomenclature (30). The neonatal treatment of 22 CB20 mice led, for all of them (Fig. 1), to the progressive establishment of a complete suppression of Igh-1b expression. The production of the other Igh^b allotypes (Igh-2b and Igh-6b) tested in their serum was unaffected by this neonatal treatment (not shown for Igh-2b; and see Fig. 1 for the quantification of Igh-6b). Moreover, cytofluorographic studies of splenocytes collected from Igh-1b-suppressed animals killed at two different ages revealed that the Igh-5b (IgD of Igh^b haplotype) expres-

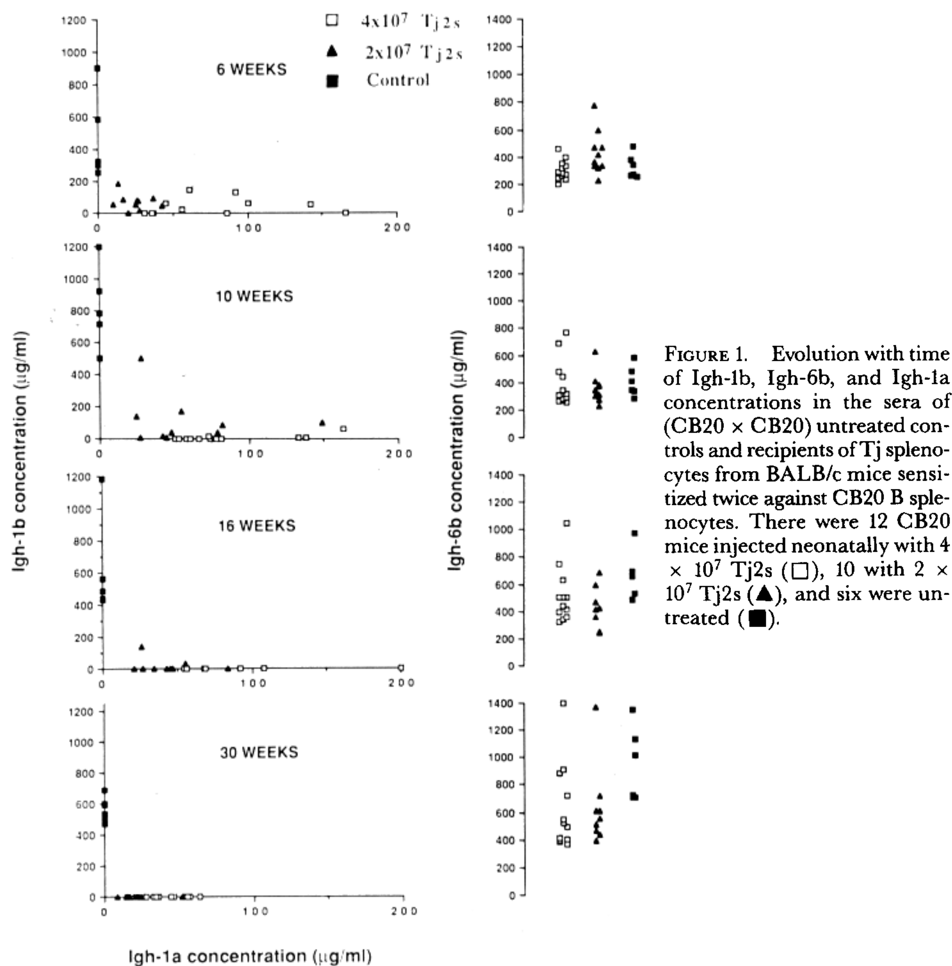

FIGURE 1. Evolution with time of Igh-1b, Igh-6b, and Igh-1a concentrations in the sera of (CB20 × CB20) untreated controls and recipients of Tj splenocytes from BALB/c mice sensitized twice against CB20 B splenocytes. There were 12 CB20 mice injected neonatally with 4×10^7 Tj2s (□), 10 with 2×10^7 Tj2s (▲), and six were untreated (■).

sion was also unaffected compared with splenocytes from untreated controls coming from the same offsprings (Table I). The suppression was demonstrated to be chronic as it persisted for >32 wk without any break in all the treated animals and even for >1 yr in the remaining 10 mice, which were not killed. The progressive establishment of the suppression is slightly different between the two groups of treated animals (Fig. 1). Of those that had received 4×10^7 Tj, none were found to express Igh-1b as of 16 wk of age, whereas, among the mice injected with half of cells, the same result was only reached at the next bleeding, that is, 2 mo later.

Induction of Allotype Suppression in C57BL/6 Mice. One further induction experiment was attempted in homozygous C57BL/6 mice. The same pattern of chronic allotype suppression was indeed induced in C57BL/6 homozygous mice following a similar procedure. C57BL/6 (Igh^b) newborns received 4×10^7 Tj splenocytes from BC8 (Igh^a) mice sensitized either once or twice against C57BL/6 B splenocytes. Like in the experiments with CB20 mice (described in Fig. 1), Igh-1b suppression was established at 16 wk of age in all the 13 animals belonging to the 4×10^7 Tj2s–recipient group (Table II) and did not affect the production of the other Igh^b allotypes tested in their sera (not shown for Igh-2b and Igh-6b). The percentages of cells positive for surface Ig, Igh-5a, and Igh-5b were estimated on splenocytes harvested at 10 wk of age from four C57BL/6 controls (51.4 ± 1.3 Ig^+; 0.4 ± 0.1 Igh-$5a^+$; and 43.1 ± 3.2 Igh-$5b^+$) and from four Igh-1b-suppressed C57BL/6 mice (54.2 ± 3.5 Ig^+; 0.5 ± 0.1 Igh-$5a^+$; and 41.8 ± 7.6 Igh-$5b^+$). These data show that the neonatal injection did not modify Igh-5b expression. Interestingly, the five recipients of 4×10^7 Tj1s continued to express Igh-1b (tested until 32 wk of age) (Table II).

Expression of Donor Igh^a Allotypes in Hosts. Further studies in both systems (CB20 and C57BL/6) revealed, by means of a sensitive ELISA, that a weak Igh-1a production occurred in Igh-1b-suppressed mice but did not in untreated mice. With CB20 mice, it was observed that the levels of serum Igh-1a concentrations seemed to be correlated with the number of injected T splenocytes (Fig. 1). In addition, the Igh-1a production seemed to decrease with time (Fig. 1). As this work was being completed, an anti-Igh-6a mAb (RS.31; reference 18) became available and allowed us, using

TABLE I

Levels of Ig, Igh-5a, and Igh-5b Expressions on Splenocytes from (CB20 × CB20) Untreated Mice and Igh-1b-suppressed Mice

Mice	*n*	Age	Percent Ig^+	Percent Igh-$5a^+$	Percent Igh-$5b^+$
		wk			
Untreated	4	32	57.2 ± 2.3	0.4 ± 0.0	41.2 ± 1.9
Igh-1b-suppressed	4	32	57.4 ± 2.5	0.8 ± 0.2	46.2 ± 3.1
Untreated	2	38	48.8 ± 3.2	0.4 ± 0.1	34.3 ± 9.9
Igh-1b-suppressed	6	38	51.0 ± 2.7	0.4 ± 0.3	34.0 ± 5.7

Untreated CB20 mice and Igh-1b-suppressed CB20 mice were killed at two different ages. Half of these allotypically suppressed mice belonged to the 4×10^7 Tj2s recipient group and the other half belonged to the 2×10^7 Tj2s recipient group studied in Fig. 1. Results of the FACScan analysis are expressed as mean values (and their SE) of percentages calculated in the indicated group of mice individually analyzed. At both ages, no significant difference was observed between the splenocytes from 4×10^7 Tj2s recipients and the 2×10^7 Tj2s recipients, therefore, their data were grouped.

TABLE II
Expression of Igh-1b in (C57BL/6 × C57BL/6) Untreated Controls and Recipients of Tj Splenocytes from BC8 Mice Sensitized either Once or Twice against C57BL/6 B Splenocytes

Age	Number of mice showing production of Igh-1b		
	Recipients (5) of 4 × 10^7 Tj1s*	Recipients (13) of 4 × 10^7 Tj2s	Untreated mice (20)
wk			
6	5	8	20
9	5	5	20
13	5	3‡	16‡
16	5	0	16
19	5	0	16
22	5	0	16
27	ND	0	16
32	5	ND	ND

Suppression establishment within treated mice was sex independent.
* Cells were injected intraperitoneally into the mice between 24 and 72 h after their birth.
‡ At 10 wk of age, four Igh-1b-suppressed mice (recipients of 4 × 10^7 Tj2s) and four untreated controls were killed for cytofluorographic analysis of their splenocytes.

RIA, to show that Igh-6a (IgM of Igh^a haplotype) production occurred in both Igh-1b-suppressed C57BL/6 and CB20 mice at similar levels to the Igh-1a production. Nevertheless, no serum Igh-2a (IgA) production was revealed, probably because of the low sensitivity of our immunodiffusion technique, and no significant percentages of Igh-5a$^+$ splenocytes were found in untreated and treated mice (see Table II and above). These weak Igh^a productions (Igh-1a and Igh-6a) were ascribed to engraftment and differentiation of donor hematopoietic stem cells known to be present in splenocyte preparations and to have the capacity of reconstituting an immune system in irradiated hosts (31, 32).

It was previously reported (33) that BALB/c Igh^b congenic mice (ICR CB17, the mother strain of our CB20 strain) produced not only Igh-1b but also transiently Igh-1a under certain critical conditions. Therefore, the Igh-1a production observed in our Igh-1b-suppressed CB20 mice could involve not only the differentiation of donor Igh^a stem cells but also the expression of an endogenous Igh-1a gene under the critical condition created by the Igh-1b suppression. We wonder whether or not our CB20 strain carries duplicated γ2a genes encoding for Igh-1a and Igh-1b (a situation previously observed in a wild mouse, 34). Southern blot analysis of genomic DNA from our CB20 strain (Fig. 2) demonstrated that it possesses the b allele of the γ 2a gene but lacks the a allele. We also verified that Igh^a C57BL/6 congenic strain, BC8, carries the gene encoding for Igh-1a but lacks the gene for Igh-1b.

Abrogation of the Igh-1b Suppression by In Vivo Treatment with Anti–T Cell Subset mAb. Among the 10 remaining Igh-1b-suppressed CB20 mice (described in Fig. 1) of 1 yr of age, four received two intravenous injections of partially purified ascitic fluid containing a rat anti–mouse CD4 mAb (produced by the GK1.5 clone), four others received two intravenous injections of partially purified ascitic fluid containing a

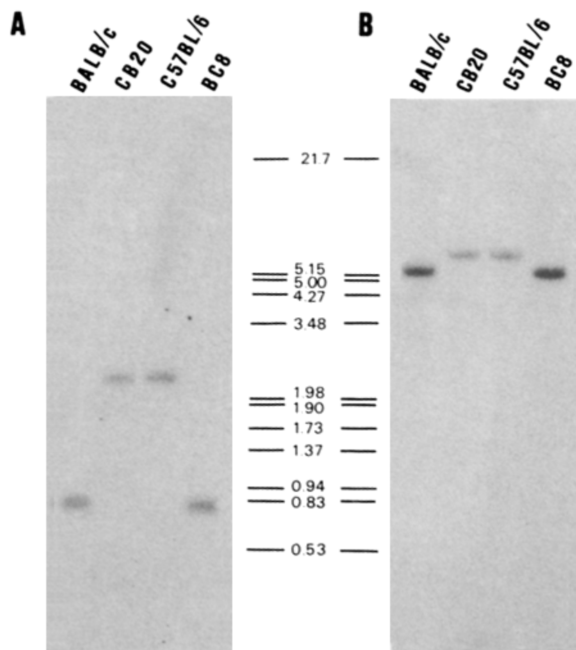

FIGURE 2. Southern analysis of liver genomic DNA from Igh^a and Igh^b mouse strains. (*A*) The genomic DNA of indicated mouse strain origin was digested by Pvu II. (*B*) The genomic DNA of indicated mouse strain origin was digested by Bgl II. Genomic DNA from spleen gave the same patterns than genomic DNA from liver and therefore are not shown. Numbers indicate the length of fragments in kilobases. The two restriction endonucleases used (Pvu II and Bgl II) show a site polymorphism between the a and b allele located in the first intron (ISV1) of the γ 2a gene. After electrophoretic migration of the digests on agarose gel and their transfer on Genescreen membrane, this polymorphism was revealed by means of a radiolabeled probe of 380-bp length and essentially covering the first intron of the γ 2a gene of BALB/c (Igh^a).

rat anti-mouse CD8 mAb (produced by the H.35-17.2 clone), and the two remaining CB20 mice were kept as controls. These two mAbs are of the IgG2b rat isotype, which was demonstrated to be the most cytotoxic in vivo on mouse cells (35). As can be seen in Table III, one out of the four mice treated with the anti-CD8 mAb exhibited stable production of Igh-1b as of 2 wk after the end of the treatment (>3 mo after treatment, we found 125 μg of Igh-1b per ml of serum). Another mouse from this group expressed up to 60 μg of Igh-1b per ml of serum 28 d after treatment; although weak, this production persisted and was of 12 μg per ml of serum at the last bleeding, that is >3 mo after treatment. Chronic Igh-1b suppression continued to be observed in anti-CD4-treated mice.

This abrogation of suppression was repeated using chronically Igh-1b-suppressed C57BL/6 mice of 6 mo of age (described in Table II). Instead of partially purified ascitic fluid, we used culture supernatant of the same anti-CD4 and anti-CD8 mAbs producing clones. We verified in an RIA that both anti-CD4- and anti-CD8-containing supernatants gave no crossinhibition of the binding of our I^{125}-labeled anti-Igh-1b mAb onto Igh-1b (not shown). The mice received the supernatants daily by both routes (intravenously and intraperitoneally) and for 7 d. The results are summarized in Table III. The anti-CD4-treated mice were still Igh-1b suppressed at 1 and 21 d after treatment, however at day 35, these four mice died, probably because of infections. In contrast, large amounts of Igh-1b were found in the serum collected 1 d after the end of the treatment from the four anti-CD8-treated mice (three of them had ~0.5 mg of Igh-1b per ml of serum and one had 0.1 mg per ml). However, this production of Igh-1b then started to decrease rapidly, and 35 d after the last injection, these four mice had almost no more Igh-1b in their serum. We decided

TABLE III
In Vivo Effects of Anti-CD4 or Anti-CD8 mAb on the Chronicity of Allotypic Suppression

Mouse type	Treatment	Number of mice[‡] studied	Serum Igh-1b concentration on day*							
					− 14	+ 14	+ 28	+ 77	+ 105	
							μg/ml			
Igh-1b-suppressed CB20[§]	anti-CD8	1			0	0	0	0	0	
		1			0	133	124	241	125	
		1			0	6	0	0	0	
		1			0	42	60	23	12	
	anti-CD4	4			0	0	ND	0	0	
	none	2			0	0	ND	0	0	
			− 7	+ 1	+ 21	+ 35	+ 41	+ 48	+ 49	+ 63
Igh-1b-suppressed C57BL/6[‖]	anti-CD8	1	0	567	55	9	New treatment[¶]		189	31
		1	0	471	29	0			116	7
		1	0	546	63	12			285	69
		1	0	119	6	0			156	21
	anti-CD4	2	0	0	0	–**				
		2	0	0	–	–				

* J0 = day of the last injection (of the first treatment when there are two).
‡ Mice were individually tested and the data obtained were grouped when identical.
§ These 1-yr-old mice belonged to the experiment described in Fig. 1. They were given two intravenous injections of either a GK1.5 (anti-CD4) or an H35-17.2 (anti-CD8) ascitic fluid partially purified.
‖ These 6-mo-old mice belonged to the experiment described in Table II. They received seven daily injections of culture supernatant of either GK-1.5 clone (anti-CD4) or H-35-17.2 clone (anti-CD8).
¶ These four mice had the same 7-d anti-CD8 treatment from day + 42– + 48 than before (from day – 6–0).
** The considered mice were dead at this time.

to subject them again to exactly the same 7-d anti-CD8 mAb treatment. It led once more to the abrogation of Igh-1b suppression as 1 d after the end of the second treatment, the serum of these four mice contained large amounts of Igh-1b (from 0.1–0.3 mg per ml of serum). Here, again, the Igh-1b production decreased rapidly (Table III).

Experiments were carried out to estimate the lymphocyte depletion induced in situ in different organs by the anti-T cell subset treatments. Normal C57BL/6 mice, littermates of those described in Table III and therefore age matched, were used. Two were kept as controls, two had the anti-CD4 mAb treatment, and two had the anti-CD8 mAb treatment as described in Table III. 1 d after the last injection, the mice were killed, and their peripheral blood lymphocytes, splenocytes, and thymocytes were prepared and stained with appropriate immuno-conjugates in order to determine the percentages of positive cells for surface CD4 or CD8 molecules, respectively. The stained cells were analyzed in a cytofluorograph, and the data obtained are presented in Table IV. For these estimations of cell percentages, we used excess amounts of biotin-labeled anti-CD4 or anti-CD8 mAb to displace eventual injected mAb already bound to the corresponding cell surface antigen (see Materials and Methods). It can be seen (Table IV) by comparison with the data acquired with the two controls that both treatments had no effect on thymocytes but were very effective in spleen and in blood. The anti-CD4 treatment led (without affecting $CD8^+$ cell percentage) to a severe depletion of the corresponding cells in peripheral blood and in spleen (86 and 66% depletion, respectively). The anti-CD8 treat-

TABLE IV

In Vivo Effects of Anti-CD4 or Anti-CD8 mAb on Spleen, Thymus, and Peripheral Blood Lymphocyte Subsets in C57BL/6 Normal Mice

In vivo treatment of normal C57BL/6 mice*	Spleen		PBL		Thymus	
	Percent $CD8^+$	Percent $CD4^+$	Percent $CD8^+$	Percent $CD4^+$	Percent $CD8^+$	Percent $CD4^+$
None	12.5 ± 1.0	20.7 ± 0.3	18.9 ± 0.3	19.1 ± 0.3	65.9 ± 2.3	92.6 ± 0.7
Anti-CD8	0.5 ± 0.1	20.5 ± 2.1	0.4 ± 0.1	17.6 ± 0.5	69.8 ± 9.7	92.5 ± 0.4
Anti-CD4	14.3 ± 1.2	6.9 ± 2.5	17.9 ± 3.9	2.6 ± 0.2	75.8 ± 1.3	92.8 ± 0.6

* These mice were controls belonging to the experiment described in Table II. Therefore, they were taken at the same age (6 mo old) as those described in Table III. Treatments with mAbs (two mice per group) were achieved as in the experiment described in Table III. The mice were killed the day after the end of the treatment. Blood, thymus, and spleen were harvested from each mouse individually, and lymphocyte suspensions were prepared. After staining, the cells were analyzed in a FACScan system. Results are expressed as mean values and their SE of percent of viable cells bearing the indicated surface antigen.

ment did not modify $CD4^+$ cell percentage but resulted in 98% depletion of $CD8^+$ cells in peripheral blood, and this depletion reached 96% in spleen.

Discussion

In the current study, we have demonstrated the possibility of obtaining chronic suppression of Igh-1b expression (IgG2a of Igh^b haplotype) in homozygous $Igh^{b/b}$ mice. For this purpose, we followed the procedure we have previously used to induce Igh-1b suppression in heterozygous $Igh^{a/b}$ mice (15). It consisted here of injecting homozygous $Igh^{b/b}$ newborn mice (CB20 or C57BL/6) with T splenocytes from $Igh^{a/a}$ congenic adult donors (BALB/c or BC8 mice, respectively) twice sensitized against congenic Igh^b B splenocytes. The totality of the treated homozygous mice developed, from 16–24 wk of age, a chronic Igh-1b suppression. This suppression can be compared with that induced in heterozygous $Igh^{b/a}$ mice having maternally inherited the Igh^b haplotype (15), because in both situations, the newborns have at the time of treatment Igh^b allotypes of maternal origin transmitted through the placenta. In both cases, the time necessary for the establishment of suppression is nearly the same, and the pattern of suppression induced by injection of T cells from twice sensitized mice (Tj2s) is very similar in that a chronic suppression of Igh-1b expression was obtained without affecting the other productions tested of IgM, IgD, and IgA of Igh^b haplotype. In contrast to the homozygous recipients of 4×10^7 Tj2s, which all developed a chronic Igh-1b suppression, all the homozygous mice having received the same number of Tj1s were still expressing Igh-1b at 30 wk of age. These data show that the second sensitization significantly enhanced the suppression inducer activity within the Igh^a donor's T splenocytes. In the same manner, we had previously shown with heterozygotes that this activity was strongly increased in Tj from Igh^a mice sensitized once compared with normal Igh^a mice (15).

While we were establishing our data, a report on allotypic suppression induced by total spleen cells neonatally injected in heterozygous mice was published (36) in which the authors state that "neonatal $IgG2a^b$ suppression was harder to achieve in homozygous CB20 mice, and titers scattered. However, at the age of 1.5 yr, seven out of eight animals did not have detectable allotype b levels anymore. Allotype a titers were high instead (data not shown)."

An important face of the current report is to present the first example of in situ experimental breaking of mouse chronic allotypic suppression. In vivo treatments of Igh-1b-suppressed mice were performed with anti-CD4 or anti-CD8 mAb. In both systems (CB20 and C57BL/6), abrogation of the suppression was observed with anti-CD8 treatment only. In a first series of experiments, we used mAbs partially purified from ascitic fluids. Three out of the four mice that had received two intravenous injections of anti-CD8 preparation were shown to express serum Igh-1b 2 wk after the last injection. Two of these three mice permanently expressed this allotype (meaning, during >3 mo, date of the last bleeding tested), while the Igh-1b production of the other mouse was only transient. We have to emphasize that during all our studies we never observed any spontaneous break of Igh-1b suppression.

In a second series of experiments, 1-wk in vivo treatment with culture supernatant containing the anti-CD8 mAb was sufficient to abrogate the chronic suppression as judged by the detection of large amounts of Igh-1b (up to 0.5 mg/ml) in the serum of the treated mice 1 d after the end of the treatment. At this time, $CD8^+$

cell percentages were severely reduced in spleen and in blood (96 and 98% of depletion, respectively), while $CD4^+$ cell percentages remained similar to those found with untreated controls. Concerning the specificity of the anti-CD4 mAb, the converse situation was observed in 1-wk-treated mice. We verified that the observed decreases of cell percentages were not due to shielding of CD4 and CD8 antigenic determinants by the injected mAbs, but reflected effective cell depletion. This depletion of $CD8^+$ cells in homozygous allotypically suppressed mice was sufficient to quickly reverse the equilibrium between the helper and suppressive effects upon the Igh-1b producers.

This induced Igh-1b production shows that the maintenance of the suppression was not the result of a clonal deletion but an active $CD8^+$ lymphocyte (suppressor or cytotoxic cell) -dependent mechanism that is reversible. In contrast, experiments carried out with heterozygous mice (manuscript in preparation) show that the capacity of sensitized T cells to induce Igh-1b suppression is abolished by both either anti-CD4 or anti-CD8 treatments. In addition, the Igh-1b production induced by anti-CD8 treatment seems too rapid and too large to be ascribed to B lymphocytes derived from remote stem cells after the mAb treatment. It could be proposed that Igh-1b producer lymphocytes were, before the in vivo mAb treatment and, thus, during the life of the Igh-1b-suppressed mice, very close to producing this allotype (an assumption under current investigation). This implies that these Igh-1b producer cells permanently trigger the involved $CD8^+$ cells, which agrees with our previous observation that T splenocytes from sensitized $Igh^{a/a}$ mice and from Igh-1b-suppressed $Igh^{a/b}$ mice had the same efficiency in inducing suppression in heterozygous F_1 mice (15), whereas, normal T cells from $Igh^{a/a}$ mice had a much lower one (14).

Nevertheless, after the appearance of large amounts of serum Igh-1b in these anti-CD8-treated mice, the quantities started to decrease, and 35 d after the last injection, all four mice had almost no Igh-1b in their serum. The difference observed between the two experiments in the duration of Igh-1b expression after the anti-CD8 treatment might be due to the fact that mice from the first experimental series were 6 mo older than mice from the second one and/or might reflect the difference in mAb concentration in ascitic fluids compared with culture supernatants, which were used for the treatments. This concentration was indirectly estimated by thymocyte staining giving in the first case an immunofluorescence titer of 1:8,000 and of 1:240 in the second case.

In animals having suffered the anti-CD8 culture supernatant treatment, this Igh-1b suppression was then abrogated once more by a similar 1-wk anti-CD8 treatment. From these data, we propose that after the clearance of the injected anti-CD8 mAb containing culture supernatant, new T cells are raised with an activity directed against the reappearing Igh-1b producers, establishing again the observed suppression. Further, these T cells could derive from precursors (whatever their origin: Igh^a donors or Igh^b hosts) coming from the thymus (an organ unaffected by the mAb treatment) which have matured in $CD8^+$ cells.

Summary

We report here the successful induction of allotype suppression in homozygous $Igh^{b/b}$ mice (CB20 or C57BL/6) by neonatal injection of T splenocytes from Igh^a congenic sensitized mice (BALB/c or BC8, respectively). The sensitization of the

T cell donors was achieved by two intravenous injections of B splenocytes from Igh^b congenic mice. Treated homozygous $Igh^{b/b}$ mice developed, as of 16–24 wk of age, a chronic suppression of Igh-1b expression (IgG2a of Igh^b haplotype). The other productions tested (IgM, IgD, and IgA) of Igh^b haplotype were unaffected.

In vivo treatment with cytotoxic anti-CD4 or anti-CD8 mAb of mice subjected to chronic Igh-1b suppression clearly showed that $CD8^+$ lymphocytes (suppressor or cytotoxic cell) were essential for the maintenance of the suppression. The suppression was indeed abrogated after a 1-wk treatment with anti-CD8 mAb containing culture supernatant, whereas, the anti-CD4-treated mice continued to be subjected to suppression. This anti-CD8 in vivo treatment was shown to have no effect on thymus but to severely reduce the percentages of $CD8^+$ cells in spleen and in peripheral blood without affecting the percentages of $CD4^+$ cells, leading to a large and rapid Igh-1b expression (up to 0.5 mg per ml of serum, the day after the end of the treatment). This suppression abrogation, and thus the Igh-1b expression, was either transient or permanent. When it was transient, a second 1-wk treatment with anti-CD8 mAb containing culture supernatant induced once again a rapid and significant production of Igh-1b (up to 0.3 mg of Igh-1b per ml of serum).

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