

## A ROLE FOR CLONAL DOMINANCE IN THE MAINTENANCE OF ALLOTYPE SUPPRESSION?\*

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Allotype suppression in the rabbit is characterized by a deficiency in immunoglobulin of a particular allotype and a compensatory increase in immunoglobulin carrying an alternate allotypic marker (1). Suppression is induced by perinatal treatment of the animal with antibody specific for the allotype that is to be suppressed, and it is perpetuated for months or years by mechanisms that remain to be elucidated. Among the possible causes of the lasting suppression are clonal elimination as a direct consequence of the treatment used in the induction of suppression, and an active autoimmune response that follows the phase of passive suppression and which might be mediated by specific suppressor cells. Although the former possibility has not been totally eliminated as a contributing factor in the early stages of suppression, there exists no evidence for cytotoxicity of anti-allotypic antibodies, and this mechanism would not explain an important attribute of chronic suppression, namely the increasing number of cells bearing the suppressed allotype on their membranes during a period when serum concentrations of the suppressed allotype remain profoundly depressed (2).

Positive and direct evidence for the existence of suppressor cells in the chronically suppressed rabbit, such as has been obtained in the suppressed mouse (3) is still lacking although there exists some indirect evidence for that mechanism in rabbits (4). In the work to be described we have explored a third possible mechanism, that of clonal dominance. This mechanism would not entail any form of specific suppression but rather would result from a positive and selective pressure by antigens upon clones of cells that produce the originally operational alternate allotypes. Although, under natural conditions, this role would be assigned to environmental antigens, the present study confines itself to a model in which immunological memory is established to selected defined antigens during passive suppression. The data show that this procedure tends to restrict subsequent recall responses to antibodies of the nonsuppressed type at a time when recovery from suppression has progressed sufficiently to allow antibodies of the suppressed and nonsuppressed types to be formed in response to antigens not previously encountered.

### Materials and Methods

*Rabbits.* Rabbits suppressed with respect to the b5 kappa-chain allotypic determinant were produced by mating females of a1/b9 or a1a2/b9 allotypes which had been intensively immunized against a1/b5 gamma globulin with males of the a1/b5 allotype. The resulting a1/

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(b5)b9 or a1a2/(b5)b9 young were immunized with 2–5 mg amounts of ovalbumin (OA),<sup>1</sup> horse spleen ferritin, keyhole limpet hemocyanin, or with 10<sup>9</sup> plaque-forming units of T2 bacteriophage. The proteins were given in saline intramuscularly, the phage was injected intravenously. The time and frequency of injections is shown in Figs. 1 and 2.

*Quantitation of b5 and b9 Immunoglobulins.* Concentrations of Ig of a given allotypic specificity in sera or purified antibody solutions were measured either by hemagglutination inhibition (HI) as previously described (5) or by single radial immunodiffusion (SRID). For this purpose the original procedure (6) was slightly modified by incorporating 2% polyethylene glycol (mol wt 6,000–7,500) into 1.5% Noble agar. Circular wells of 35-mm diameter were charged with 14- $\mu$ l aliquots of appropriately diluted specimens or standard Ig preparations and the diameters of precipitate rings were measured after 3–4 d of incubation. The anti-b5 serum used in these assays was one produced in b9 rabbits and the anti-b9 reagent was from a b5b6 animal.

Solid-phase radioimmunoassays (RIA) for the direct estimation of antibodies with b5 or b9 markers in unfractionated sera employed 96-place hemagglutination trays in which the wells had been coated with OA or HC. This was accomplished by overnight incubation at 37°C with solutions of 0.1–1 mg/ml antigen. After removal of nonattached antigen by washing, the wells were charged with 25  $\mu$ l amounts of appropriate dilutions of sera to be tested or standard b5 and b9 antisera, followed by 4 h of incubation at 4°C, washing, and the addition of 25  $\mu$ l amounts of anti-b5, anti-b9, or goat anti-rabbit Ig, each trace labeled with <sup>125</sup>I. After overnight at 4°C the wells were washed, cut, and counted in a gamma counter. Standard sera against OA and HC, made in b5 and b9 homozygous rabbits and calibrated by quantitative precipitation tests for their specific antibody content, provided the reference curves from which the antibody content of unknowns was estimated.

*Purification of Antibodies.* To obtain purified antibody preparations, antisera were passed over cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N.J.) to which either OA or FER had been linked. After exhaustive washing with buffered saline, pH 7.3, the antibody was eluted with 0.17 M glycine-HCl buffer, pH 2.5, neutralized with 1 N NaOH and dialyzed against phosphate-buffered saline, pH 7.3. The volume was usually adjusted to  $\sim$ 1/10 of that of the antiserum applied.

*Phage Neutralization.* The procedure used was exactly as previously described in which anti-allotype serum is used to amplify the feeble activity of early IgM antibodies and in doing so identifies the allotypic marker of the anti-T2 antibodies. The 30% endpoints have been validated as statistically highly significant (7).

*Membrane-bound Ig.* To enumerate peripheral blood lymphocytes with bound Ig of the b9 or b5 allotypes a rosetting assay was employed which used sheep erythrocytes to which purified anti-b5 or anti-b9 had been linked covalently. Details of the test have been previously described (8).

## Results

*Allotypic Specificity of Antibodies Produced During Spontaneous Recovery From Suppression.* In the experiment to be described we employed a litter of seven rabbits of the genotype a1/b5b9 which were suppressed for b5. Four rabbits, making up group A, were given a single dose of 5 mg OA at the age of 6 wk and three (group B) received 5 mg FER at that age. When these animals were 9–10 mo old and had partially recovered from suppression, they were immunized, first with an antigen they had not previously encountered, namely FER for group A and OA for group B, then with a recall dose of the antigen they had received at 6 wk, and finally with yet another antigen, bacteriophage T2. This design allowed each animal to serve as its own control with respect to its selection of cells producing Ig of the alternate allotypes for the production of antibodies against each of the several antigens. One rabbit in group A

<sup>1</sup>Abbreviations used in this paper: FER, horse spleen ferritin; Hc, keyhole limpet hemocyanin; OA, ovalbumin.

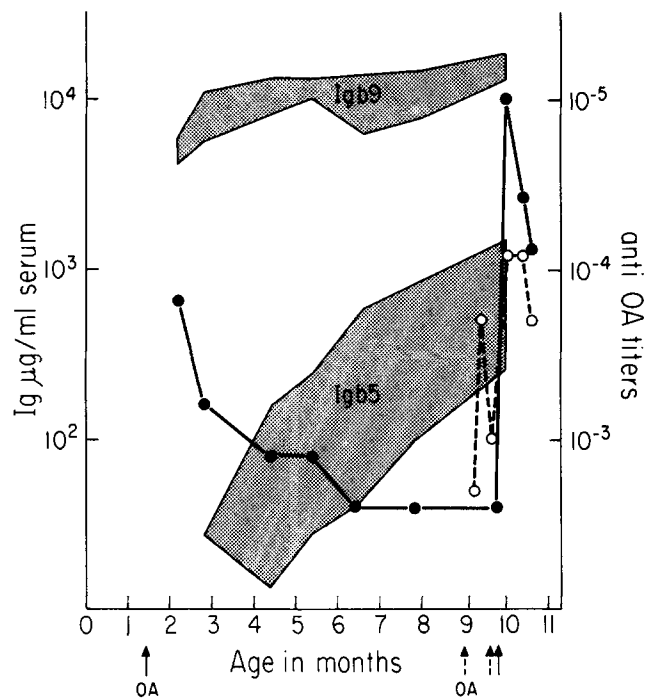


FIG. 1. Spontaneous recovery from suppression and antibody responses to ovalbumin. —, OA injections and responses in rabbits of group A; ----, OA injections and responses in rabbits of group B.

remained profoundly suppressed at the age of 10 mo and therefore was eliminated from further consideration.

The kinetics of recovery from suppression in this group of animals are depicted in Fig. 1. Immunoglobulin with the b5 marker appeared in the sera when the rabbits were 3–4 mo old and its concentration increased gradually to 0.3–1.5 mg/ml at 9–10 mo. The nonsuppressed Igb9 remained at a rather constant level of 10–20 mg/ml during the entire period. Cellular recovery from suppression is documented in Table I where the previously noted differences in the rates and extent of cellular and humoral recovery are seen. Because nonsuppressed b5b9 rabbits generally have b9/b5 ratios of  $\sim 0.3$  both in serum Ig and in blood lymphocytes carrying these markers it is apparent that recovery was incomplete at 10 mo.

Also shown in Fig. 1 are the responses of rabbits in group A to the first and to the recall injections of OA at 6 wk and 10 mo, respectively, and the anti-OA responses of rabbits in group B to 2 injections of OA at 9–10 mo of age. The data show that injection at 6 wk elicited an antibody response that also effectively primed the animals for a brisk secondary response at 10 mo. Rabbits given their first injection of OA at 9 mo did not produce significantly more antibody than did those injected at 6 wk and they were given a second injection to yield the amounts of antibody required for allotypic analysis.

The responses to FER are not shown because injection of this antigen into the 6-wk-old rabbits failed to prime effectively and, indeed, resulted in partial tolerance. It was noted, nevertheless, that in their secondary response to FER the animals of group

TABLE I  
*Spontaneous Cellular and Humoral Recovery from Suppression*

	Age			
	3	4	6	8
	<i>mo</i>			
Serum Ig				
b5 (mg/ml)	<0.02	0.08 (0.01-0.15)	0.23 (0.05-0.6)	0.45 (0.1-0.8)
b9 (mg/ml)	7.9 (5.5-11.3)	11.3 (7.2-13.2)	11.6 (6.2-13.8)	12.2 (7.8-14.5)
b9/b5		282 (80-593)	87 (23-240)	40 (15-77)
Blood lymphocytes				
b5 (percent)	1.3 (0.5-3.1)	2.8 (1.9-5.1)	10 (7.2-18)	11 (8.7-16.3)
b9 (percent)	37 (27-50)	40 (34-46)	50 (40-59)	49 (43-64)
b9/b5	40 (29-56)	16 (8-24)	5.1 (2.9-7.8)	4.8 (3.1-7.4)

Data are means and, in parentheses, ranges determined by hemagglutination inhibition (serum) and rosetting (cells) techniques, respectively.

TABLE II  
*Allotypes of Anti-OA Made by Rabbits during Spontaneous Recovery from Allotype Suppression*

Group	Rabbit	Primed with	Purified antibody		Ratio b9/b5	
			b5	b9	Purified Ab	Serum
			$\mu\text{g/ml}$			
A	198	OA	2.4	5,350	2,230	7
	199		2.4	5,990	2,500	5
	201		<2.4	3,850	>1,600	10
B	202	FER	402	3,000	8	10
	203		50	1,600	32	40
	205		152	2,350	15	40

Rabbits primed at 6 wk of age as indicated; injected or reinjected with OA at 9-10 mo. Data based on hemagglutination-inhibition assays.

B made antibody that was exclusively b9 whereas the controls of group A made predominantly b9 antibodies but also measurable responses expressed in b5 molecules.

The results of allotype analyses of anti-OA antibodies made by these rabbits are shown in Table II. It will be noted that the anamnestic response of group A was restricted to b9, the allotype of Ig made by cells that were active at the time of first exposure of these rabbits to OA. The anti-OA made by rabbits of group B consisted of b5 and b9 molecules, and the proportion of antibodies with these specificities closely resembled the ratio of b5 and b9 in the sera of these animals. It is apparent, therefore, that in the partially recovered rabbits there exists a pool of cells capable of making anti-OA of the b5 type.

Further evidence for the availability of cells capable of producing antibodies of the b5 type in our animals at the age of 10 mo was obtained in a study of their responses to T2 phage. Using a single injection, followed by bleeding 5 d later, and applying a neutralization assay with amplification by antiallotype sera, we obtained the data shown in Table III. It will be noted that the IgM anti-T2 response (8) consisted of

TABLE III  
*Allotypes of Anti-T2 Phage in Rabbits During Spontaneous Recovery From Allotype Suppression*

Group	Rabbit	Primed with	Neutralization titer of anti-T2 with allotype		Ratio of b9/b5	
			b5	b9	Neutr. Ab	Serum
A	198	OA	1,600*	9,600	6	7
	199		1,600	9,600	6	5
	201		150	4,800	32	10
B	202	FER	300	6,400	21	10
	203		200	9,600	48	40
	205		200	4,800	24	40

\* IgM responses, amplified with anti-b5 or anti-b9, expressed as NT30 (30% neutralization titers). Responses listed are those observed 5 d after a single injection of T2 at 10 mo of age.

both b5 and b9 antibodies, and that the ratio of these allotypes in the antibody closely resembled the ratio of the allotypes in total serum Ig. The b5 responses of all rabbits to T2 phage and the b5 responses of group B animals to OA are in sharp contrast to the exclusively b9-anti-OA responses of rabbits in group A and suggest a causal relationship between the priming of group A animals with OA during total b5 suppression and the restricted b9-anti-OA response of these animals after partial recovery from b5 suppression.

*Allotypes of Antibodies Produced by Rabbits Released from Suppression.* Because the slow rate of spontaneous recovery from suppression retarded progress in this investigation we sought to take advantage of rescue maneuvers used by others and by us in previous studies (9, 10). A litter of nine rabbits genotypically a1/b5b9 or a1a2/b5b9 and suppressed for b5, was divided into two groups (C and D). Because the injection of Ig of the suppressed type effects accelerated release from suppression only when given before the 5th wk of life, the time for the first (priming) injection with antigen was advanced to the 14th d of life and the dose was reduced to 2 mg. This amount of Hc was given to group C rabbits, and a similar amount of OA to rabbits of group D. The releasing injection consisted of 7 ml of a2a3/b5 serum; the a3 was to serve as an indicator of the disposition of this serum in the recipients.

Shown in Fig. 2 are the responses of a representative rabbit of group C. The b5 serum levels seen reflect the results of the injection of the releasing serum into 3.5-wk-old b5-suppressed rabbits. The initial rise and decline of b5 closely parallel increase and decrease of the other donor marker, a3; the subsequent plateau and increase of b5 alone represent b5 synthesis by the recipient and show that by the age of 3 mo suppression had been relieved sufficiently to allow production of 1 mg/ml serum Igb5. Also seen in Fig. 2 is the primary antibody response to Hc which peaked 1 mo after immunization of the 2-wk-old rabbit. The secondary response to Hc suggests that adequate priming had been attained. The response to OA, first injected at 3 mo, was similar to that seen in rabbits injected with this antigen at 1.5 or 9 mo of age (Fig. 1).

The allotypic composition of anti-Hc responses is shown in Table IV. It will be noted that at 17 wk, when serum ratios of b9/b5 averaged 7 ( $\pm 2$ ), the Hc-primed rabbits made anti-Hc which was almost exclusively of the b9 allotype whereas the OA-primed animals made anti-Hc of the b5 as well as of the b9 allotypes. In this

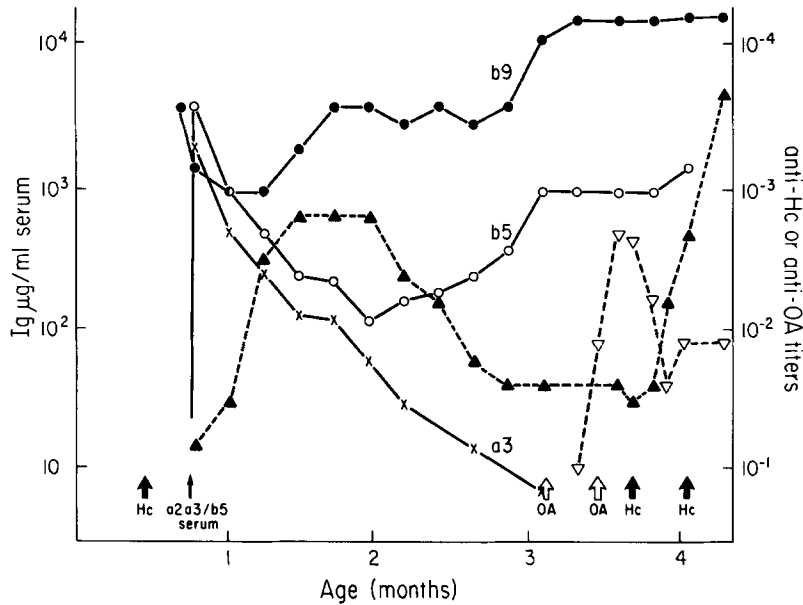


FIG. 2. Responses to releasing injection and to antigens. —●—●—, b9; —○—○—, b5; —×—×—, a3; —▲—▲—, Anti-Hc; —▽—▽—, Anti-OA. Rabbit 296, of allotype a1/(b5)b9, injected with hemocyanin and ovalbumin as indicated, before or during recovery from b5-suppression accelerated by the injection of a2a3/b5 serum at 3.5 wk of age. Concentrations of Ig determined by hemagglutination inhibition.

experiment (Tables IV and V) allotype analyses were done not only on purified and concentrated antibody preparations which were examined by hemagglutination-inhibition or single radial diffusion but also on anti-Hc or anti-OA in unfractionated sera. This duplication was considered desirable to minimize the shortcomings inherent in each of the methods and to guard against possible bias introduced by incomplete recovery of antibodies from immunoadsorbents. It will be seen that concordant results were obtained by these diverse procedures. The b9/b5 ratios for anti-Hc were in excess of 65 for group C, the Hc-primed group, and 4 for group D, the controls. Also shown in Table IV are the results obtained after more intensive immunization, followed by bleeding at 23 wk of age. Although the differences between experimental and control animals diminished, those of group C still engaged in the preferential synthesis of b9-anti-Hc whereas animals of group D made anti-Hc in which the b9/b5 ratio was 1 or 2.

Anti-OA made by rabbits in these groups once again followed the pattern of dominance of the allotype that was operational at the time of first exposure to the antigen. The data in Table V show that the OA-primed animals of group D responded at 18 wk with antibody that was predominantly b9, whereas controls made anti-OA which in its b9/b5 ratio closely resembled whole serum Ig. Rabbit 297 responded so poorly to OA at 18 wk that its response could not be properly analyzed. After hyperimmunization and at 27 wk of age the differences between experimental and control animals still persisted. It is noteworthy that at both ages the responses of OA-primed animals to OA were significantly less than those of controls, suggesting that 2 mg OA at 2 wk had not only evoked antibody formation and memory, but had also

TABLE IV  
*Allotypes of Antibodies to Hemocyanin in Rabbits Released from Suppression*

Group	Rabbit	Anti-Hc antibody		b9/b5		Serum b9/b5‡
		b5 (µg/ml)*	b9 (µg/ml)*	RIA*	SRID‡	
C HC-primed 17 wks	294	0.6	98	165	55	9
	295	0.9	38	42	44	7
	296	1.7	79	46	44	8
	300	<1	100	>100	54	5
	301	3.4	123	35	60	4
D OA-primed 17 wks	297	34	105	3	3	6
	298	16	125	8	6	7
	299	52	158	3	2	4
	302	43	223	5	3	7
C 23 wks	294	27	496	18	23	4
	295	89	655	7	8	4
D 23 wks	297	670	1,010	2	4	3
	298	416	437	1	3	4

\* Radioimmunoassay for anti-Hc of the b5 or b9 allotype, using whole sera.

‡ Single radial immunodiffusion on purified anti-Hc antibody preparations or whole sera.

induced hyporesponsiveness. This finding resembles that of a diminished anti-ferritin response in rabbits primed with ferritin at 6 wk (group B).

### Discussion

The data presented here show that the administration of antigens during total allotype suppression determines the allotypic distribution of recall responses to these antigens during recovery from suppression. The most probable mechanism is the establishment and proliferation of antigen-specific memory cells committed to the production of Ig of the originally operational allotype. Subsequent challenges with the antigen would reinforce this process and yield antibody disproportionately enriched in molecules of the nonsuppressed allotype even when recovery has sufficiently advanced to allow expression of responses to newly introduced antigens in which the ratio of nonsuppressed to suppressed allotypes in the antibody fraction resembles that found in serum. This interpretation invokes the phenomenon of clonal dominance, previously observed in the serial transfer of a myeloma cell line (11), the distribution of idiotypes (12) and also invoked in an attempt to explain the preponderance of kappa over lambda Ig in mice (13). An earlier study had shown that some a1a3 rabbits, neonatally suppressed for a1 and immunized from the 4th wk on, continued to make antibodies deficient in a1 molecules even after partial recovery from suppression had occurred. The authors invoked specific feedback and space restrictions as possible causes (14).

Because it has been shown that antibody responses of heterozygous rabbits to certain antigens can be markedly skewed (15, 16), those used in the present investigation were deliberately chosen from a group of complex antigens and the data proved this selection to be adequate. There is a slight suggestion that anti-OA

TABLE V  
*Allotypic Distribution of Anti-ovalbumin in Rabbits Released from Suppression*

Group and Age	Rabbit	Anti-ovalbumin			Serum b9/b5
		b5	b9	b9/b5	
C (HC-primed) 18 wk	294	15*	46	3	9
	295	6	17	3	7
	296	11	37	3	8
	300	7	16	2	5
	301	6	23	4	4
D (OA-primed) 18 wk	297	(1)	(5)	(5)	6
	298	(1)	32	(32)	7
	299	(1)	12	(12)	4
	300	(1)	48	(48)	7
C 27 wk	294	245	333	1	4
	295	141	268	2	4
D 27 wk	297	11	94	9	3
	298	8	74	9	4
	290	8	79	10	3

\* Microgram = specific antibody per milliliter serum. Values in parentheses are estimated. Data are based on the results of radioimmunoassays.

responses in the partially recovered (b5)b9 rabbits of the control groups (not primed with OA during total suppression) were slanted toward a preferential engagement of b5-producing cells but not to a degree sufficient to obscure the results.

For control purposes we have also injected nonsuppressed b5b9 rabbits with hemocyanin at 2 wk of age and have analyzed sera and antibodies for their allotypic composition. The data showed close agreement between the ratios of the two allotypes in antibody and in serum.

It is of interest to note from the anti-OA responses of rabbits in groups C and D that the injection of OA at 2 wk of age rendered recipients hyporesponsive to OA (Table V) and yet established clonal dominance. The peak responses of these animals to the first injection were modest (HA titers of 1:100 to 1:300) and although their responses to a second injection at 18 wk were less than that of controls, it had the kinetic aspects of a recall response. It seems possible that in these animals the primary injection engendered both B-cell memory and T-cell suppressor responses.

The demonstration of clonal dominance in rabbits undergoing recovery from allotype suppression suggests a possible causal relationship between clonal dominance and chronic suppression. If a substantial portion of normal Ig were antibody directed against environmental antigens, and if exposure to such antigens during the neonatal period of total suppression resulted in the fixation of antibody responses to these antigens in clones of cells committed to production of the nonsuppressed type, one could imagine a major role for this mechanism in perpetuating apparent suppression even in the absence of an actively suppressing process. Experiments designed to test the merit of this suggestion have been initiated with representative antigens.

One additional observation recorded in this report calls for attention because it suggests that clonal dominance alone fails to account for all the attributes of chronic



allotype suppression in the rabbit. The antibodies produced in response to select antigens injected for the first time after partial recovery from suppression contain molecules bearing the nonsuppressed and suppressed markers in a proportion that closely resembles that prevailing in whole serum and differs from that of lymphoid cells carrying these markers on their membranes. The similarity of allotypic distribution in the serum and in purified antibody made by the recovering rabbit has previously also been noted (17). Because it seems more likely that the immediate precursors of cells destined to produce antibody against OA belong to the class of B cells not yet engaged in the secretion of Ig, it would seem to follow that whatever mechanism blocks differentiation and maturation of B cells toward secretion of the suppressed allotype also affects the subclass of such B cells which could respond with anti-OA synthesis and secretion. Obviously further studies are needed to elucidate the relative roles of clonal dominance and of other mechanisms in the maintenance of allotype suppression.

### Summary

The establishment of immunological memory during the early and complete phase of allotype suppression in the young rabbit has been shown to lead to the preferential production of antibodies with the nonsuppressed allotypic specificity in response to recall injections given after spontaneous or induced release from suppression. It is suggested that this manifestation of clonal dominance, applied to stimulation by environmental antigens, may contribute to the long lasting persistence of allotype imbalance in allotype suppressed rabbits.

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