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IN VITRO STUDIES OF THE SUPPRESSION OF DELAYED HYPERSENSITIVITY BY THE INDUCTION OF PARTIAL TOLERANCE*

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Partial immunologic tolerance, which is produced by antigen treatment of newborn or adult guinea pigs, is manifested by depressed or absent delayed hypersensitivity in animals which produce normal amounts of certain classes of antibodies (1–4). In the present studies, the cellular basis of partial tolerance was investigated utilizing in vitro techniques. Normally, peritoneal exudate cells (PEC)[1] obtained from sensitized guinea pigs are inhibited from migrating out of capillary tubes by sensitizing antigen (5, 6). The inhibition of migration is due to a soluble material, migration inhibitory factor (MIF), which is elaborated by sensitized lymphocytes in contact with the appropriate antigen (7, 8). We found that peritoneal exudate cells from partially tolerant guinea pigs behave as cells from nonsensitized animals, i.e. their migration is not affected by addition of the tolerance-inducing antigen. This lack of in vitro responsiveness of peritoneal exudate cells from partially tolerant animals is accompanied by a failure of their lymphocytes to elaborate MIF when incubated with the tolerance-inducing antigen.

Materials and Methods

Animals.—Newborn and adult Hartley strain guinea pigs weighing 400–600 g were used for the induction of partial tolerance or sensitization. Albino guinea pigs weighing 250–350 g were used for passive cutaneous anaphylaxis.

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[1] *The following abbreviations are used in this paper:* BGG, bovine gamma globulin; BPA, bovine plasma albumin; DNPS, 2,4-dinitrophenylsulfonic acid; MIF, migration inhibitory factor; OCBC, ϕ-chlorobenzoyl chloride; and PEC, peritoneal exudate cells.

Antigens.—2,4-Dinitrophenylsulfonic acid (DNPS) (Eastman Kodak Co., Rochester, N. Y.), twice recrystallized, was coupled to bovine plasma albumin (BPA) (Armour Pharmaceutical Co., Kankakee, Ill.) and ϕ-chlorobenzoyl chloride (OCBC) (Eastman Kodak Co.) was coupled to bovine gamma globulin (BGG) as previously described (9, 10). Briefly, 500 mg of BGG was dissolved in 50 ml of distilled water containing 5 ml of 5% Na_2CO_3. To this was added, drop by drop, 2 ml of a mixture containing 0.4 ml of OCBC in 7.6 ml of dioxane. This mixture was centrifuged and 1 N HCl was added to the supernatant until a maximum precipitate was obtained. After incubation for about 3 hr at room temperature, the precipitate was spun down and resuspended in a small volume of distilled water. The precipitate was brought into solution by adding 1 N NaOH. It was then extensively dialyzed with distilled water made alkaline to pH of 7.8–8.0 with Na_2CO_3.

Induction of Partial Tolerance in Adult Guinea Pigs.—Adult animals were treated with a total of 468 mg/kg of OCBC-BGG in saline given subcutaneously in eight equal daily doses. After a rest period of 3 wk, the animals were injected with 200 μg of OCBC-BGG, and 200 μg of DNP-BPA. Both antigens were mixed 1:1 with complete Freund's adjuvant (Difco H37Ra), and each animal received a total volume of 0.4 ml, 0.1 ml/footpad. For controls, animals not treated with OCBC-BGG in saline were injected with the two antigens in complete Freund's adjuvant.

Induction of Tolerance in Adult Guinea Pigs with a Single Intravenous Dose of Aggregate-Free Antigen.—A solution of OCBC-BGG or DNP-BPA (18–30 mg/ml) in 0.15 M NaCl was centrifuged at 40,000 rpm in a Spinco Model L using a No. 40 rotor. 10 animals were injected intravenously with 1 ml (23.3 mg/ml) of the supernatant, which was presumed to be aggregate-free OCBC-BGG and 10 animals were similarly injected with 1 ml (17.7 mg/ml) of supernatant, aggregate-free DNP-BPA. All these animals, together with untreated controls, were injected with OCBC-BGG and DNP-BPA in complete Freund's adjuvant, as described above, immediately after the intravenous antigen injection.

Induction of Tolerance in Newborn Animals.—Guinea pigs were injected peritoneally during the 1st wk of life with a total of 32 mg of DNP-BPA divided into eight equal daily doses. After a rest period of 3 wk, these animals, together with noninjected control litter mates, were injected with 200 μg of DNP-BPA and 200 μg of OCBC-BGG in complete Freund's adjuvant as above.

Skin Tests.—All animals were skin tested with 50 μg of each antigen in saline 10 days after their sensitization with antigen in complete Freund's adjuvant. Readings were made 24 hr later.

Antibody Determination.—At the time of the in vitro studies the animals were bled and their sera assayed by passive hemolysis for γ_2 antibody and by quantitative passive cutaneous anaphylaxis (PCA) for γ_1 antibody (11, 12).

In Vitro Test for Cellular Hypersensitivity.—Peritoneal exudate cells from normal, sensitized, and tolerant guinea pigs were induced by an intraperitoneal injection of light mineral oil 2 wk after the skin test, and collected as previously described (9, 13). The cells were washed in balanced salt solution and made to a 10% suspension by volume in Eagle's minimum essential medium[3] with 15% normal guinea pig serum, 85 units of penicillin/ml, and 85 μg of streptomycin/ml. Capillary tubes were filled with the cell suspension, sealed with wax, and centrifuged. The tubes were cut and the portion containing the cells placed in Mackaness-type chambers, two per chamber. Cells from each animal were assayed as follows: two chambers for each of the antigens and two chambers with media, but no antigens. The antigen concentration was 100 μg/ml.

The chambers were incubated for 19 hr at 37°C and the area of migration was measured by planimetry as described previously (6). In calculating the data from these experiments, the

following formula was used:

$$\frac{\text{Average migration with antigen}}{\text{Average migration with no antigen}} \times 100 = \text{per cent migration.}$$

Production of Migration Inhibitory Factor.—Lymphocytes, obtained by teasing regional lymph nodes from guinea pigs sensitized with antigen in complete Freund's adjuvant, were incubated in minimum essential medium (Eagle's) without serum at a concentration of 2.4×10^7 cells/ml with and without specific antigen (100 μg/ml). After 24 hr of incubation, the cell suspensions were centrifuged at 12,000 rpm, and the supernatants removed and made to contain 15% guinea pig serum. The supernatants were tested for MIF activity (8).

RESULTS

Macrophage Migration Inhibition Assay Using Cells of Animals in Which Delayed Hypersensitivity was Suppressed In Vivo.—Adult guinea pigs pretreated with large intravenous or subcutaneous doses of OCBC-BGG in saline and subsequently injected with this antigen and an unrelated antigen, DNP-BPA, in complete Freund's adjuvant had markedly diminished delayed hypersensitivity to OCBC-BGG. However, delayed hypersensitivity to DNP-BPA was unimpaired. In addition, γ_2 antibody formation to OCBC-BGG was diminished or suppressed, while γ_1 antibody formation remained unaffected. Similar results were obtained regardless of whether tolerance was induced by neonatal injection, by multiple doses of antigen given subcutaneously, or by a single dose of aggregate-free antigen given intravenously. Results of skin tests and antibody titers on animals that were further studied by the in vitro assay are shown in Table I.

Peritoneal exudate cells from animals with suppressed delayed hypersensitivity skin tests to OCBC-BGG were assayed in vitro with that antigen and with the unrelated antigen, DNP-BPA. The results of these experiments can be seen in Table II. The peritoneal exudate cells from each of the 10 experimental animals were not inhibited from migrating in vitro by OCBC-BGG, the antigen used to suppress delayed hypersensitivity in vivo; the average migration of cells from these animals was 112.4%. In contrast, peritoneal exudate cells from the same 10 experimental animals were readily inhibited in vitro by the unrelated antigen (DNP-BPA), which also elicited delayed hypersensitivity in vivo; the average migration in the presence of this antigen was 46.1%. The peritoneal exudate cells of control animals that were sensitized to both antigens were inhibited in vitro by both antigens. The average migration of control cells was 45.5% with OCBC-BGG and 35.5% with DNP-BPA. The peritoneal exudate cells of normal, nonsensitized animals were not inhibited from migrating by either antigen. Similar results were obtained in experiments using DNP-BPA to suppress delayed hypersensitivity and OCBC-BGG as the unrelated antigen. In these experiments, the average migration of the peri-

toneal cells of four animals was 105% with DNP-BPA and 57% with OCBC-BGG.

Experiments with Migration Inhibitory Factor.—These experiments were

TABLE I

Partial Tolerance: Results of Skin Tests and Antibody Formation

Animals	Skin tests		Antibody formation to OCBC-BGG	
	OCBC-BGG	DNP-BPA	γ_2 passive lysis $\log_2$ titer	γ_1 PCA $\log_2$ titer
Experimental*:	*mm*	*mm*		
1	5 × 6‡	20 × 25	0	10
14	8 × 6	20 × 25	0	10
85	0	10 × 10	0	9
94	7 × 6	18 × 19	4	11
95	5 × 5	20 × 17	0	11
96	10 × 7	22 × 25	5	11
29§	6 × 6	16 × 18	4	11
32	6 × 6	20 × 20	4	11
34	8 × 8	15 × 20	5	11
40	7 × 7	20 × 20	4	11
Control‖				
52	15 × 15	15 × 15	6	8
55	20 × 25	15 × 15	6	8
1c	11 × 12	22 × 15	6	9
2c	20 × 20	18 × 17	8	11
3c	15 × 16	15 × 20	8	11
0c	15 × 15	13 × 18	7	11
37¶	15 × 15	20 × 20	7	11
43	15 × 15	20 × 20	6	10
48	20 × 15	15 × 15	6	10

* Experimental animals 1–96 made partially tolerant by multiple subcutaneous injections of antigen.

‡ Average skin tests of 10 normal unsensitized animals with 50 μg OCBC-BGG (4 × 6 mm) (2 animals 0 × 0 mm; 1 animal 8 × 12 mm).

§ Experimental animals 29–40 made partially tolerant by a single intravenous injection of aggregate-free antigen.

‖ Control animals for experimental animals 1–96.

¶ Control animals for experimental animals 29–40.

designed to determine if an alteration in the behavior of macrophages or lymphocytes was responsible for the absence of inhibition of migration by the antigen used to induce tolerance. The finding that macrophages of animals tolerant to one antigen (OCBC-BGG) were inhibited by another antigen

(DNP-BPA), to which the animals also displayed delayed hypersensitivity in vivo, demonstrated that, at least to this second antigen, the macrophages respond normally in vitro, i.e. they could be inhibited by MIF produced when

TABLE II

Partial Tolerance Studied by In Vitro Inhibition of Cells Migration

Animals	Migration	
	OCBC-BCG	DNP-BPA
	%	%
Experimental*:		
1	99	45
14	100	50
85	119	37
94	104	31
95	155	62
96	106	28
29‡	111	33
32	111	51
34	88	66
40	131	58
Average	112.4	Average 46.1
Control§:		
52	45	41
55	40	37
1c	51	27
2c	55	33
3c	43	26
0c	41	20
48‖	39	40
37	34	50
43	63	46
Average	45.5	Average 35.5

* Experimental animals 1–96 made partially tolerant by multiple subcutaneous injections of antigens.

‡ Experimental animals 29–40 made partially tolerant by a intravenous single injection of aggregate-free antigen.

§ Control animals for experimental animals 1–96.

‖ Control animals for experimental animals 29–40.

DNP-BPA sensitive lymphocytes reacted with DNP-BPA. In addition, macrophages from animals rendered tolerant by neonatal injection of DNP-BPA were still capable of being inhibited when incubated with MIF produced

by the interaction of sensitive DNP-BPA lymphocytes and DNP-BPA (Table III). Of interest was the finding in two experiments that lymph node lymphocytes from OCBC-BGG tolerant guinea pigs, when incubated with OCBC-BGG, did not produce MIF (migration 93 and 98%). In one experiment, it was not possible to detect an inhibitor of MIF by mixing supernatants obtained from OCBC-BGG tolerant lymphocytes that had been incubated with OCBC-BGG with active MIF produced by sensitized OCBC-BGG cells.

DISCUSSION

The results of the studies described above indicate that suppression of delayed hypersensitivity in vivo is correlated with failure of the tolerant animal's lymphocytes to produce MIF on exposure to the same antigen used to induce partial tolerance. Moreover, the suppression of delayed hypersensitivity is specific, whether tested in vivo or in vitro. Peritoneal exudate cells from animals tolerant to one antigen are readily inhibited from migrating in vitro when exposed to a second unrelated antigen to which the animal is sensitized. This finding indicates that the macrophages from tolerant animals respond normally in vitro to MIF and suggests that they are not responsible for the lack of inhibition of migration observed in the presence of the antigen used to induce tolerance. This interpretation is supported by results of experiments with migration inhibitory factor, which indicate that the lymphocytes are the cells involved in the alteration of delayed hypersensitivity reactions in tolerant animals. No MIF was produced when lymph node cells from partially tolerant animals were incubated with the antigen used to induce tolerance. However, the migration of peritoneal exudate cells obtained from tolerant animals was inhibited by MIF obtained from control animals sensitized with the antigen used to induce tolerance. The normal behavior of macrophages in these studies is consistent with findings by others of normal functions of macrophages obtained from tolerant animals (14, 15, 16). In addition, the suppression of γ_2

TABLE III

Inhibition of Migration of Tolerant Peritoneal Exudate Cells by MIF

Source of PEC	Migration	
	DNP-BPA*	MIF‡
	%	%
Normal	85	43
Tolerant to DNP-BPA by neonatal antigen injection	75	32
Sensitized to DNP-BPA	25	25

* DNP-BPA in media, 100 μg/ml.

‡ MIF made from DNP-BPA sensitized cells in media.

antibody was also observed, as previously reported (1, 2). However, there is no direct correlation between the suppression of delayed hypersensitivity and the suppression of γ_2 antibody (4).

This study shows that antigen suppression of delayed hypersensitivity results in the absence of at least one of the soluble mediators of this immune phenomenon. The mechanism by which induction of partial tolerance suppresses production of migration inhibitory factor is unknown.

SUMMARY

Suppression of delayed hypersensitivity in vivo is correlated in vitro with the absence of macrophage migration inhibition in the presence of the antigen used to induce partial tolerance. The suppression of delayed hypersensitivity is antigen-specific in vivo as well as in vitro. The lymphocytes, and not the macrophages, are the cells involved in the induction of tolerance in terms of delayed hypersensitivity which is characterized by an absence of migratory factor activity.

BIBLIOGRAPHY

1. Dvorak, H. F., J. B. Billote, J. S. McCarthy, and M. H. Flax. 1965. Immunologic unresponsiveness in the adult guinea pig. I. Suppression of delayed hypersensitivity and antibody formation to protein antigens. *J. Immunol.* **94:**966.
2. Asherson, G. L., and S. H. Stone. 1965. Selective and specific inhibition of 24 hour skin reactions in the guinea pig. *Immunology* **9:**205.
3. Borel, Y., M. Fauconnet, and P. A. Miescher. 1966. Selective suppression of delayed hypersensitivity by the induction of immunologic tolerance. *J. Exp. Med.* **123:**585.
4. Borel, Y., M. Fauconnet, and P. A. Miescher. 1967. Dissociation of immune responses by the induction of partial unresponsiveness. *J. Immunol.* **98:**881.
5. George, M., and J. H. Vaughan. 1962. *In vitro* cell migration as a model for delayed hypersensitivity. *Proc. Soc. Exp. Biol. Med.* **111:**514.
6. David, J. R., S. Al-Askari, H. S. Lawrence, and L. Thomas. 1964. Delayed hypersensitivity *in vitro*. I. The specificity of the inhibition of cell migration by antigens. *J. Immunol.* **93:**264.
7. Bloom, B. R., and B. Bennett. 1966. Mechanism of a reaction *in vitro* associated with delayed hypersensitivity. *Science* (*Washington*). **152:**80.
8. David, J. R. 1966. Delayed hypersensitivity *in vitro*. Its mediation by cell free substances formed by lymphoid cell antigen interaction. *Proc. Nat. Acad. Sci. U.S.A.* **56:**72.
9. Eisen, H. N., M. D. Carsten, and S. Belman. 1954. Studies of hypersensitivity to low molecular weight substances. III. The 2,4-dinitrophenyl group as determinant in the precipitin reaction. *J. Immunol.* **73:**296.
10. Ovary, Z., and B. Benacerraf. 1963. Immunological specificity of the secondary response with dinitrophenylated proteins. *Proc. Soc. Exp. Biol. Med.* **114:**72.
11. Ovary, Z. 1958. Immediate reactions in the skin of experimental animals provoked by antibody-antigen interaction. *Progr. Allergy.* **5:**460.

12. Bloch, K. J., F. Kourilsky, Z. Ovary, and B. Benacerraf. 1963. Properties of guinea pig 7S antibodies. III. Identification of antibodies involved in complement fixation and hemolysis. *J. Exp. Med.* **117:**965.
13. David, J. R., and S. F. Schlossman. 1968. Immunochemical studies on the specificity of cellular hypersensitivity. *J. Exp. Med.* **128:**1451.
14. Nossal, G. J. V., and G. L. Ada. 1964. Recognition of foreignness in immune and tolerant animals. *Nature (London).* **201:**580.
15. Harris, F. 1967. Macrophages from tolerant rabbits as mediators of a specific immunological response *in vitro*. *Immunology.* **12:**159.
16. Friedman, H. 1968. The nature of immunogenic RNA antigen complexes in immune and tolerant mice. *In* Nucleic Acid Immunology. O. J. Plescia and W. Braun, editors. Springer-Verlag New York Inc., New York. 505.