Brief Definitive Report

RELEASE FROM MATERNALLY-INDUCED ALLOTYPIC SUPPRESSION IN RABBIT BY NOCARDIA WATER-SOLUBLE MITOGEN

By C. BONA* and P. A. CAZENAVE

(From The Centre National de Recherche Scientifique, and the Pasteur Institute, Paris, France)

In vivo allotypic suppression in rabbits has been described in newborn heterozygotes which were offspring of mothers immunized against the father's immunoglobulin allotype (1) or which were injected at birth with antiserum directed against the father's allotype (2). This suppressed allotype is either entirely unexpressed or expressed in very low amounts (1).

In rabbit in vitro systems, anti-allotypic antibodies induce blast transformation (3) and inhibit the synthesis of allotypes (4) whereas Nocardia water-soluble mitogen (NWSM) is a B-cell mitogen for several mammalian species (5, 6) as polyclonal activator induces the differentiation of rabbit small lymphocytes to plasma cells and their polyclonal activation (7).

The aim of the present work was to study the synthesis of allotypes by lymphocytes from suppressed rabbits subsequent to in vitro stimulation by NWSM.

Materials and Methods

Animals. 4 day, 3, 6, and 12-wk-old offspring (b4/b5) were obtained from b4/b4 mothers immunized against the paternal allotype b5/b5 according to methods described by Dray (1), 12-mo old offspring which had escaped from maternally allotypic suppression and 12-mo old normal heterozygous b4/b5 rabbits (Pasteur Institute, Garches, France) were used in these experiments.

Reagents. Rabbit antiallotypic sera directed against the a and b series allotypes were obtained according to Oudin (8) NWSM from *Nocardia opaca* was prepared according to Ciorbaru et al. (9) Concanavalin A (Con A) was obtained from Miles Yeda Ltd. and [^{3}H]thymidine with 1 Ci/mM sp act was obtained from Commissariat a l'Energie Atomique, Saclay, France.

Blast Transformation Assay. Single cell suspensions were obtained from spleens according to a previously described technique (7). Lymphocytes (1.5×10^6 were cultured for 3 days in 1 ml RPML-1640 medium (Grand Island Biological Company, Grand Island, N. Y.) supplemented with 15% heat inactivated b6/b6 rabbit serum. The cultures were performed in plastic tubes which were incubated in an incubated model 1-H 100 Gallenkamp under a continuous flow mixture of 5% CO_2 and 95% Air. [^{3}H]thymidine (1 μCi) was added to each culture tube 12 h before harvesting the cells and incorporation of radioactivity was measured by liquid scintillation counting (7).

Measure of In Vitro Synthesis of Allotype. Splenic lymphocytes (25×10^6) were cultured for 72 h in 5 ml RPML-1640 medium supplemented with 10% fetal calf serum (Flow Laboratories, Inglewood, Calif.) in the same conditions described above.

The amount of immunoglobulin in 72-h supernatant cultures was measured by a quantitative inhibition radioimmunoassay. IgG fractions were prepared from b4/b4 and b5/b5 homozygous rabbits by DEAE cellulose chromatography (10) and were labeled with ^{125}I, according to Green-

* Present address: Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md. 20014

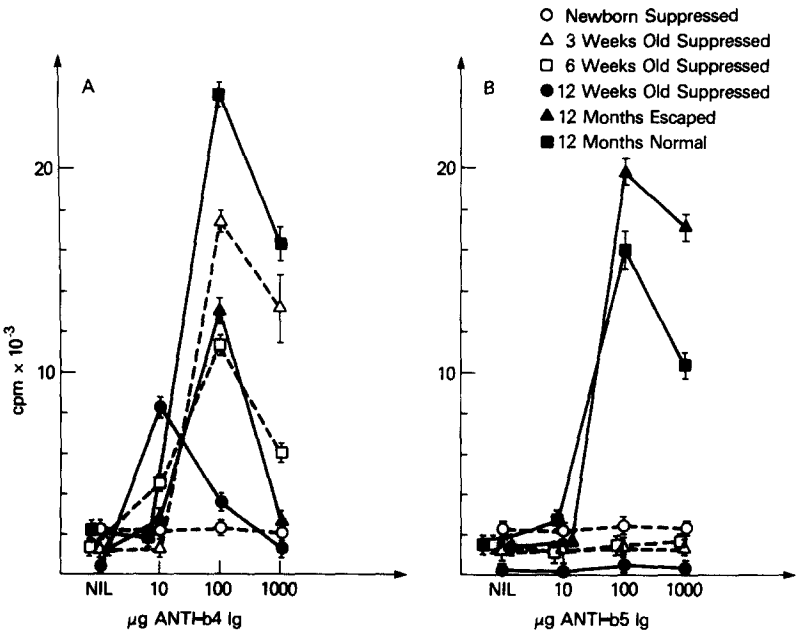

FIG. 1. Stimulation of rabbit lymphocytes by anti-b4 and anti-b5 anti-allotypic antibodies. (A) Cells incubated with anti-b4 anti-allotypic serum. (B) Cells incubated with anti-b5 anti-allotypic serum. 1.5×10^6 lymphocytes were incubated for 72 h in 1 ml RPMI medium supplemented with 10% b_6/b_6 serum with various concentrations of anti-allotypic antibodies. Lymphocytes originated from suppressed rabbits b4(b5), normal adult b4/b5, and adult rabbit b4/b5 escaped from allotypic suppression.

wood and Hunter (11). The rabbit anti-allotypic sera were polymerized by using ethyl-chloroformate according to Avrameas and Ternynck (12). In the inhibition assays known amounts of unlabeled IgG carrying a given allotype or various dilutions of culture supernate were used to inhibit the binding of ^{125}I IgG (carrying the same allotype) by anti-allotype immunoadsorbent. The inhibition of radioimmunoassay was performed according to Landucci-Tosi and Mage (13).

Results

The proliferative response of lymphocytes from b4/b5 offspring suppressed for paternal allotype b5/b5 was studied and compared to lymphocytes of offspring that had escaped from allotypic suppression as well as lymphocytes from normal b4/b5 rabbits. We have previously studied the blast response induced by anti-allotype antibodies and NWSM which stimulate B cells and by Con A which stimulates T cells (14).

As can be seen in Fig. 1 A, anti-b4 allotype antibodies (i.e., against nonsuppressed allotype) induce a significant [^{3}H]thymidine incorporation excepting the lymphocytes from 4-day-old suppressed rabbits which developed a weak blast response. This low response of neonatal suppressed rabbits is related to the age of the animal rather than to the state of suppression since we have previously found a very weak blast response in the normal newborn rabbits.[1] Very few cells

[1] P. A. Cazenave, D. Juy, and C. Bona. Ontogeny of lymphocyte functions during embryonic life of rabbit, manuscript submitted for publication.

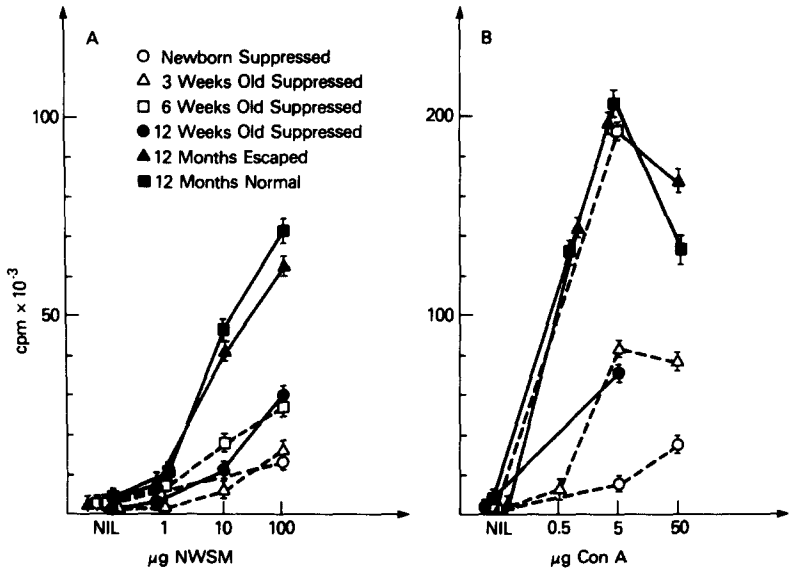

FIG. 2. Stimulation of rabbit lymphocytes by NWSM and concanavalin A. (A) Cells incubated with NWSM. (B) Cells incubated with Con A. 1.5×10^6 lymphocytes were incubated for 72 h in 1 ml RPMI medium supplemented with 10% b_6/b_6 serum with various concentrations of mitogens.

can be stained by anti-rabbit μ-antibodies in neonatal rabbits (R. Mage, personal communication).

Anti-b5 antibodies did not stimulate [^{3}H]thymidine incorporation in the case of rabbits suppressed for b5 allotype but induced significant stimulation in normal adult rabbits or in those that had escaped suppression (see Fig. 1 B). In all cases, the nonspecific B- or T-cells mitogens NWSM and Con A, induced a strong proliferative response dependent upon the dose of mitogen and the age of donor rabbit lymphocytes (see Fig. 2 A and B). The in vitro synthesis of allotypes was studied by incubation of lymphocytes for 18 h with mitogens. The lymphocytes were then washed three times and cultured an additional 72 h. The data presented in Table I show that only NWSM induced an increased synthesis of allotype of a and b series, whereas anti-allotype antibodies inhibited specifically the synthesis of correspondent allotype. Since only NWSM had the ability to induce increases of synthesis of immunoglobulin in normal rabbits, this mitogen was used to study the synthesis of allotype in the suppressed rabbits. In the following experiments, the optimal amount of mitogen was used, i.e., 10 μg NWSM for 5×10^6 cells/ml.

The synthesis of allotypes was studied on the lymphocytes from 4 day, 3, 6, and 12-wk-old suppressed rabbits and it was compared to those of adult normal rabbits or those rabbits which had escaped from allotypic suppression.

As can be seen in Table II, NWSM has increased the synthesis of b4 (nonsuppressed) allotype in suppressed, escaped from suppression, or normal rabbits. The most striking observation of our study was that this mitogen also induced the synthesis of b5 allotype in the case of suppressed rabbits. However, the synthesis of suppressed allotype was weaker in the case of 12-wk-old suppressed rabbits as compared with that of neonate or 3- and 6-wk-old animals.

TABLE I

*Influence of NWSM and Anti-Allotypic Sera on the In Vitro Synthesis of Allotypes by Rabbit Lymphocytes**

Lymphocytes‡ incubated with	Synthesis of allotypes expressed in ng/culture§		
	b4	b5	a3
Nil	3,500 ± 500	2,600 ± 200	2,400 ± 400
NWSM 1 μg	18,200 ± 2,600	7,300 ± 100	5,200 ± 200
10 mg	42,000 ± 6,000	10,000 ± 800	8,000 ± 100
100 μg	36,000 ± 4,000	17,300 ± 3,000	36,000 ± 800
Anti-b4 Ab 10 μg	740 ± 20	ND‖	960 ± 160
100 μg	720 ± 120	2,250 ± 550	960 ± 40
1,000 μg	320 ± 50	9,200 ± 400	5,400 ± 200
Anti-b5 Ab 10 μg	2,700 ± 350	132 ± 14	740 ± 50
100 μg	2,800 ± 200	126 ± 14	1,300 ± 80
1,000 μg	5,800 ± 200	76 ± 12	1,600 ± 50
Anti-a3 Ab 10 μg	1,400 ± 100	500 ± 20	240 ± 20
100 μg	2,800 ± 250	820 ± 210	440 ± 20
1,000 μg	3,800 ± 200	940 ± 15	2,460 ± 200

Nil, no mitogen added.
* Rabbit donor was b4/b5/a3/a3.
‡ 1.5 × 10^6 lymphocytes/ml.
§ 25 × 10^6 lymphocytes/culture.
‖ ND.

TABLE II

Influence of NWSM on In Vitro Synthesis of Allotype by Lymphocytes from Rabbits with Maternally-Induced Allotypic Suppression

Origin of spleen cells	Number of rabbits tested	Lymphocytes incubated with	Synthesis of allotypes expressed in ng/cultures	
			b4	*b5*
4-day-old suppressed rabbits	3	Nil	1,350 ± 65	<10
		NWSM*	6,500 ± 250	875 ± 10
3-wk-old suppressed rabbits	3	Nil	2,400 ± 400	<10
		NWSM	23,000 ± 1,000	2,200 ± 300
6-wk-old suppressed rabbits	2	Nil	2,600 ± 400	<10
		NWSM	21,500 ± 150	650 ± 50
12-wk-old suppressed rabbits	3	Nil	3,650 ± 100	<10
		NWSM	15,000 ± 450	70 ± 10
12-mo-old rabbit escaped from suppression	1	Nil	15,600 ± 4,400	5,200 ± 800
		NWSM	42,000 ± 6,000	17,000 ± 200
12-mo-old normal rabbit	2	Nil	4,120 ± 390	1,500 ± 100
		NWSM	13,300 ± 3,500	13,500 ± 200

Nil, no mitogen added.
* 5 × 10^6 cells/ml were incubated with 10 μg of NWSM.

Discussion

Our data demonstrate that NWSM which is a T-independent, B-cell mitogen in rabbits induces the in vitro synthesis of an allotype suppressed in vivo. In contrast to cells from normal b4/b5 rabbits, the cells from b5 suppressed animals were unable to synthesize b5 immunoglobulins in control, unstimulated cultures. Moreover, they were not stimulated to proliferate by anti-b5 allotype serum but were stimulated by anti-b4 antibodies.

The release from suppression by NWSM indicates that in the early phases of maternally induced allotypic suppression (i.e. up to 12 wk of age) there exist precursors of b5 producing cells which are unable to mature into Ig-secreting cells. Thus, NWSM which is known to induce the in vitro differentiation of resting lymphocytes into plasma cells (7) is able to break the maturational blockade of suppressed lymphocytes. Our data are in agreement with the

hypotheses advanced by Harrison et al. (15) who considered that allotype suppression in rabbits is due to a blockade of the maturation of suppressed cells. These authors demonstrated that during release from suppression, lymphocytes exist which actively synthesize the suppressed allotype on the surface but are unable to secrete it or to be stimulated by the corresponding anti-allotypic serum.

In the case of 12-wk-old suppressed rabbits, NWSM-induced synthesis of suppressed allotype was lower (70 ng/culture) when compared to that of the younger suppressed animals. These observations suggest that in the chronic phase of allotypic suppression other mechanisms participate in the regulatory process. Thus, the generation of suppressor T cells (16) or the production of anti-allotype antibodies (17) resembling Jerne's lymphocyte network (17) could be involved.

Summary

The in vitro synthesis of allotypes of b4/b5 offspring obtained from b4/b4 mothers immunized against paternal allotype b5/b5 was studied in comparison to similar offspring that had escaped from suppression and normal heterozygous b4/b5 rabbits.

Nocardia water-soluble mitogen—a rabbit B-cell mitogen which is known to induce the differentiation of small lymphocytes into plasma cells and polyclonal activation of Ig, was able to break in vitro the allotypic suppression induced in vivo.

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References

1. Dray, S. 1962. Effect of maternal isoantibodies on the quantitative expression of two allelic genes controlling γ-globulin allotypic specificities. *Nature (Lond.)*. **195**:677.
2. Mage, R. E., and S. Dray. 1966. Persistence of altered expression of allelic γG-immunoglobulin allotypes in heterozygous rabbits exposed to isoantibodies in fetal and neonatal life. *J. Immunol.* **95**:525.
3. Sell, S., and P. G. H. Gell. 1965. Studies on rabbit lymphocytes in vitro I. Stimulation of blast transformation with antiallotype serum. *J. Exp. Med.* **122**:423.
4. Schuffler, C., and S. Dray. 1974. *In vitro* immunoglobulin allotype synthesis by spleen cells of heterozygous rabbit. Specific suppression by anti-allotype antibody. *Cell. Immunol.* **10**:267.
5. Bona, C., Ch. Damais, and L. Chedid. 1974. Blastic transformation of mouse spleen lymphocytes by a water soluble mitogen extracted from Nocardia. *Proc. Natl. Acad. Sci. U.S.A.* **71**:1602.
6. Brochier, J., C. Bona, R. Ciorbaru, J. P. Revillard, and L. Chedid. 1976. A human T independent B lymphocyte mitogen extracted from Nocardia. *J. Immunol.* **117**:1434.
7. Bona, C., L. Chedid, C. Damais, R. Ciorbaru, P. N. Shek, S. Dubiski, and B. Cinader. 1975. Blast transformation of rabbit B-derived lymphocytes by a mitogen extracted from Nocardia. *J. Immunol.* **114**: 348.
8. Oudin, J. 1960. Allotype of rabbit serum protein. I. Immunochemical analysis leading to the individualization of seven main allotypes. *J. Exp. Med.* **112**:107.
9. Ciorbaru, R., A. Adam, J. F. Petit, E. Lederer, C. Bona, and L. Chedid. 1975.

Isolation of mitogenic and adjuvant active fractions from various species of Nocardiae. *Infect. Immun.* **11**:257.

10. Levy, H. B., and H. A. Sober. 1960. A simple chromatographic method for preparation of gamma globulin. *Proc. Soc. Exp. Biol. Med.* **103**:250.
11. Greenwood, F. C., and W. M. Hunter. 1963. The preparation of ^{131}I-labeled human growth hormones of high specific radioactivity. *Biochem. J.* **89**:114.
12. Avrameas, S., and T. Ternynck. 1967. Biologically active water-insoluble protein polymers. I. Their use for isolation of antigens and antibodies. *J. Biol. Chem.* **242**:1651.
13. Landucci-Tosi, S., and R. G. Mage. 1970. A method for typing rabbit sera for A14 and A15 allotypes with cross-linked antisera. *J. Immunol.* **105**:1046.
14. Bona, C., B. Cinader, and S. Dubiski. 1977. Cellular immunology of the rabbit. *Ann. Immunol. (Inst. Pasteur)* **128C**:38.
15. Harrison, M. R., G. J. Elfenbein, and R. G. Mage. 1974. Defective activation of b5 bearing lymphocytes in rabbits recovering from b5 allotype suppression. *Cell. Immunol.* **11**:231.
16. Herzenberg, L. A., K. Takamura, and C. H. Metzler. 1975. Regulation of immunoglobulin and antibody production by allotype suppressor T cells in mice. *Transplant. Rev.* **27**:57.
17. Jerne, N. K. 1974. Towards a network theory of the immune system. *Ann. Immunol. (Inst. Pasteur)* **125C**:373.