THE COMPARTMENTALIZATION OF ANTIGEN-REACTIVE LYMPHOCYTES IN DESENSITIZED GUINEA PIGS*

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Injection of large doses of antigen into an animal with delayed hypersensitivity can render the animal specifically unreactive to subsequent intradermal challenge with that antigen (1–3). The unresponsiveness is short-lived, lasting 3–10 days, and related to the dose of antigen given. Desensitization results in an antigen-specific depression of delayed skin reactivity except for the first few hours after the desensitizing injection when anergy to unrelated antigens is seen. Little is known of the cellular basis for antigen-specific desensitization other than the failure to transfer delayed hypersensitivity with leukocytes from desensitized human beings (4).

In the present studies, the cellular basis for antigen-specific desensitization is further investigated utilizing the in vitro techniques of antigen-induced thymidine incorporation and macrophage-migration inhibition (5–8). We found that a single injection of a large dose of antigen specifically obliterates the delayed skin test to that antigen. In addition, lymphocytes obtained from an inflammatory peritoneal exudate from these desensitized animals either responded poorly or not at all in antigen-induced thymidine incorporation or the macrophage-migration inhibition assays. On the other hand, lymph node lymphocytes from "desensitized" animals were readily induced by antigen to incorporate thymidine and behaved like cells from nondesensitized animals. Retention of antigen-reactive lymphocytes in lymph nodes and depletion of antigen-reactive peripheral lymphocytes at inflammatory exudates or skin may explain the short-lived nature of the desensitization phenomenon and further demonstrates the compartmentalization of lymphocytes (9–12).

Materials and Methods

Preparation of Antigens.—A mixture of oligolysine peptides with an average chain length of 14 lysine residues, each containing a single dinitrophenyl (DNP)[1] substitution on the ε-amino

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[1] *Abbreviations used in this paper:* BBS, Hanks' balanced salt solution; DNP, dinitrophenyl;

group of the C-terminal lysyl (Lys) residue, was prepared by Yeda, Rehovot, Israel, as previously described (13). The mixture was separated by the chromatographic methods previously described and those peptides containing 7–10 lysine residues were pooled and designated ϵ,DNP-Lys_{7-10} (14).

Spectrophotometry.—A Gilford spectrophotometer (Gilford Instrument Co., Oberlin, Ohio) with silica cells of 1-cm light path was used for all spectrophotometric determinations. The ϵ,DNP-lysine content of these peptides was determined in 0.01 M sodium phosphate-saline buffer, pH 7.0 at 360 nm (E_{360nm} = 17,400).

Immunization, Skin Testing, and Desensitization.—Inbred strain 2 guinea pigs weighing 300–500 g were sensitized to ϵ,DNP-Lys_{7-10}. Each animal received 90 mμmole of ϵ,DNP-Lys_{7-10} diluted in 0.3 ml of saline and emulsified with an equal volume of Freund's complete adjuvant containing *Mycobacterium tuberculosis* H37Ra 2 mg/ml (Difco Laboratories, Detroit, Mich.). The emulsion was divided equally among the four footpads. 11–13 days after immunization the animals were tested by intradermal injection of 2.4 and 16.0 mμmole of ϵ,DNP-Lys_{7-10} dissolved in 0.1 ml of buffered saline and with either 0.2 or 5 μg of purified protein derivative (PPD) in 0.1 ml of buffered diluent (Merck Sharp and Dohme, West Point, Pa.). The test sites were observed at 3–6 hr and at 24 hr, and the extent of induration and erythema was measured. Arthus-type reactions (3–6 hr) were either weak or not seen. 72 hr after the initial intradermal skin tests, the guinea pigs were injected intraperitoneally with 700 mμmole of ϵ,DNP-Lys_{7-10} in 0.5 ml of saline. 1 hr later the animals were challenged as above by intradermal injection of ϵ,DNP-Lys_{7-10} and PPD. 24 hr later skin tests were measured and the animals sacrificed for lymph node cultures. Similarly treated, but nondesensitized animals served as controls. Three additional groups of guinea pigs were immunized and skin tested as above but desensitized with 700 mμmole of ϵ,DNP-Lys_{7-10} given by intracardiac injection. 1 hr after intracardiac injection of antigen, these animals, along with sensitized and nonimmunized controls, were injected intraperitoneally with 30 ml of sterile mineral oil. Animals were skin tested 48 hr after desensitization with ϵ,DNP-Lys_{7-10} and PPD as described above and the test sites measured 24 hr later. The peritoneal exudates were then harvested for cell culture. Similar groups of animals were sacrificed at 72 hr for lymph node cell cultures.

Lymph Node Cell Cultures.—Lymph node lymphocytes from individual animals were obtained as previously described (15) and placed in tissue culture medium (TCM) minimal essential medium (MEM, Grand Island Biological Co., Grand Island, N.Y.) containing 13% normal guinea pig serum; penicillin, 50 units/ml; streptomycin, 50 μg/ml; L-glutamine and nonessential amino acids. Living cells were counted in a hemocytometer by trypan blue exclusion. Additional TCM was added to provide a concentration of 2×10^7 viable cells/ml. Cell viability was consistently greater than 70%. 1 ml of TCM, 0.1 ml of antigen to be tested, and 0.5 ml of lymph node cells in TCM containing 1×10^7 cells were added to rubber stoppered 16 × 125 mm disposable glass culture tubes. Duplicate or triplicate tubes were prepared for every dose level. Cultures were incubated vertically at 37°C for 24 hr and then pulsed with 0.2 μCi of thymidine-2-^{14}C (New England Nuclear Corp., Boston, Mass.). After an additional 24 hr, cultured cells were centrifuged, washed with Hanks' balanced salt solution (BSS) (Grand Island Biological Co.), dissolved in NCS reagent (Nuclear Chicago, Des Plaines, Ill.), and counted in a liquid scintillation counter. In each experiment the antigen-induced incorporation of thymidine-2-^{14}C (*s*) was compared with the amount of thymidine-2-^{14}C incorporated (*c*) into antigen-free cultures prepared from the same animals. The data is presented as *s/c* ratios and absolute counts per minute incorporated in baseline (*c*) cultures.

Peritoneal Lymphocyte Cultures.—Oil-induced peritoneal exudate cells (PEC) were harvested and pooled from individual groups of sensitized and desensitized animals. A partially

MEM, minimal essential medium; MIF, migration inhibitory factor; PEC, peritoneal exudate cells; PPD, purified protein derivative; TCM, tissue culture medium.

purified lymphocyte population was obtained by incubating the cells at 37°C for 30 min in tubes containing cotton (16). From this procedure, 3.0–3.5 $\times$ 10^6 cells/0.4 ml packed cell volume were recovered containing 80% lymphocytes that were essentially 100% viable. The cells were washed three times in BSS and resuspended at a concentration of 1.5 $\times$ 10^6 cells/1.5 ml of TCM as described above. Cells with antigen (1.5–58 mμmole of ϵ,DNP-Lys_{7-10} and 10 μg PPD/ml of cell suspension) and without antigen were cultured for varying periods of time (48–96 hr) and harvested daily in order to determine the peak response. Maximal stimulation was reproducibly found after 96 hr of culture. At 72 hr 0.5 μCi of thymidine-^{3}H (Amersham-Searle Corp., Chicago, Ill.) (specific activity 5.0 mCi/mM) was added to each tube. After an additional 24 hr, the cultures were terminated and DNA-labeled material was obtained by trichloroacetic acid precipitation, washed with methanol, and solubilized with NaOH as previously described (17). The data is presented as s/c ratios (stimulation index) and baseline counts per minute (c). As with lymph node cell cultures s/c equal to or greater than 2 are considered positive.

Migration-Inhibition Assays.—Peritoneal exudate cells from desensitized, sensitized, and nonimmunized animals were obtained as previously described (6, 18). The cells were washed in BSS and suspended to 10% by volume in media consisting of MEM with 100 units of penicillin and 100 μg of streptomycin/ml and made to contain 15% normal guinea pig serum. 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 tested at each concentration and two chambers with media with no antigens. The antigen concentration ranged from 0.16–16 mμmole ϵ,DNP-Lys_{7-10}.

The chambers were incubated during 18 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:

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

RESULTS

Effect of DNP-Oligolysines on Lymph Node Cells from Desensitized Animals.—All strain 2 guinea pigs developed delayed skin reactivity to ϵ,DNP-Lys_{7-10} 11–13 days after sensitization with 90 mμmole of ϵ,DNP-Lys_{7-10} in complete Freund's adjuvant (Table I). Animals D1–D4 were desensitized by intraperitoneal injection of 700 mμmole of ϵ,DNP-Lys_{7-10} and 1 hr later skin tested again. No observable skin reactivity to either 2.4 or 16 mμmole of ϵ,DNP-Lys_{7-10} was seen in desensitized animals (Table I). In contrast, sensitized controls S1–S3 were strongly reactive. Intradermal challenge with a low dose of PPD (0.2 μg) evoked a response in sensitized control animals which was greater after the second intradermal skin test than after the first. Desensitized animals, on the other hand, had a slightly diminished response to PPD after the second test injection. Lymph node cells for culture were obtained from both desensitized and sensitized controls. Despite the absence of skin tests to ϵ,DNP-Lys_{7-10}, lymph node cells from desensitized animals were readily triggered to incorporate thymidine-2-^{14}C by varying doses of ϵ,DNP-Lys_{7-10} (Table II). Both the magnitude of the stimulation index and the response to various doses

of ϵ,DNP-Lys_{7-10} were as great or greater for desensitized cells as for control sensitized cells.

Effect of DNP-Oligolysines on Peritoneal Exudate Cells from Desensitized Animals.—The dissociation between thymidine-2-^{14}C incorporation and skin

TABLE I

Delayed Hypersensitivity Skin Reactions to ϵ, DNP-Lys_{7-10} and PPD

Treatment of animals	Animal No.	Cutaneous reactions before desensitization*			Cutaneous reactions after desensitization*		
		ϵ, DNP-Lys_{7-10}		PPD 0.2 μg	ϵ, DNP-Lys_{7-10}		PPD 0.2 μg
		2.4 mμmole	16 mμmole		2.4 mμmole	16 mμmole	
		mm	*mm*	*mm*	*mm*	*mm*	*mm*
Desensitized	D1	7/7	13/13	4/5	0	0	5/5
	D2	14/14	17/17	7/7	0	0	0
	D3	10/10	20/25	0	0	0	6/6
	D4	10/10	25/25	15/15	0	0	10/10
Sensitized (controls)	S1	10/10	20/20	0	15/15	25/25	15/15
	S2	6/6	15/15	0	10/10	17/20	10/10
	S3	3/3	17/17	7/7	9/9	16/16	10/8

* Size of skin reaction (erythema) obtained at 24 hr.

TABLE II

Antigen-Induced Thymidine Incorporation in Lymph Node Lymphocytes: the Effect of Intraperitoneal Injections of ϵ, DNP-Lys_{7-10}

Treatment of animals	Animal No.	Stimulation index (*s/c*) mμmole ϵ, DNP-Lys_{7-10} added							Baseline (cpm)*
		1.7	3.4	17	34	58	115	170	
Densensitized	D1	1.6	—	5.0	11.9	8.1	—	27.8	88
	D2	1.8	4.7	11.0	22.5	—	41.9	48.8	143
	D3	2.7	6.8	13.9	38.1	39.9	50.3	41.0	75
	D4	3.6	6.9	22.1	33.1	47.2	51.6	48.8	112
Sensitized (controls)	S1	2.5	—	4.6	—	3.6	—	6.9	3255
	S2	4.1	6.3	7.0	7.9	7.9	11.5	12.8	1153
	S3	1.9	8.8	8.5	21.3	22.4	37.0	24.7	125

* Thymidine incorporation (cpm) in cells cultured without antigen (*c*).

reactivity prompted further studies of the immune potential of cells in a desensitized animal. It was of importance to determine whether the antigen-induced inhibition of migration assay for cellular hypersensitivity was also affected by desensitization. For these studies, animals were desensitized by intracardiac injection of 700 mμmole of ϵ,DNP-Lys_{7-10}. Mineral oil was then

injected intraperitoneally and three days later the PEC was harvested and tested. 24 hours before harvesting the peritoneal exudate, animals were skin tested with 16 mμmole of ϵ,DNP-Lys_{7-10}. Negative skin reactions were seen only in desensitized animals D5–D8 (Table III). The reduction of skin reactivity in sensitized control animals S4–S6 may have resulted from mineral oil injection. As shown in Table III, PEC from control animals exhibited marked inhibition of migration in the presence of antigen. The dose response curve was similar to that previously described with other DNP-oligolysines (18). In contrast, no inhibition of migration of PEC from desensitized animals D5–D8 was

TABLE III

The Effect of Intracardiac Injection of ϵ, DNP-Lys_{7-10} on Delayed Hypersensitivity Skin Reactions and Antigen-Induced Inhibition of Migration of Peritoneal Exudate Cells

Treatment of animals	Animal No.	Cutaneous reaction to 16 mμmole ϵ, DNP-Lys_{7-10}*		Per cent migration of peritoneal exudate cells mμmole ϵ, DNP-Lys_{7-10} added		
		Before desensitization	After desensitization	16	1.6	0.16
		mm	*mm*			
Desensitized	D5	18/18	0	70	98	102
	D6	15/15	0	75	87	102
	D7	20/20	0	76	92	102
	D8	22/22	0	74	100	113
Sensitized (controls)	S4	23/23	8/8	24	52	79
	S5	17/17	8/8	12	35	79
	S6	17/17	9/10	10	46	96

* Size of skin reaction (erythema) obtained at 24 hr.

observed with either 1.6 or 0.16 mμmole of ϵ,DNP-Lys_{7-10}. At 16 mμmole of antigen, inhibition of migration of desensitized peritoneal exudate cells was seen. This dose, however, is a 100-fold greater than that required to cause a similar amount of inhibition of migration of sensitized cells.

For comparison, lymph node cells were obtained from animals D9–D13, desensitized 3 days earlier by intracardiac injection of 700 mμmole of ϵ,DNP-Lys_{7-10}. As shown in Table IV, the response of these cells to in vitro antigen challenge was similar to that obtained with lymph node cells from normal sensitized controls.

Peritoneal exudate cells harvested from animals desensitized 72 hr earlier were inhibited only at the highest dose of ϵ,DNP-Lys_{7-10} whereas lymph node cells obtained at the same time or 24 hr after desensitization were readily triggered to incorporate thymidine-2-^{14}C. These findings suggest a dissociation between thymidine incorporation on the one hand and the production of medi-

ators on the other. However, this observation could be a consequence of the use of different populations of cells in the two assays, i.e., peritoneal exudates vs. lymph node cells. It was therefore necessary to determine whether peritoneal lymphocytes from desensitized animals could incorporate thymidine in response to antigenic challenge. Animals were desensitized by intracardiac injection of

TABLE IV

The Effect of Intracardiac Injection of ε, DNP-Lys$_{7-10}$ on Antigen-Induced Incorporation of Thymidine by Lymph Node Cell Cultures

Treatment of animals	Animal No.	Stimulation index (s/c) mμmole ε, DNP-Lys$_{7-10}$ added				Baseline (cpm)*
		3.4	17	85	170	
Desensitized	D9	1.4	3.4	12.0	19.0	475
	D10	1.8	2.3	5.0	6.0	1293
	D11	1.9	2.4	5.4	9.0	527
	D12	1.4	2.7	4.6	7.9	383
	D13	—	8.6	15.4	—	473

* Thymidine incorporation (cpm) in cells cultured without antigen (c).

TABLE V

The Effect of Intracardiac Injection of ε, DNP-Lys$_{7-10}$ on Antigen-Induced Incorporation of Thymidine by Peritoneal Lymphocytes

Treatment of animals	Animal No.	Stimulation index (s/c) mμmole ε, DNP-Lys$_{7-10}$				PPD 10 μg	Baseline (cpm)*
		1.5	15	29	58		
Sensitized	S7–S12 (pool)	—	—	6.7	5.1	14.5	603
	S13–S18 (pool)	—	4.7	4.1	3.0	6.7	796
	S19–S21 (pool)	1.1	5.5	4.9	—	7.7	719
Desensitized	D14–D16 (pool)	0.8	0.9	0.9	—	6.6	883
	D17–D18 (pool)	1.0	1.3	1.3	—	3.0	635

* Thymidine incorporation (cpm) in cells cultured without antigen (c).

700 mμmole of ε,DNP-Lys$_{7-10}$ and PEC from both desensitized and sensitized control animals were collected 72 hr later. Partially purified lymphocytes were obtained for antigen induced thymidine-^{3}H incorporation assays as described in Materials and Methods. As shown in Table V, peritoneal lymphocytes from sensitized animals incorporated thymidine in response to ε,DNP-Lys$_{7-10}$ and PPD. In contrast, peritoneal lymphocytes from desensitized animals were unresponsive to ε,DNP-Lys$_{7-10}$ but were still responsive to PPD. It should be noted that these desensitized animals had no observable skin tests to ε,DNP-Lys$_{7-10}$ and strongly positive delayed skin tests to 5 μg of PPD (averaging

16/16 mm). Moreover, as expected, their peritoneal lymphocytes demonstrated desensitization by the migration-inhibition assay.

DISCUSSION

In the present studies we have attempted to investigate the cellular basis for specific desensitization of delayed skin reactions. It was shown that a "desensitizing injection" of antigen obliterates the delayed skin reaction to that antigen. For the first 24 hr after desensitization with ϵ,DNP-$Lys_{7\text{-}10}$, however, there was a diminished response to PPD. Compared to specific desensitization, however, this nonspecific effect is short-lived and incomplete (2, 3). The mechanism for nonspecific anergy after desensitization to a heterologous antigen is unknown but may be related to the temporary disappearance of peripheral monocytes and macrophages from peritoneal exudates which occurs within hours after injection of large doses of antigen in animals with delayed hypersensitivity (19–21).

Lymphocytes obtained from a local inflammatory site, i.e. the peritoneal exudate from desensitized animals, show a marked depression in reactivity to specific antigen when assayed both by antigen-induced inhibition of migration and thymidine incorporation. In contrast, lymph node lymphocytes from desensitized animals can be readily triggered by antigen to incorporate thymidine. Moreover, with this assay the reactivity of lymph node lymphocytes from desensitized animals cannot be distinguished from those of sensitized animals. Retention of antigen-reactive cells in lymph nodes and depletion of antigen-reactive peripheral lymphocytes appearing at inflammatory sites is consistent with the short-lived nature of the desensitization phenomena and the concept of lymphocyte heterogeneity and compartmentalization. Recent studies by Koster et al. have shown that the lymphocytes capable of localizing in inflammatory sites, for example, the peritoneal exudate, have a short-circulating lifespan, are short-lived, and participate in cellular immune reactions (11–12). Our studies also suggest that the reactivity of lymphocytes obtained from peritoneal exudates correlates better with the reactivity of lymphocytes at a dermal delayed reaction than does that of lymphocytes obtained from lymph nodes. Whether lymph nodes from desensitized animals still contain a full complement of effector lymphocytes remains to be determined.

Although our initial studies showing reactive lymph node cells and unreactive peritoneal exudate cells suggested a dissociation between mediator production and the proliferative response, subsequent analysis of peritoneal exudate cells shows that this was not the case since both thymidine incorporation and migration-inhibitory factor (MIF) production were depressed in this common compartment of cells. Studies showing a dissociation between antigen-induced mediator production in one cell population and proliferation in another population deserve further exploration with a common cell population (22, 23).

However, a dissociation between mediator production and thymidine incorporation may be seen with a common population of cells in patients with a variety of diseases that affect cellular immunity (17) and with some antigens (24). The basis for this dissociation and its relation to lymphocyte heterogeneity and function is not clear.

The manifestations of desensitization are different from those observed in tolerant animals and suggest different mechanisms for the two phenomena. In tolerance, unreactivity to antigen is long-lived and both peritoneal and lymph node lymphocytes are affected (25, 26). Neither antigen-induced thymidine incorporation or MIF production was observed with tolerant cells despite the use of similar amounts of antigen to evoke tolerance or desensitization. Tolerance must prevent the induction of the immune response before the commitment of the thymus-derived cell whereas "desensitization" may affect a cohort of committed cells. In both tolerance and desensitization the effect is on the lymphocyte and not the macrophage since in these phenomena the macrophage is capable of responding normally to MIF when such mediators are triggered by heterologous antigen (25). The mechanism of desensitization is not clear but may result in part from specific depletion of the immunologically reactive effector lymphocytes. Perhaps large doses of antigen trigger and temporarily deplete the effector cells of mediators with recovery paralleling either the capacity of these desensitized cells to synthesize new mediators or the appearance of new effector cells derived from the lymph node population. The rapid disappearance of blood monocytes and peritoneal macrophages after injection of antigen in a sensitized animal may be the result of mediators produced by cells which have been triggered by antigen (19–21). It is not known whether the antigen receptor on the lymphocyte is blocked by antigen, or whether the effector lymphocyte is actually killed, pushed to terminal differentiation, or altered in such a way that its circulation is changed leading to its sequestration in still undefined sites. The evidence is as yet insufficient to decide on the exact cellular mechanism for desensitization by antigen.

SUMMARY

A single injection of ϵ,DNP-$Lys_{7\text{-}10}$ can render previously sensitized guinea pigs specifically unreactive to subsequent intradermal challenge with that antigen. Antigen-reactive lymphocytes, as assayed by macrophage-migration inhibition or thymidine incorporation, were depleted from the peritoneal exudates of those animals. In contrast, it was intriguing to find that lymph node lymphocytes from such animals responded normally in the antigen-induced thymidine incorporation assay. These studies demonstrate a compartmentalization of antigen-reactive lymphocytes in desensitized animals which may account for the short-lived nature of this phenomenon.

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