STUDIES ON DELAYED HYPERSENSITIVITY IN MICE
III. Evidence for Suppressive Regulatory T_1-Cell Population in Delayed Hypersensitivity*

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There is increasing evidence for cell-to-cell interactions in immune response (1, 2). While the interaction between B and T lymphocytes has been studied by many investigators both in vivo and in vitro, there is a paucity in the literature concerning subpopulations of T cells and their interactions. The nature of T-T-cell interactions is considered an important key in resolving the regulatiry mechanisms of cell-mediated immune responses (CMI).[1] Cantor et al. (3, 4) first reported the cooperation of two T-cell subpopulations, termed precursor of effector cells and amplifier cells, in the development of graft-vs.-host reactions (GVH) in mice. Suppressive actions of T cells were also demonstrated in GVH by Gershon et al. (5, 6) and by Hardin et al. (7). T-T-cell cooperations and suppression in vitro have been observed in mixed lymphocyte reactions (MLR) (8–11) and in cytotoxic allograft responses (12–15). Delayed hypersensitivity (DH) is an important phenomenon manifested by T-cell activity, yet few papers have discussed such activity.

A decline in immunocompetence with respect to both cell-mediated and humoral immunity is considered an age-related phenomenon in mammalians and this decline appears to be linked to involution of the thymus (16). In the present study using mice, we found with age, an increasing reactiveness in DH to methylated human serum albumin (MHSA). After reaching peak responses at around 10–15 mo of age, the animals showed a gradual reduction in the degree of the response. In a preceding paper,[2] we reported that foot pad reaction (FPR) as a manifestation of DH to MHSA is a phenomenon with high T-cell function dependence, and that the grade of foot pad swelling was limited by the sensitized T-cell population. The discrepancy observed in involution of the thymus and

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; CFA, complete Freund's adjuvant; CMI, cell-mediated immune response; DH, delayed hypersensitivity; FPR, foot pad reaction; GVH, graft-vs.-host reactions; HSA, human serum albumin; MHSA, methylated human serum albumin; MLR, mixed lymphocyte reaction; PBS, phosphate-buffered saline; PHA, phytohemagglutinin.

[2] Morikawa, S., M. Baba, T. Harada, and K. Yasuhira. 1977. Studies on delayed hypersensitivity in mice. II. T cell dependency of the response: limiting cells in induction of foot pad reaction. Manuscript submitted for publication.

increasing of the FPR with age suggests the existence of a suppressive regulatory mechanism related to T-cell function in DH. Cell transfer study and adult thymectomy reveal the presence of a suppressive T-cell population in regulation of DH in mice. These suppressive T cells were found to belong to the short-lived T_1 cell (17) populations.

Such age-related loss of suppressive regulation in CMI was recently reported in the study on GVH in New Zealand Black mice by Gerber et al. (18). The age-related nonspecific regulation on CMI is discussed herein.

Materials and Methods

Mice. Inbred C57BL/6J mice raised and maintained by brother and sister mating in the animal center in our laboratory were used throughout. Mice of various ages were fed a commercial laboratory chow and water ad libitum. Individual mice used for foot-pad assay of DH were identified by marks made on the ears with a metal punch.

Antigen. Four times crystallized human serum albumin (HSA), crystalline bovine serum albumin (BSA), and six times crystallized ovalbumin (OA) were obtained commercially from ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio, and Armour Pharmaceutical Co., Kankakee Ill. MHSA was prepared by the methanol-hydrochloric acid method described by Crowle et al. (19). BSA and OA were coupled with recrystallized 2,4-dinitrophenyl (DNP) sulfonic acid as hapten by the method of Eisen et al. (20). Final protein concentrations were determined by modified micro-Kjeldahl analysis (21). The number of DNP groups per molecule of carrier protein (DNP-BSA, DNP-OA) was determined spectrophotometrically. Sheep red blood cells (SRBC) were obtained in Alsever's solution from the Nikken Animal Blood Supply Center, Kyoto, Japan, and were washed three times with phosphate-buffered saline (PBS), pH 7.1, before use.

Preparation of Cell Suspensions. Suspensions of thymus, spleen, and popliteal lymph node cells were prepared according to the method described previously.[2] Viability of cells was examined by dye exclusion test with trypan blue.

Assay for Response to PHA. Spleens from two to four mice were removed, pooled, and prepared as single cell suspensions. The cell populations were adjusted to 5×10^6 viable cells/ml in medium RPMI 1640 containing 10% horse serum and 60 μg/ml kanamycin. Tubes were set up in triplicate containing 10^7 cells and 1 μg phytohemagglutinin (PHA) (Burroughs-Wellcome & Co., Tuckahoe, N. Y.) in 2 ml. This dose of PHA was shown to be optimal in preliminary experiments. The cells were cultured for 24 h, pulsed with 1 μCi tritiated thymidine (sp act 1.0 Ci/mmol) and then cultured for an additional 24 h at 37°C in an atmosphere of 5% CO_2 and 95% air. After 48 h incubation, the cells were harvested by centrifugation and repeatedly washed. The radioactivity was measured in a liquid scintillation spectrometer (Mark II; Nuclear-Chicago Corp., Des Plaines, Ill.). Data were expressed as mean counts per minute of the cultures with the standard error of the mean. The stimulation index was calculated as the mean counts per minute of PHA-stimulated cultures per the mean counts per minute of nonstimulated cultures. In this experiment, cells from mice of all ages were tested on the same day.

Thymectomy. Thymectomy was performed on groups of mice at various ages according to the method described previously.[2] Mice under 2 wk of age were anesthetized by cooling. After operation they were warmed at 37°C in an incubator.

Immunization. Immunization for foot pad DH assay was carried out by subcutaneous injection into the left hind foot pad with 0.05 ml of emulsion consisting of equal volumes of 0.5% MHSA solution in PBS and complete Freund's adjuvant (CFA).[2] The mice were immunized with DNP-BSA for the assay of antibody activity by the same precedure as MHSA described above. Immunization for antibody assay to SRBC was performed by intraperitoneal injection of 5×10^7 SRBC to mice at various ages without any adjuvant.

Foot pad Assay for DH. Foot pad measurement was made according to the method previously described (22). The difference of foot pad thickness at 24 h after and just before challenge injection was measured and expressed as FPR in 0.1 mm unit. Challenge injection with 0.02 ml of 0.1% protein solution was usually performed on the 11th or 12th day after sensitization.

Antibody Titration. Hemolysin titers were determined in microtitration trays using 0.025-ml

vol of dilutent by serial dilution of inactivated pooled sera in PBS containing 1% inactivated normal mouse serum, which was added on to an equal volume of 0.2% packed SRBC. After a 1 h incubation at 37°C, twofold diluted normal guinea pig serum was added as complement. The incubation mixtures were incubated further for 1 h at 37°C. Each titration was performed in duplicate and the mean titer was expressed in $\log_2$. Passive hemagglutination titers to DNP-hapten and BSA of serum were measured by the microtitration technique with tanned SRBC coated with DNP-OA or BSA by the method of Stavitsky and Arquilla (23).

Statistical Methods. Standard errors and the means and P values were calculated using the Student's t test.

Results

Age-Related Changes in Thymus Weight and FPR to MHSA in Comparison with Hemolysin Response to SRBC. Involution of the thymus begins around 4 wk of age in C57BL/6J mice. At that time the thymus weight of our C57BL mice had reached nearly 55 mg. Thereafter the thymus weight decreases gradually throughout the life of the mouse. In contrast, C57BL mice developed FPR to MHSA, increasing steadily in degree until around 8–10 mo of age and then showed a plateau of the response to MHSA as high as 20 in FPR for a rather long period followed by a gradual decrease in grade. Of interest was the observation that the steady increase of the degree of FPR to MHSA paralleled the same stage of drastic weight loss of the thymus, despite the fact that the FPR to MHSA is a highly T-cell-dependent phenomenon.[2] Meanwhile, C57BL/6J mice sensitized with 5×10^7 SRBC intraperitoneally produced hemolysin to SRBC and such is regarded as a T-cell-dependent humoral immune response without any age-associated change so far as tested (Fig. 1).

Effect of Aging on Responsiveness of Spleen Cells to PHA. To examine other age-related responses of T-cell function in C57BL/6J mice, stimulation of DNA synthesis was done by addition of PHA, T-axis mitogen, to spleen cell culture from variously aged mice (Table I). The highest stimulation ratio was observed in spleen cells from young adult mice 5 mo of age. A decline was seen in the responsiveness to PHA of spleen cells from old mice. On the other hand, in the youngest group, 1-mo-old mice, a low response to PHA was seen in spleen cell culture.

Effect of Additive Transfer of Thymus or Spleen Cells on FPR to MHSA. The discrepancy observed between involution of the thymus or age-related decline of responsiveness of spleen cells to PHA and age-related kinetics of FPR to MHSA leads to the consideration of two possible factors: (a) requirement of so-called immune maturation in the peripheral lymphoid system to elicit remarkable foot pad swelling to MHSA by sensitization; and (b) decrease in suppressor T-cell population regulating the degree of DH response to MHSA associated with thymic involution. To examine these possibilities, cell transfer studies were carried out between syngeneic young and old mice. As seen in Table II, in old C57BL mice in which the FPR to MHSA was considerably high, the degree of FPR was suppressed by intravenous injection of thymus cells from normal young mice 2 days before sensitization. Increasing dosages of transferred thymus cells resulted in a greater suppression in FPR to MHSA in old mice, however, thymus cells derived from mice 9 mo of age were less effective in suppression of FPR than thymus cells from young mice. This suppression of development of DH occurred only by transfer of viable thymus cells. Thymus

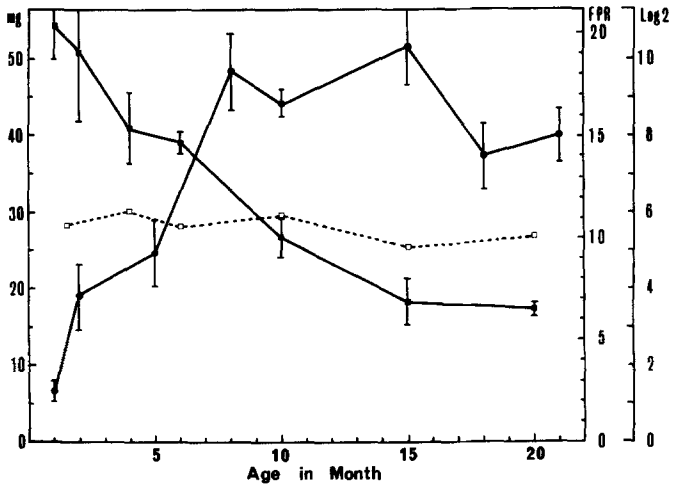

FIG. 1. Age-related changes of foot pad response to MHSA (solid line with closed circle), thymus weight (solid line with closed square), and hemolysin titer to SRBC (broken line with open square) in C57BL mice. Each group consists of five to six male mice. Vertical bars are standard errors of the mean.

TABLE I

Stimulation of Tritiated-Thymidine Incorporation by PHA in Spleen Cells from Variously Aged C57BL Mice

Age of spleen cell donor	Incorporation of ^{3}H-thymidine (mean cpm ± SE)		Stimulation index
	Without PHA	With PHA	
mo			
1	9,600 ± 670	125,000 ± 7,000	13
5	2,690 ± 190	269,000 ± 2,000	100
10	8,320 ± 460	245,000 ± 12,000	30
15	5,190 ± 360	143,000 ± 9,000	28
18	3,900 ± 640	94,700 ± 9,400	24
24	9,300 ± 1,120	218,000 ± 16,000	24

cells killed by repeated freezing and thawing and injected intravenously had no effect on the development of foot pad swelling (Table III). Cell transfer carried out 2 days before test injection in presensitized mice did not show any suppressive effect on appearance of FPR to MHSA (Table III). Complete suppression of development of FPR was never achieved herein by transfer of thymus cells from normal donors to normal recipients. Transfer of thymus cells from young donors did not suppress the degree of FPR to MHSA in young recipients, rather enhancement of the response to MHSA being occasionally observed.

As shown in Table II, 5×10^7 spleen cells from both young and old mice showed an enhancing effect on eliciting FPR to MHSA in young mice by intravenous transfer 2 days before sensitization. The spleen cells from older mice (9- to 10-mo old) were more effective for enhancing FPR to MHSA in the recipient mice than those from young mice (7-wk old), suggesting that the spleen of old mice is richer in effector cells. On the other hand, the spleen cells

TABLE II

Effect of Additional Transfer of Thymus or Spleen Cells on Development of FPR to MHSA in Old or Young C57BL Mice

Cells transferred*			Recipients		
Age of donor	Cell source	Dose	Age	No.	FPR on 12th day, mean ± SE
			mo		
	–		10–12	5	23.0 ± 0.6
9 mo	Thymus	5×10^7	10–12	4	16.8 ± 2.1
7wk	Thymus	1×10^7	10–12	5	16.6 ± 1.7‡
7wk	Thymus	10×10^7	10–12	5	9.6 ± 0.7§
	–		2–3	8	11.1 ± 1.2
7 wk	Spleen	5×10^7	2–3	5	14.8 ± 1.4
9–10 mo	Spleen	5×10^7	2–3	8	17.1 ± 0.9‖

* Viable cells from normal donor were transferred into nontreated recipients 2 days before sensitization with MHSA and CFA.
‡ $P < 0.025$.
§ $P < 0.001$.
‖ $P < 0.01$.

TABLE III

Time Effect of Thymus Cell Transfer on Suppression of FPR to MHSA in Old Mice

Day of the treatment	Cell transfer*	Recipients	
		No.	FPR on 12th day, mean ± SE
2 days before sensitization	5×10^7 viable cells	6	14.8 ± 1.9‡
2 days before sensitization	1×10^8 killed cells	4	19.5 ± 1.3
2 days before challenging	5×10^7 viable cells	6	20.7 ± 0.8
–	–	7	20.3 ± 0.7

* Thymus cells from 6- to 7-wk-old mice were transferred into 11-mo-old recipients.
‡ $P < 0.02$.

from young normal or MHSA-sensitized donors 4 days before transfer showed no significant suppressive effect on development of FPR to MHSA in recipients into whom intravenous transfer was made 2 days before sensitization (Tables IV and V). Cells from the popliteal lymph nodes from the sensitized donor also had no suppressive effect on FPR (Table V).

Effect of Thymectomy on the Induction of FPR to MHSA in C57BL/6J Mice. If the decline of thymus function with age results in an increase in the foot pad swelling by MHSA, due to a decrease in supply of suppressive cell population from the thymus, the possibility of whether or not a thymectomy at an adult age would result in an enhancement of FPR to MHSA should be investigated. Thus groups of six to seven mice were thymectomized at various ages and all were sensitized with MHSA and CFA and a foot pad assay was done when the animals were 6 mo of age. As seen in Fig. 2, in mice thymectomized 3

TABLE IV
Effect of Additional Transfer of Spleen Cells from Young Donors on Development of FPR to MHSA in Old Mice

Cells transferred*	Dose	Recipients	
		No.	FPR on 12th day, mean ± SE
–		5	12.8 ± 1.4
Spleen cells	5×10^7	4	9.8 ± 1.3
Thymus cells	2×10^7	4	4.8 ± 0.5

* Spleen and thymus cells were prepared from the same donors at the age of 6–7 wk. These cells were injected intravenously into the recipients at 9 mo of age and 2 days before sensitization.

TABLE V
Failure of Sensitized Lymphoid Cells to Suppress Development of FPR to MHSA

Cells transferred*	Recipients	
	No.	FPR on 12th day, mean ± SE
–	3	6.0 ± 1.0
1×10^8 lymph node cells	4	8.3 ± 0.9
1×10^8 spleen cells	5	8.8 ± 1.4

* Spleen and popliteal lymph node cells were obtained from young donors (3-mo old) sensitized with MHSA and CFA 4 days before sacrifice. These cells were injected into 5-mo-old recipients.

days after birth, three out of six did not develop FPR while in the others FPR to MHSA developed at same level as seen in normal C57BL mice of 6-mo old. Thymectomy performed at 1 or 2 wk of age had no effect on the degree of FPR to MHSA in the mice at 6 mo of age. Mice undergoing thymectomy at 4, 6, 8, or 16 wk of age showed a remarkable enhancement of FPR to MHSA at 6 mo of age. The degree of the enhancement was essentially similar in each group of mice thymectomized at various ages and levels were similar to those seen in old mice. Increasing the time intervel between thymectomy and immunization did not significantly alter the degree of enhancement.

The time of onset of enhanced FPR to MHSA after thymectomy was then examined. C57BL mice, 4-mo old, were thymectomized and groups of the mice were sensitized with MHSA and CFA at subsequent time intervals of 2, 4, and 6 wk. As shown in Fig. 3, enhancement of FPR to MHSA was observed in mice sensitized as early as 4 wk after thymectomy. The early occurrence of enhanced FPR after thymectomy suggests that the effect is due to the disappearance of suppressive T-cell populations which are short lived and are continuously supplied by the thymus.

Effect of Aging, Adult Thymectomy, and Additionally Transferred Thymus Cells on Humoral Antibody Response to Hapten and Carrier in C57BL/6J Mice. To determine whether or not a similar effect of T-cell regulation is working on humoral antibody response, groups of 6- and 12-mo-old C57BL mice

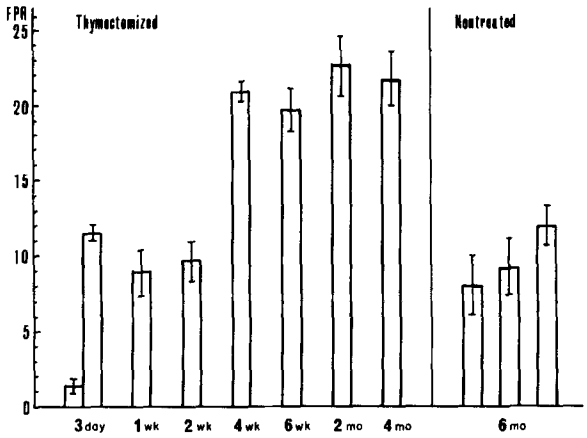

FIG. 2. Levels of 24-h FPR to MHSA in 6-mo-old C57BL mice which had been thymectomized at various ages. The histograms of control groups show the levels of FPR to MHSA assayed at different times in the 6-mo-old mice not thymectomized. Vertical bars are standard errors of the mean.

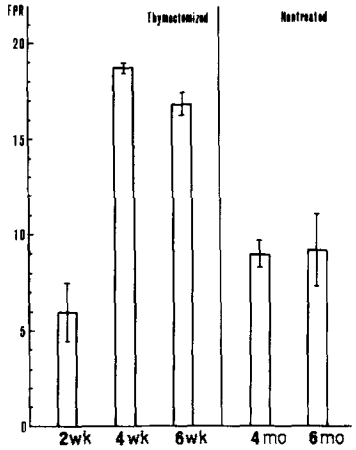

FIG. 3. Levels of 24-h FDR to MHSA in C57BL mice sensitized and assayed at different intervals after thymectomy at the age of 4 mo. Control groups show the level of FPR to MHSA in nonthymectomized mice at the ages of 4 and 6 mo. Vertical bars are standard errors of the mean.

were immunized with 125 μg of DNP-BSA and CFA by the same procedure employed in sensitization with MHSA. Some adult animals were thymectomized and/or an additional transfer of thymus cells before immunization was made. All mice were bled from the retro-orbital plexus on the 12th day after immunization. As seen in Table VI, groups of 6- and 12-mo-old C57BL mice produced only a minimal humoral response to BSA. No enhancing effect was observed with aging. Moreover, transferred thymus cells from young donors showed a tendency to an increased antibody response in old recipients. An increased humoral response to hapten DNP was more apparent in 12-mo-old mice as compared with those in mice 6 mo of age. Transferred thymus cells did not suppress antibody response to DNP in either old or adult thymectomized mice.

TABLE VI

Effect of Adult Thymectomy and Thymus Cell Application on Humoral Antibody Response to Hapten and Carrier in C57BL Mice

Age	Treatment	No. of animals	Antibody titer (log_2)	
			Anti-DNP	Anti-BSA
mo				
6	–	5	3.9 ± 0.6	2.0 ± 0.2
6	Thymectomy*	5	4.4 ± 0.7	2.9 ± 0.6
6	Thymectomy + thymus cell‡	5	5.8 ± 0.8	2.5 ± 0.3
12	–	4	5.6 ± 0.3	2.0 ± 0
12	Thymus cell‡	6	5.7 ± 0.5	2.9 ± 0.4

* Thymectomy was performed 8 wk before sensitization.

‡ 5×10^7 thymus cells from young donors were injected intravenously into the recipients 2 days before sensitization.

Discussion

In this work we found that two distinctively different T-cell populations are involved in the DH response, one of the T-cell manifestation of CMI; one regulatory T cells and another antigen-reactive T cells, effector T cells. The latter are stimulated by antigens and proliferate to develop a hypersensitive state in mice. The former work as a regulator on unprimed antigen-reactive T cells to prevent an excess proliferation, as transferred thymus cells could suppress FPR to MHSA when injected into the old recipients before sensitization, and not before challenge. Moreover they did not suppress the FPR when the recipients were young mice which developed poor FPR to MHSA. This regulation suppresses induction of extremely high sensitive states in individuals.

Although the mechanism of this regulation on the DH response by T cells is still obscure, it is likely that the suppressive regulation occurs by direct cell-to-cell interactions between two T-cell subpopulations. Presensitized spleen and lymph node cells lacked the suppressive effect on developing delayed foot pad swelling when these cells were injected intravenously before sensitization. Moreover the foot pad swelling with MHSA in mice is a highly T-cell-dependent phenomenon in which the contribution of humoral antibody is almost exclusively eliminated.[2] These observations suggest that the suppressive regulation in DH to MHSA is not due to an increased production of humoral antibody and/or contra-sensitizing antibody, such as was reported by Crowle and his colleagues (24, 25) and Mackaness et al. (26) in mice. Early appearance of the enhanced effect of adult thymectomy on DH response also supports the conception of T-T-cell interactions, because the effect of thymectomy in adult life on humoral antibody appears significantly after a period of 9–11 mo in mice. (27, 28).

More recently, presence of suppressor T cells in the DH response of the rat (29) and in contact sensitivity of mice (30) were reported. However, the donor animals of suppressor T cells in those experiments were highly immunized by an unusually high dose of antigen or by repeated immunization. Therefore, the possibility that these suppressor T cells were active as the results of excess

antigen and/or antigen-antibody complex has to be considered (31). Bullock et al. (32) also reported the suppressive effect of sensitized T cells with a small molecular weight antigen on development of the DH skin reaction to carrier protein conjugated with this low molecular compound, when the animals were sensitized with the antigen and incomplete Freund's adjuvant and tested by the carrier protein. The antigen-specific suppressor cells did not work, however, when the animals were sensitized in the presence of CFA. Thus, in addition to the mechanism suggested by the authors herein, there appears to be another regulatory mechanism in T-cell function such as observed in anti-hapten antibody responses (33, 34).

According to our observations, suppressor T cells in DH response have a life span of less than 4 wk and require a constant derivation from the thymus, as the decline of suppressive activity is associated with involution of the thymus and is often observed to be rapid after adult thymectomy. In addition to the weight loss of the thymus with age, the precursors of such T cells seem to be abundant in young mouse thymus and few in that of old mice, and there is little distribution in the peripheral lymphoid system.

Short life span and scanty in aged animals of suppressor T-cell population were reported in regulation of GVH (18) and in in vitro CMI response (13, 14, 35). Besides CMI, such short-lived suppressor T cells were found to participate in regulation of primary humoral antibody response to thymus-independent antigen (36–40). These suppressor T cells including those working in DH response may interact directly with antigen-reactive T cells or B cells at their proliferating stage by recognition of the idiotypic difference and/or autoantigenic determinants of possible surface markers. Such functions of short-lived T-cell populations no doubt play an important role in the immune surveillance system.

Effector T cells are not affected by adult thymectomy for long intervals between treatment and sensitization and seem to be low responders to PHA stimulation in vitro. As there was no enhancement of FPR to MHSA in 6-mo-old mice, which had been thymectomized at less than 2 wk of age, a period of time may be required to develop effector T-cell function in the peripheral lymphoid system under the state of a functioning thymus. In CMI, such long-lived effector T cells were also observed in development of GVH response (3, 4, 41) in vivo and in expression of T-cell-mediated cytotoxicity (12) and MLR (8) in vitro.

Finally, three different subsets of immune competent cells are involved in DH response just as in GVH and humoral antibody responses in vivo. The cells consist of one specifically antigen-reactive T cell; one suppressive regulatory T cell; and one bone marrow-derived cell, a macrophage, which responds to a chemical mediator from sensitized effector T cells and develops a DH skin lesion. On the other hand, two types of specifically reactive T cells, precursors of antigen-reactive cells and amplifier cells, and one suppressive regulatory cell participate in GVH reaction (3, 4). As for thymus-dependent humoral antibody response, two subsets of specifically reactive T cells, helper T cell and suppressor T cell (36, 42–44), and one specifically reactive B cell, precursor of antibody-forming cell, are observed in the cell interaction system. Although B cells in humoral antibody response differ in specificity and in mediation, nevertheless, amplifier T cells in GVH response and monocytes in DH response work equally as effector cells which produce various immune phenomona in vivo.

Summary

T-T-cell interactions involved in delayed hypersensitivity (DH) response have been studied by employing delayed foot pad assay to methylated human serum albumin in C57BL/6J mice. The DH response, one of the T-cell manifestations of cell-mediated immune response is suppressively regulated by T cells and such observation was based on studies of age-associated kinetics of foot pad reaction and effects of cell transfer and adult thymectomy on developing DH response. These suppressively regulatory T cells in DH have a life span of less than 4 wk and a constant derivation from the thymus is required. Such cells are numerous in the young mouse thymus and few in the spleen and thymus of old mice. On the one hand, the presence of a long-lived effector T-cell population was suggested in DH. These cells are numerous in the spleen and are low responders to phytohemagglutinin in vitro. It is assumed that these suppressive T cells interact with antigen-reactive cells at their proliferating stage by recognition of the iodiotypic difference through surface receptors. As in the case of graft-vs.-host and humoral response in vivo, three different subsets of immune competent cells participate in the DH response. These cells consist of one specifically antigen-reactive T cell, one suppressive regulatory T cell, and one bone marrow-derived cell, a macrophage that responds to a chemical mediator from sensitized effector T cells and that develops a DH skin lesion nonspecifically.

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