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Studies on Delayed Hypersensitivity in Mice II. T-Cell Dependency of the Response: T Cell; Limiting Cells in Induction of Delayed Footpad Reaction

(delayed hypersensitivity/footpad reaction/T cell/limiting cell)

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Cells involved in DH response in mice were investigated by footpad assay in sensitized mice with methylated human serum albumin (MHSA) and complete Freund's adjuvant (CFA). Lymphoid cells responsible for eliciting footpad swelling in sensitized mice are sensitive to ATS and relatively resistant to radiation, and ferritin, macrophage toxic agent, completely suppressed eliciting footpad swelling. However, ATS administration to mice before sensitization failed to effectively suppress induction of DH to MHSA in mice. It was also confirmed by means of cell transfer experiment in adult thymectomized and lethally irradiated recipients that cells triggering delayed skin response are θ-positive T cells.

Reconstitutive capacity of various individual and mixed lymphoid cell populations to induce DH response in thymectomized and lethally irradiated mice was examined using dose response studies, to elucidate the role of cells involved in establishment of DH state to MHSA. A good correlation between the transferred thymus or spleen cell dosages and the degree of FPR in the recipients was seen. On the other hand, injection of anti-θ treated bone marrow cells to mice given moderate doses of thymus cells did not affect the degree of FPR within a wide range of bone marrow cell doses. Thus, DH response to MHSA in mice requires the cooperation of both thymus and bone marrow cell populations. However, as T cells are limiting cells determining the degree of the sensitive state of DH, such may reflect the reactivity of sensitized T cells producing DH lesion in the skin. DH in mice is a highly T-dependent immune phenomenon both in afferent and efferent limbs.

It is an established fact that delayed hypersensitivity (DH) is a form of manifestation of cell-mediated immunity (CMI), mainly due to expression of specific T-cell activity. By means of cell transfer experiments it was demonstrated that the lymphoid cells responsible for eliciting DH reaction in the recipient animals are anti-θ antiserum or anti-thymus antisera sensitive (1, 2,

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3). On the other hand, Lubaroff and Waksman (4) showed the essential participation of bone marrow cells for the production of tuberculin skin reactions. Thus, thymus and bone marrow cell synergism as observed in humoral antibody response (5) was also utilized to develop DH reaction against oxasolone (6), tuberculin (7) and methylated human serum albumin (MHSA) in mice.

Today, footpad assay becomes one of the widely used test in the quantitative study on DH in mice (1, 8, 9, 10) and in rats (11). However, a few papers concern which kind of cell, T or B, is the limiting cell in determining the degree of delayed footpad response (FPR) in sensitized animals. By means of reconstitution experiments of DH, Eidinger *et al.* (8) suggested that the thymus cell was the antigen reactive cell and that the effector cell limiting the degree of response was of bone marrow origin.

In this report, T cell dependency of delayed FPR in both inducing and eliciting stage was confirmed in mice using MHSA as antigen. Dose response studies on reconstitutive capacity of various individual and mixed lymphoid cell populations to induce DH responsiveness in thymectomized and lethally irradiated mice revealed that the cell limiting the degree of response was of thymus origin. Macrophages, bone marrow cells, were also essential for developing footpad swelling.

MATERIALS AND METHODS

Mice

Inbred male C3H/He mice raised and maintained by brother-sister mating at Kyoto University Animal Center (Kyoto) were used mainly. In some experiments, (C57BL/6 × C3H) F_1 hybrid (BC3) were used. All animals were 10—12 weeks of age unless stated otherwise. Individual mice were identified by ear marks made with a metal punch.

Antigen

Four times crystallized human serum albumin (HSA) was obtained from Nutritional Biochemicals Co. (Cleveland, USA). Methylated human serum albumin was prepared by methanol-hydrochloric acid method described by Crowle, Hu & Patrucco (12). Heat-aggregated HSA (⊿HSA) was prepared by incubation of 200 mg HSA in 10 ml of phosphate buffered saline at 85°C for 20 minutes. The incubated solution was then spun down by ultracentrifuge at 105,000 g for 120 minutes. The pellet was resuspended in phosphate buffered saline.

Immunization and Footpad Assay for DH

Each mouse was sensitized by an injection into the left hind footpad with 0.05 ml of an emulsion of equal volumes of a MHSA solution (5 mg/ml) in phosphate buffered saline (pH 7.1) and complete Freund's adjuvant (CFA) containing 3 mg/ml of killed *Mycobacteria* H37Rv. At a specified time, usually 11—12 days later, the sensitized mice were challenged with 0.02 ml of 0.1% MHSA in phosphate buffered saline given intradermally into the contralateral hind footpad. The thickness of the footpad was measured just before and at

different times after challenge, usually 24 hr for DH assay. The difference in the thickness of footpad before and after challenge was expressed in 1/10 mm as footpad reaction (FPR). An increase of 0.2 mm in thickness was regarded as positive (13).

Irradiation

The irradiation source was cobalt 60 delivered at a rate of 78 rads/min for whole body irradiation. The mice housed in a lucite chamber were generally irradiated with a given dose of radiation each of which is indicated accordingly herein. Lethally irradiated recipients were usually irradiated with 800 R.

Adult Thymectomy

The mice aged 7 to 9 weeks old were thymectomized under sodium pentobarbital anesthesia. Following a skin incision, the upper part of the sternum was split longitudinally to expose the thymus gland. Each lobe of the gland was separated freely from the surrounding tissue by using a tooth-pick cottoned at the tip. The lobes were then extirpated by using fine forceps. The skin wound was closed with surgical cement.

Preparation of Cell Suspensions

Suspensions of popliteal lymph nodes, spleen and thymus cells from normal or sensitized mice were prepared by gently teasing the tissue apart with a pair of fine forceps in an ice-cold medium RPMI-1640 containing 1 % normal mouse serum and kanamycin (60 μg/ml). Following repeated gentle pipetting, the cell suspensions were placed in glass centrifuge tubes with a coned bottom and left to stand for 15 minutes in order that the tissue particles would settle. The resulting cell suspension free from tissue fragments was washed three times with medium RPMI-1640 containing only kanamycin and was then resuspended in a cold medium to the proper concentration. Bone marrow cells were collected by injecting and aspirating a small amount of medium twice through the shaft of the femur or tibia using a 1 ml plastic syringe and a needle (22G or 25G). Viability as examined by dye exclusion test with trypan blue was more than 90 % in each cell suspension.

Antisera

Anti-thymocyte antisera (ATS) was obtained from white rabbits given two intravenous injection of C3H thymus cells two weeks apart according to the method of Levey and Medawar (14). ATS was heat-inactivated at 56°C for 30 minutes and absorbed three times with acetone powder from C3H mouse kidney and then once with mouse erythrocytes. AKR anti-C3H θ antiserum (anti-θ) was produced by weekly injections of CBA thymocytes into AKR mice according to the method of Reif and Allen (15). The pooled serum had a cytotoxic titer for normal C3H thymocytes greater than 128 in 50 % killing of 1×10^7 cells.

Cells for treatment with anti-θ or normal AKR mouse serum were incubated in a concentration of 10^8 cells/ml with the same amount of undiluted serum for 30 min at 37°C. After washing, the cells were incubated in complement, a two-fold diluted normal guinea pig serum absorbed by agarose (Nakarai

Chemicals Ltd., Kyoto).

Treatment with Ferritin

Six times crystallized ferritin from horse spleen was purchased from Pentex Biochemicals, Kankakee, USA. A ferritin solution was dialysed repeatedly against PBS in order to be free from cadmium salt. Sensitized mice were injected intraperitoneally with 0.2 ml of 0.1 % ferritin solution in PBS.

Statistical Methods

Standard errors and the means and P values, if necessary, were calculated using the Student's t-test.

RESULTS

1. Effect of an Interval between Sensitization and Challenge on FPR against MHSA

C3H mice were immunized with 0.125 μg of MHSA with CFA. At different intervals after immunization, groups of four to five mice were tested with 20 μg of MHSA and FPR was measured 24 hr later. Following a latent period of 2 days, FPR was positive on day 4 and increased steadily until the 12th day. The sensitizing state appeared stable and persisted until at least the 252 day after sensitization (Fig. 1).

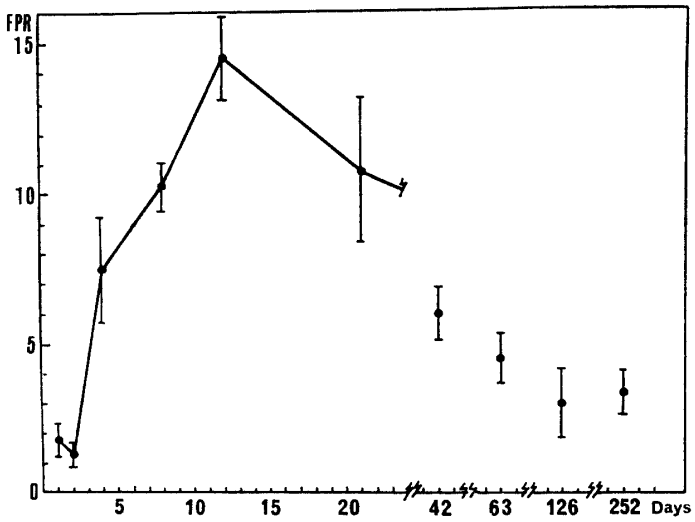

Fig. 1. Levels of 24 hr FPR to MHSA in C3H mice at various intervals after sensitization with MHSA and CFA. Mean of 4 to 5 mice ± standard error.

2. Delayed Response of FPR against MHSA in Mice

Footpad swelling following a challenge injection is usually complicated by a proceeding Arthus hypersensitivity, which first appears at about 2 hr, peaks at 3 to 5 hr and disappears within 12 hr after challenge (16, 17). In contrast, a typical footpad swelling with DH occurs first at about 6 hr after challenge, peaks at 18 to 24 hr, and is still much in evidence at 48 hr. Table I shows one of the typical results of occurrence of footpad swelling to MHSA, compared with footpad swelling by native HSA and heat-aggregated

HSA in mice. Mice sensitized with MHSA showed a minimal footpad swelling at 5 hr and all animals developed large FPR, mean 13.7, at 24 hr. The group of mice sensitized in the same way with heat-aggregated HSA which evoked an adequate antibody response, resulted in considerable footpad swelling at 5 hr and a lesser response at 24 hr after challenge with 0.02 ml of 0.1 % heat-aggregated HSA suspension in PBS.

TABLE I. *Immediate and Delayed Hypersensitivity Induced in Mice by HSA, Heat-Aggregated HSA and MHSA with CFA and Assayed by Footpad Swelling*

Antigens		Number of animals	FPR on 12th day Mean ± S. E.	
Sensitizing	Test		5 hr	24 hr
HSA	HSA	9	—	2.0±0.2
HSA	△HSA	5	6.6±0.7	3.2±0.9
△HSA	HSA	5	7.4±2.3	1.0±0.5
△HSA	△HSA	5	13.2±0.9	7.2±0.2
MHSA	MHSA	5	2.7±0.2	13.0±1.2

Male C3H mice were sensitized with 125 μg of one of the antigens and challenged 12 days later.

3. Suppressive Effect of ATS on Development of FPR

Three groups of mice were sensitized with MHSA on day 0. One group was injected i. v. three times with each 0.1 ml of absorbed ATS, on day 6, 8, and 10. Another groups was given i. v. 0.1 ml of normal rabbit serum three times according to the same schedule as noted above. Serum was not injected

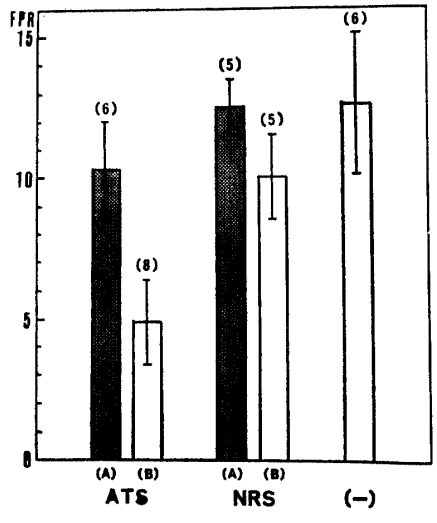

Fig. 2. Levels of 24 hr FPR to MHSA in C3H mice treated with ATS before sensitization (A) or before challenge (B), mice treated with NRS before or after sensitization, and non-treated mice. Numbers in parentheses indicate number of animals. Vertical bars are standard errors of the means.

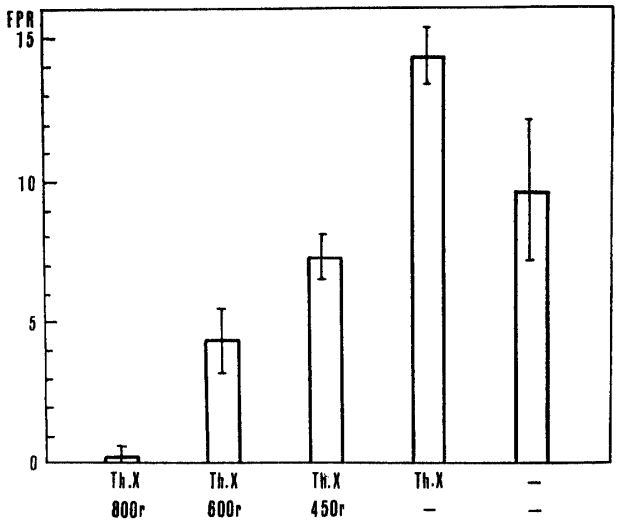

Fig. 3. Levels of 24 hr FPR of thymectomized MHSA-sensitized adult mice and various dosages of irradiation between sensitization and challenge. Each group included six to eight mice. Vertical bars are standard error of the means.

into the other group. All mice were challenged on day 12 and FPR was measured 24 hr later. As shown in Fig. 2, the mice treated with ATS developed suppressed FPR mean 4.9 ($p<0.005$), as compared with a mean FPR of 10.0 in normal rabbit serum- (NRS) or mean FPR of 12.0 in the non-treated mice. ATS from the same lot showed no remarkable suppression of induction of DH in mice, when 0.1 ml was administered i. v. 3 times daily before sensitization (Fig. 2).

4. Effect of Irradiation on the Established DH State Induced by MHSA

To examine the radiosensitivity of the effector limb of DH, groups of previously sensitized mice were exposed to increasing doses of whole body irradiation on day 10 of sensitization and were challenged on the 12th day. Four groups were thymectomized 3 days after sensitization to minimize the effect of regeneration and/or resensitization. The animals undergoing thymectomy and 800 R irradiation were restored by 10^7 normal bone marrow cells given i. v. on the day of irradiation to prevent high mortality rates. As observed in Fig. 3, irradiation suppressed the 24 hr FPR dose-dependently. Under these conditions, 800 R irradiation completely abolished the development of FPR despite restoration with bone marrow derived cells. In mice given 600 R irradiation, even a low degree of 4.4 (mean value) of FPR developed. In mice exposed to 450 R a moderate level of FPR developed.

5. Effect of in vitro Treatment of Immune Lymphoid Cells with Anti-θ Antiserum on Transfer of DH to MHSA

DH as well as other CMI can be transferred to normal recipients by various lymphoid cells from previously sensitized donors (1, 3, 18). Anti-θ sensitivity of the effector limb of delayed FPR to MHSA was examined by the adoptive transfer system in order to confirm the participation of T cells. $BC3F_1$ mice were sensitized with 250 μg of MHSA and CFA in both hind footpads 12 days before sacrifice. Spleen and popliteal lymph node cells were obtained from these primed mice. Mixture of 1 part of lymph node cells and 2 part of spleen cells from sensitized donors were divided into two groups of cell suspensions. Either of them were incubated with anti-θ antiserum and complement or normal

TABLE II. *Effect of Anti-θ Treatment on Adoptive Transfer of Delayed FPR to MHSA by Sensitized Lymphoid Cells* into Normal Recipients*

Treatment of lymphoid cells	Cell dose	No. of recipients	FPR** in recipients (mean ± S. E.) 6 hr	12 hr	24 hr
normal AKR serum and complement	10^8	5	4.0±0.6	3.4±0.7	1.0±0.3
anti-θ serum and complement	10^8	5	2.0±0.3	0.4±0.2	0.2±0.2

* Mixture of 1 part of lymph node cells and 2 part of spleen cells from sensitized donor with MHSA and CFA 12 days before sacrifice were incubated with anti-θ antiserum or normal AKR serum and complement, and then injected intravenously into normal recipients.

** Recipients of sensitized lymphoid cells were challenged with 20 γ of MHSA in PBS at left hind footpad 2 days later, and then FPR was estimated at various intervals after challenge.

AKR serum and complement. Cell transfers were accomplished by intravenous injection of 10^8 incubated cell mixture per each normal recipient. The recipients were challenged by the standardized method 2 days later, and then FPR of each recipient was estimated at various intervals. As shown in Table II, anti-θ treatment of immune lymphoid cells resulted in a loss of capacity to transfer DH to MHSA. Normal AKR serum and complement were ineffective on transfer. Preliminary study using C3H mice showed the same results as above. These results confirmed that the cells play an important role in effector limbs of FPR to MHSA are T cells.

6. Effect of Ferritin on Eliciting Delayed Footpad Response by MHSA

In order to clarify the participation of macrophages in developing delayed footpad swelling, sensitized $BC3F_1$ mice aged 8 months received 0.2 ml of ferritin solution intraperitoneally just before challenge injection on the 12th day of sensitization. Our previous work (13) revealed that ferritin was as selectively toxic to macrophage as was carrageenan and that the former was more potent than the latter. As shown in Table III, delayed FPR was suppressed in ferritin treated mice as completely as in 800 R irradiated mice, while this treatment appeared to have no effect on the general condition of mice.

TABLE III. *Effect of Intraperitoneal Injection of Ferritin Prior to Challenge Injection on Development of Delayed Footpad Reaction to MHSA in Mice**

Pretreatment** with ferritin	No. of animals	FPR on 12th day Mean±S. E.
no	5	11.8±0.6
yes	5	0.8±0.6

* (C57BL×C3H) F_1 hybrid (8 m. o.).
** 0.2 mg of ferritin was injected intraperitoneally just before challenge injection.

7. Cells Involved in Establishment of DH against MHSA

The reconstitutive capacity of various individual and mixed lymphoid cell population in thymectomized and lethally irradiated recipients were examined by FPR to MHSA. As shown in Fig. 4, a preliminary study revealed a synergism between thymus and bone marrow cells in DH. Transferred 2×10^7 bone marrow cells did not elicit FPR in the recipients. The recipients restored by 5×10^7 spleen cells developed moderate FPR and the mice restored by 5×10^7 thymus cells and 2×10^7 bone marrow cells showed a rather strong FPR.

In order to elucidate the relationship of thymus and bone marrow cells in establishment of DH state, dose response studies on FPR to MHSA were carried out in the immunologically suppressed host restored by employing three mixed lymphoid cell populations, each consisting of different cell dosages. The bone marrow cells employed in this experiment were treated with anti-θ and complement to eliminate contamination of a small number of T cells in this cell population. As can be seen in Table IV, DH response increased in the footpad swelling according to the increasing dosages of transferred thymus or spleen cell populations.

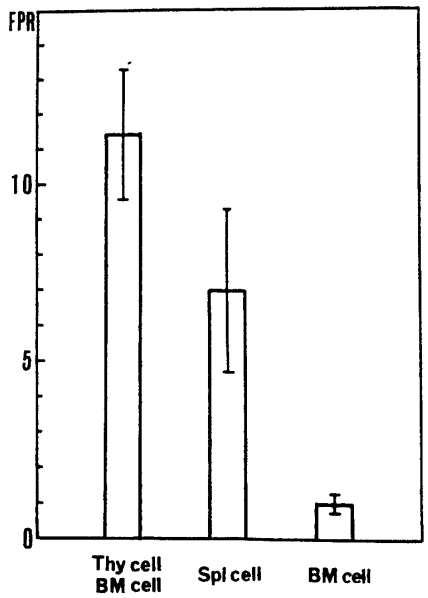

Fig. 4. Levels of 24 hr FPR to MHSA in adult thymectomized and lethally irradiated recipients reconstituted with 10^7 bone marrow cells (BM cell). 5×10^7 spleen cells (Spl cell), and 5×10^7 thymus cells (Thy cell) and 10^7 bone marrow cells. Mean of 5 mice ± standard error.

TABLE IV. *Dose Effect of Transferred Lymphoid Cells on the Grade of FPR to MHSA in Thymectomized and Irradiated Recipients*

Exp.	Populations of transferred cells			Number of animals	FPR** on 12th day Mean±S. E.
	Thymus cell	Spleen cell	Bone marrow* cell		
C	—	—	10^7	3	1.3±0.7
	—	—	2×10^7	8	1.0±0.2
Exp. I	10^5	—	10^7	3	0.3±0.3
	10^6	—	10^7	5	0.4±0.2
	10^7	—	10^7	6	4.7±1.4
	10^8	—	10^7	6	13.7±0.6
Exp. II	—	10^6	10^7	6	2.5±0.9
	—	10^7	10^7	5	5.5±1.4
	—	10^8	10^7	5	16.7±1.5
Exp. III	5×10^7	—	10^5	5	5.2±1.0
	5×10^7	—	10^6	5	7.0±1.3
	5×10^7	—	10^7	5	7.0±2.3
	5×10^7	—	10^8	5	7.2±1.8

* Bone marrow cells were treated with anti-θ and guinea pig complement just before transfer.

** Recipients were thymectomized at 7 weeks of age, and 800 R irradiated at 9 weeks. Various lymphoid cells were transferred intravenously on the same day of irradiation. Each recipient was sensitized with MHSA and CFA 2 days later.

At least 10^7 thymus cells or 10^6 spleen cells were required to elicit FPR to MHSA in the recipients restored with 10^7 bone marrow cells which did not

develop FPR to MHSA. In contrast, addition of increasing dosages of bone marrow cells to mice given 5×10^7 thymus cells did not affect the degree of FPR within a wide range of dosage of bone marrow cells.

Thus footpad response to MHSA in mice requires the synergistic activity of thymus and bone marrow cell populations. However, the limiting cells in the degree of sensitive state of DH in mice are T cells, which may react with the antigen specifically, and not B cell, which may reflect the reactivity of sensitized T cells with antigen to produce DH lesion in the skin.

DISCUSSION

An increasing number of investigators employ footpad swelling as devised by Gray and Jennings (19) for the assay of DH in rodents as this method is both reproducible and convenient. Accumulating evidence suggests that the footpad swelling is a reaction possessing characteristics of classical delayed skin reaction immunobiologically as well as histopathologically (13, 20). However, it must be pointed out that an increase in footpad thickness even at the delayed stage does not necessarily eliminate the possible contribution of an Arthus type reaction due to antibody.

Previous reports provided evidence that protein antigens (11, 12, 20), as well as heterologous erythrocytes (22) evoked DH response rather preferentially as compared to humoral antibody response by chemical modification such as methylation, acetoacetylation *etc.* Indeed, specific antibody forming cells to MHSA were not demonstrated by an indirect immunofluorescent technique in the regional lymph nodes and the spleens from mice sensitized with MHSA and CFA 8 or 12 days prior to sacrifice (13). The minimal footpad swelling observed at 5 hr in MHSA-sensitized mice might account for the low ability of this antigen to elicit humoral antibody response, compared with the high or moderate 5 hr footpad response in animals sensitized with heat-aggregated HSA or native HSA, respectively (Table I). Moreover, 800 R whole body irradiation completely suppressed the FPR in presensitized mice, even though they were given 2×10^7 bone marrow cells after irradiation. Thus the experimental condition employed herein excludes the role of antibody in developing delayed footpad swelling, including a role of cytophilic antibody which sensitized unprimed bone marrow derived cells passively to evoke delayed type skin lesion (23, 24).

Development of FPR to MHSA in the 450 R or 600 R irradiated mice suggests the relative radioresistance of presensitized lymphoid cells participating in FPR and that these cells are a subpopulation of T cells, not B cells nor promonocytes. The latter types are considered to be more radiosensitive than some subsets of T cell populations (7, 25). Youdim *et al.* (26) also observed that transfer of DH to *Listeria monocytogenes* in mice was achieved by the radioresistant T cell population. Increasing doses of irradiation resulted in the gradual suppression of development of FPR and only 800 R irradiation could completely eliminate the eliciting of FPR. On the other hand, one administration of 0.2 mg ferritin suppressed delayed footpad swelling completely. These

observations might support the conception that macrophages are also essential for developing delayed FPR in mice.

Eidinger *et al.* (8) and Ackerman and Eidinger (27) suggested the presence of T-B cell interaction for induction of delayed footpad swelling to MHSA, as observed in the case of humoral antibody response. They also concluded that in DH the T cell was akin to the antigen-reactive and that the cell limiting the degree of response, the effector cell, was of bone marrow origin. They observed that increasing the number of bone marrow cells increased the degree of reactivity to MHSA in the recipient mice (27). It could not be ruled out, however, that a small number of contaminating T cells in the bone marrow cell population was responsible for such a dose dependent response. In the present work, the recipient mice reconstituted by a moderate number of thymus cells did not show any increasing degree of FPR to MHSA according to addition of increasing dosages of bone marrow cells, when the latter were treated with anti-θ and complement. Indeed, 10^6 normal spleen cells, 3 to 4 $\times 10^5$ T cells in the spleen, were sufficient to induce FPR to MHSA in the immunologically suppressed recipient restored by 10^7 anti-θ treated bone marrow cells (Table IV). On the other hand, there were good correlations between the transferred thymus or spleen cell dosages and the degree of FPR in the recipients.

The amount of chemical mediators released from presensitized T cells by stimulating with antigen (28, 29, 30) and acting upon non-sensitized monocytes as the chemotactic factor resulting in development of DH skin lesion (4, 31) must be dependent on the population of specific sensitized T cells reacting with antigen, when the effect of preformed antibody in experimental system can be eliminated. Youdim *et al.* (26) observed in their irradiation study on established DH state that DNA synthesis of monocytes in the skin DH lesion was also a phenomenon of T cell dependency. The same observation was reported by North and Mackaness (32). The T cell population capable of encountering antigen molecules in the skin may reflect the entire population of specific sensitized T cells in individual mice. Thus under the conditions provided by our experimental system, T cells were shown to be the limiting cells for determining the degree of the sensitive state of DH, and such data may demonstrate the reactive state of sensitized T cells in sensitized animals.

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