CYCLOPHOSPHAMIDE ELIMINATES SUPPRESSOR T CELLS IN AGE-ASSOCIATED CENTRAL REGULATION OF DELAYED HYPERSENSITIVITY IN MICE

By AKIO MITSUOKA,* SHIGERU MORIKAWA, MITSUO BABA, AND TAKAYUKI HARADA

From The Departments of Thoracic Surgery and Pathology, Chest Disease Research Institute, Kyoto University, Japan, and the Department of Pathology, Shimane Medical School, Japan

It has become increasingly apparent that subclasses of thymus-derived lymphocytes suppressively regulate cell-mediated immune phenomena such as graft-versus-host reaction (GvH)[1] (1, 2), cytotoxic allograft response (3), in vitro mixed lymphocyte reaction (MLR) (4), and contact delayed hypersensitivity (5). Recently, we reported on the T-T-cell interaction in delayed hypersensitivity (DH) response in mice induced by immunization with methylated human serum albumin (MHSA) in complete Freund's adjuvant (CFA), as revealed by cell transfer study and adult thymectomy (6). We suggested that the suppressor T cells regulated the DH in the induction stage. The suppressor activity was detected in such conventionally immunized mice, prominent in the young mouse thymus cells, but rare in the old mouse thymus cells or peripheral lymphocytes (6).

On the other hand, DH seems to be regulated by humoral antibodies. There is an apparent inverse relationship between induction of humoral antibody response and DH reactivity (7, 8). Moreover, DH induction is inhibited by antibodies such as gamma$_1$-contrasensitizing antibodies (9, 10). Pretreatment of guinea pigs with cyclophosphamide (CY), an alkylating drug, was also reported to increase DH intensity associated with a reduction in gamma$_1$-antibody production (11). Recently, treatment of animals with CY has been a popular method of investigating the suppressive role of humoral antibodies in DH, for this drug has been considered to affect B cells rather selectively (12–14).

In our preliminary work (15), we attempted to determine the effect of CY on the DH to MHSA. The DH was augmented by pretreatment of mice with CY, and it was suggested that augmentation of DH was attributed to damage of the suppressor T cells but not the B cells. The present work reconfirms our previous suggestion and an analysis is made of the cellular regulation involved in the mouse DH with application of CY.

* Requests for reprints should be sent to: Department of Thoracic Surgery, Tenri Hospital, Tenri (632) Japan.

[1] *Abbreviations used in this paper*: CFA, complete Freund's adjuvant; CY, cyclophosphamide; DH, delayed hypersensitivity; FPR, footpad reaction; GvH, graft-versus-host reaction; MHSA, methylated human serum albumin; MLR, mixed lymphocyte reaction; PBS, phosphate-buffered saline, SRBC, sheep erythrocytes.

[2] S. Morikawa et al. Manuscript in preparation.

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Materials and Methods

Mice. Inbred C57BL/6 mice were used throughout. C57BL/6J mice were raised and maintained by brother and sister mating in the animal house of Chest Disease Research Institute, Kyoto University. C57BL/6Cr mice were purchased from the Experimental Animal Cooperative Association of Shizuoka (Hamamatsu), and C57BL/6N were purchased from the Charles River Japan Co., Ltd. (Atsugi).

Antigen. Human serum albumin four times crystallyzed was obtained from Nutritional Biochemicals Co. (Cleveland, Ohio). MHSA was prepared by the methanol-hydrochloric acid method described by Crowle et al. (7).

Preparation of Cell Suspension. Suspensions of thymus, spleen, and bone marrow cells were prepared according to the method described elsewhere (16).

Thymectomy. Adult thymectomy was performed at various ages according to the method described previously (16).

Splenectomy. Mice were anesthetized with pentobarbital sodium given intraperitoneally. After skin incision on the left side of the abdomen, laparotomy was performed by incising the abdominal muscles and peritoneum with scissors. The spleen was grasped by two forceps. While pressing branches of the splenic artery and vein with one forceps, the spleen was removed by tearing off from those vessels with the other forceps. Heated forceps were used for coagulation of the torn vessels. The abdominal wall and the skin wounds were closed with silk thread, respectively.

Sensitization. Sensitization for footpad DH assay was carried out by subcutaneous injection into the left hind footpad with 0.05 ml of emulsion consisting of an equal vol of 0.5% MHSA solution in phosphate-buffered saline (PBS), pH 7.1, and CFA.

Footpad Assay for DH. Footpad measurement was made according to the method described previously (17). Briefly, the difference in footpad thickness at 24 h after and just before challenge was measured and expressed as footpad reaction (FPR) in 0.1-mm units. Challenge with 0.02 ml of 0.1% MHSA solution in PBS was routinely performed on 11–13 d after sensitization.

CY Treatment. Mice were given CY treatment intraperitoneally, (Endoxan, Shionogi Co., Osaka), 150 mg/kg body weight, before sensitization. The interval of 4 d between CY treatment and sensitization was most commonly adopted. A reproducible enhancement occurred at this dose and interval (data not shown). In some experiments, CY treatment was made after sensitization.

Results

Age-Related Changes of DH to MHSA and Thymus Weight in Normal and CY-Treated C57BL/6 Mice (Fig. 1). In normal C57BL/6J mice, the level of DH to MHSA increased with increase in age of the mice from 5 wk to 8 mo, a result which was consistent with our previous data (6). Pretreatment of young mice with CY 4 d before sensitization enhanced the DH markedly, whereas the pretreatment of mice aged 6 or 8 mo did not. As a result, the level of DH in variously aged mice which had been given CY was much the same, and also similar to the level seen in nontreated aged mice.

Decrease in thymus weight was apparent up to 6 mo of age in nontreated mice. Hence, the upper age limits in CY-enhancing phenomenon and in drastic thymus involution appeared to be much the same. The effect of CY on the DH in C57BL/6Cr mice was somewhat decreased but comparable to that in C57BL/6J mice.

Effect of Antigen Dose on the DH in Normal and CY-Treated Mice. Both young adult (3 mo) and old (10 mo) mice were sensitized with varying doses of MHSA, followed by challenge 12 d later. FPR at 24, 48, and 96 h was measured (Table I). In young mice, a slight FPR at 24 h was seen with 5, 25, and 125 μg MHSA. With 625 μg MHSA, a more evident FPR was seen at 24 h but consequently diminished thereafter and a low

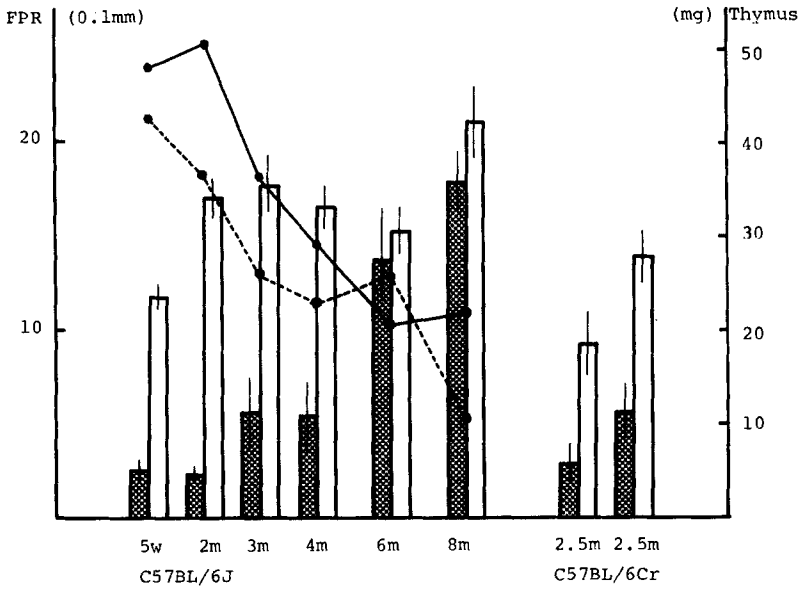

FIG. 1. Age-associated changes of DH to MHSA in normal (dotted column) or CY treated (open column) mice, and thymus weight in normal (solid line) or CY treated (broken line) mice. Mice were given CY, 150 mg/kg i.p., 4 d before sensitization. Each group included four to six mice. Vertical bars are standard errors of the means. W, weeks; m, months.

TABLE I

Dose-Response of DH to MHSA and the Effect of CY

Age	CY	MHSA	FPR after challenge on 12th d		
			24*	48	96
mo		*μg*		*h*	
3	−	5	3.0 ± 0.5	2.0 ± 0.3	1.0 ± 0.3
	−	25	4.0 ± 0.7	2.8 ± 0.4	1.2 ± 0.2
	−	125	3.8 ± 0.4	2.6 ± 0.7	1.4 ± 0.4
	−	625	7.4 ± 1.7	3.8 ± 0.8	1.0 ± 0.4
	+	25	11.8 ± 2.6	10.8 ± 3.1	1.0 ± 0.4
	+	625	10.3 ± 2.7	4.3 ± 1.6	1.0 ± 0.4
10	−	5	9.3 ± 2.4	8.1 ± 2.4	3.9 ± 1.5
	−	25	5.3 ± 2.1	6.5 ± 1.8	1.5 ± 0.8
	−	125	15.8 ± 3.1	15.3 ± 4.7	6.5 ± 2.5
	−	625	15.0 ± 1.3	8.5 ± 1.8	2.5 ± 0.7

Female C57BL/6Cr mice were sensitized with varying doses of MHSA in CFA. Mice were given CY, 150 mg/kg i.p., 4 d before sensitization. Each group included four to seven mice.

* Mean ± standard error.

FPR was seen at 48 h. When CY was given 4 d before sensitization in such young mice, FPR at 24 h was enhanced to the same level in lower and higher-dosed sensitized mice. However, CY did not block a decline of FPR at 48 h in the higher-dosed sensitized mice.

In old mice, the optimal sensitizing dose of MHSA appeared to be 125-625 μg. The level of DH reaction was higher in old mice. The decline of DH reaction at 48 h in old mice sensitized with 625 μg MHSA was also marked.

TABLE II
Suppressive Effect of Thymocytes on the Enhanced DH by Cyclophosphamide Treatment

CY	Thymocytes	No.	DH on day 11
−	−	5	2.8 ± 1.0
−	+	6	3.0 ± 0.9
+	−	5	9.2 ± 1.7
+	+	6	3.5 ± 1.2

Male C57BL/6Cr mice aged 2.5 mo were given CY, 150 mg/kg i.p., on day −4, and 2.5 × 10^7 thymus cells were transferred from normal syngeneic young mice on day −2 of sensitization.

From results in Fig. 1 and Table I, it appears that DH reactivity assumes the pattern seen in old mice when young mice are treated with CY.

Suppressive Effect of Normal Thymocytes on the Enhanced DH in Mice Pretreated with CY. In our previous work (6), we suggested that the DH induction is strongly regulated by suppressor T cells in young C57BL/6 mice but that the suppressor T-cell activity diminishes in aged animals. It is possible, hence, that enhancement of DH due to CY treatment of these young mice is attributed to elimination of such suppressor activity. For elucidation, young mice were given CY followed by a normal young thymocyte transfer before sensitization. As shown in Table II, administration of normal thymocytes did eliminate the DH enhancing effect of CY treatment. Thus, our preliminary suggestion (15) was confirmed.

Effect of Thymocytes or Splenocytes Transfer from Normal or CY-Treated Young Mice on the Enhanced DH in CY-Treated Mice. To determine whether there is a difference in T-cell (or precursor) activities, effector or suppressor, between T-axis cells in the thymus stage of nontreated and CY-treated mice, donor cells were also obtained from CY-treated mice. As shown in Fig. 2, enhanced DH due to CY treatment was suppressed by normal thymocytes but not by thymocytes from mice which had been given CY 7 or 13 d before the cell transfer. Thymocytes from mice treated with CY 7 d previously further enhanced the DH. Spleen cells never suppressed the DH reaction, and normal spleen cells enhanced the DH in this experiment. These data suggest that there are different subpopulations of thymocytes and that lymphocytes in the nonsensitized mouse spleen bear little, if any, suppressor activity in DH.

Suppression of DH by Treatment with CY after Sensitization and its Reconstitution with Lymphoid Cells. We previously found that treatment of mice with CY at 4 or 9 d after sensitization suppressed the DH on day 12 of sensitization (15). To determine what cell populations were injured by post-treatment with CY, mice were given CY 6 d after sensitization (5 d before challenge) followed by cell transfer of normal lymphoid cells 1 d thereafter (4 d before challenge) (Table III). Bone marrow cells alone or thymus cells alone could not restore the DH reactivity. However, with application of both of these cells, the mice were rendered full reactivity of DH response, as the level of DH was comparable to that of DH on day 4 in normal mice (16).

Effect of Cy Treatment on DH in Different Time Schedules for DH Induction. Our preceding data indicate the age-dependent difference in the kinetics of DH reactivity after sensitization; in old C57BL/6 mice, the DH reactivity showed its peak during

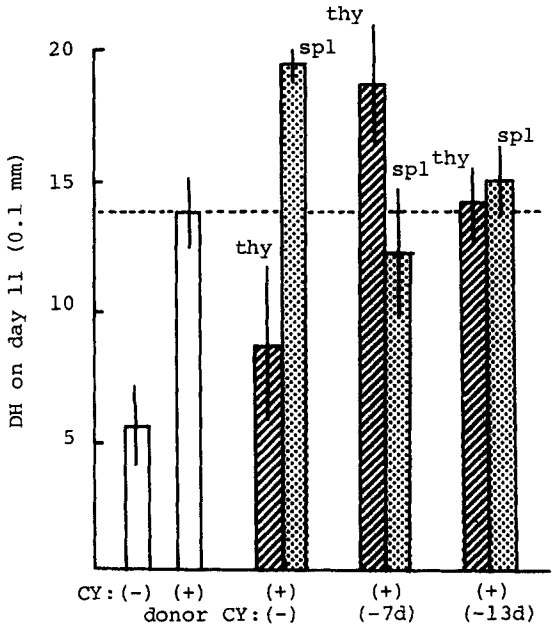

FIG. 2. Effect of cell transfer on the enhanced DH in CY treated mice. Recipient mice were treated with CY 4 d before sensitization. 1 d after CY treatment, the mice were transferred with 2×10^7 thymocytes (hatched column) or 5×10^7 splenocytes (dotted column) from normal mice or mice which had been treated with CY 7 d or 13 d before cell transfer, as indicated above. Two control groups, nontreated and CY-treated mice without cell transfer are shown as open columns. All animals used in this experiment were male C57BL/6Cr mice aged 2.5 mo. Each group included five to seven mice.

TABLE III

Reconstitution with Lymphoid Cells of Immunodepressed Mice by Treatment with Cyclophosphamide

CY	Cells transferred	No.	DH on day 11
−	No	5	10.6 ± 2.5
+	No	5	0.8 ± 0.6
+	Marrow cells	6	1.8 ± 0.6
+	Thymus cells	5	2.4 ± 0.2
+	Marrow cells and thymus cells	6	5.2 ± 1.0

Male C57BL/6J mice aged 5 mo were given CY, 150 mg/kg i.p., 6 d after sensitization and 1×10^7 bone marrow cells and 2.5×10^7 thymus cells from normal syngeneic mice were given 2 d thereafter.

12–16 d after sensitization, gradually decreasing in its reactivity afterward (16), and in young C57BL/6 mice, the peak DH reactivity was seen 8–12 d after sensitization and was followed by a rapid loss of reactivity (A. Mitsuoka, unpublished data). Earlier peak DH reactivity in young mice might also be the result of more potent regulation by a suppressor mechanism in young mice.

For clarification, the effect of CY on DH was investigated in different time schedules. In 8-mo-old mice, DH reactivity was generally high, even on day 16 after sensitization. CY treatment 4 d before sensitization did not augment the DH in this

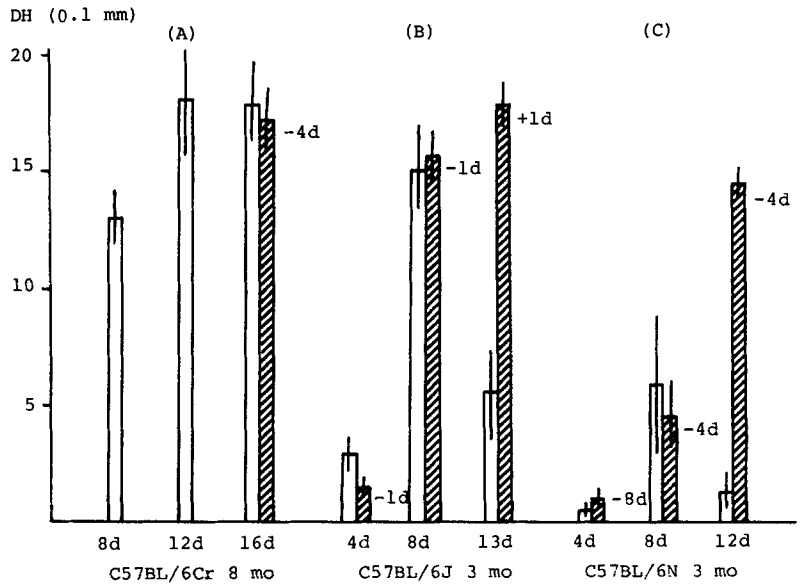

FIG. 3. Kinetics of DH reactivity on various days after sensitization in normal (open column) or CY-treated (hatched column) mice of various ages. The timing of CY treatment is indicated on the left shoulder of the hatched column, where minus means before, and plus means after sensitization. Each group included five animals.

group of mice (Fig. 3 A). In young mice (3 mo), DH reactivity was less intense and the peak response was seen on day 8. When CY was given to these mice, the DH on day 12 or 13 was markedly enhanced. The DH on day 8 was, however, neither enhanced nor suppressed with CY (Fig. 3 B and 3 C).

Influence of Previous Surgical Treatments of Mice on the DH in Nontreated and CY-Treated Mice. The foregoing data indicate that CY treatment of mice damages not only suppressor T cells but effector T cells and bone marrow-derived monocytes. Recovery from the damage of effector T cells and monocytes is considered to be more rapid and/or more complete than that of suppressor T cells. If this is the case, one of the problems is the location of the recovery site of effector T cells in CY-treated young mice. Thus, mice were surgically prepared before CY treatment (Table VI).

Mice which had been thymectomized 5 wk before showed a somewhat lesser degree of DH reaction as compared with intact mice. The difference was not significant. CY treatment before sensitization enhanced the DH in intact mice but not thymectomized ones (Table IV). As shown in Table V, splenectomy or sham operation did not significantly affect the DH level. CY treatment enhanced the DH in both intact and splenectomized mice. Similar results are shown in Table VI, in which previously splenectomized mice but not thymectomized mice given CY postoperatively exhibited a more intense DH than the nontreated mice.

Discussion

Suppressor T cells involved in DH against MHSA in mice decrease in activity through aging processes linked with physiological involution of the thymus (6). Results of CY treatment before sensitization, in relation to aging, indicate the strong

TABLE IV

Effect of Cyclophosphamide on the DH to MHSA in Intact or Thymectomized Mice

CY	ThX	DH on day 12	Thymus weight
—	—	11.6 ± 3.2	22 ± 4 (mg)
−3 d	—	14.6 ± 2.1	18 ± 5
−15 d	—	22.3 ± 2.3	33 ± 5
−30 d	—	16.3 ± 0.3	25 ± 2
—	−5 wk	7.3 ± 2.9	
−3 d	−5 wk	8.6 ± 2.3	
−15 d	−5 wk	10.3 ± 0.5	

Male C57BL/6Cr mice were thymectomized 5 wk before and then given CY, 150 mg/kg i.p., 3–30 d before sensitization as indicated. Age of mice was 4 mo at sensitization. Each group included four to five animals.

TABLE V

Effect of Cyclophosphamide on the DH to MHSA in Intact or Splenectomized Mice

CY	SpX	DH on day 13	No.
No	No	2.8 ± 0.9	5
Yes	No	12.8 ± 2.0	6
No	Yes	3.4 ± 1.0	5
Yes	Yes	16.4 ± 1.6	5
No	No*	4.3 ± 1.2	3

Female C57BL/6N mice were sensitized at the age of 2.5 mo. Splenectomy was performed 2 wk before, and CY was given 4 d before sensitization.

* Sham operation.

TABLE VI

Effect of Thymectomy or Splenectomy on the DH to MHSA in Mice Treated with CY

CY	Surgical treatment	No.	DH on day 12
−	—	4	2.3 ± 0.4
+	—	4	16.8 ± 1.1
+	SpX	3	10.7 ± 4.4
+	ThX	6	2.5 ± 0.9

Male C57BL/6Cr mice aged 2.5 mo were splenectomized or thymectomized on day −11, and then given CY, 150 mg/kg i.p., on day −4 of sensitization.

suppressive regulation of DH working in normal young C57BL mice, and the absence of regulation in CY-treated mice as well as in nontreated-aged ones (Fig. 1). This DH enhancement with CY was more apparent at the lower dose sensitization (Table I). Moreover, the antigen, MHSA, is one which does not induce a detectable antibody production (17). Therefore, it is unlikely that this DH enhancement with CY is due to inhibition of B cells which modulate DH, as was suggested in other experimental systems (11, 18, 19).

In fact, enhancement of DH with CY was eliminated by the transfer of thymocytes from normal syngeneic young mice, thereby suggesting the involvement of T cells in

suppressive regulation of DH (Table II). Further studies with cell transfer (Fig. 2) indicate that, in the thymus stage, T-axis lymphocytes are differentiated into distinct subpopulations for later further differentiation in the peripheral organs. Spleen-localized cells have no suppressive effect on the DH, which is also consistent with our previous observations (6, 15).

Our results suggest that DH enhancement with CY can be attributed to elimination of suppressor T cells. The alternative interpretation, however, is that the apparent enhancement is solely a reflection of delayed kinetics of DH reactivity because of poor and slow sensitization with antigen by remaining lymphoid cells after CY-induced damage. A drastic damage of these cells after CY treatment is shown in Table III, which is consistent with findings of others (19, 20). Results in Fig. 3 eliminate the possibility of the alternative interpretation. Effect of CY treatment is not the sole shift of the kinetics curve of DH reaction but rather both increase in intensity and persistency of DH reactivity. This means increase in the integral calculus of the time-response curve, indicating the net increased activity of effectors.

The enhancement of DH with CY requires sufficient recovery of effector T cells and macrophages from damage induced by CY treatment. It can be postulated that in such a situation, sensitization of effector T cells might effectively occur in the absence of suppressor T cells whose recovery appears to be more limited. Experiments of thymectomy shortly before sensitization show that overshooting recovery site for effector T cells is mainly the thymus, and splenectomy does not significantly affect the DH (Table IV). The relatively lesser importance of the spleen and the greater importance of the thymus in the development of DH is, however, limited to the young (A. Mitsuoka, data to be published). We previously reported that thymectomy of young C57BL mice at appropriate intervals before sensitization causes a profound enhancement of DH, suggesting the short life-span character of suppressor T cells (6). Hence, in the young, outflux of precursor T effector cells from the thymus to the peripheral organs appears to be continuous. At the same time, release of suppressor cell precursors from the thymus is probably required for the maintenance of homeostasis. The suggestive central regulation in cell-mediated immunity appears most apparent in C57BL mice.[2]

Recently, an increasing number of papers have dealt with elimination of suppressor cells with CY in enhancement of contact DH in guinea pigs (21) or in mice (22, 23). In these systems in mice (22, 23), two different antigen-specific suppressor cells are involved. One has the T-cell nature, is induced by intravenous pretreatment with antigen, and is resistant to CY. The other does not have the T-cell nature, is induced with supraoptimal dose of the antigen and in the latter period after sensitization, and sensitive to CY. These seem to be categorized as a peripheral regulation.

As to the peripheral regulation, we found antigen-specific suppressor T cells in DH to MHSA, and these cells are generated by pretreatment with the antigen from peripherally localized precursor cells with a long life-span, and are nylon-wool adherent and resistant to CY (S. Morikawa et al., data to be published).

Another example is that the DH induced by intravenous sensitization with a supraoptimal dose of sheep erythrocytes (SRBC) is shown to be enhanced with CY, and this was attributed to selective inhibition of B cells with CY (18). More recently,

[2] S. Morikawa et al. Manuscript in preparation.

a similar phenomenon was reported to be because of elimination of the antigen-specific suppressor T cells localized in the spleen (24–26). Even if such DH to heterologous erythrocytes is substantially of the tuberculin type, sensitization kinetics and the regulatory mechanisms of this DH seem different from those of DH to MHSA as previously discussed (27). Enhancement of DH to SRBC with CY occurs only at a supraoptimal dosed antigen sensitization (18, 26). This is also the case in contact DH as was shown and discussed by Sy et al. (23). On the other hand, the DH induced by an optimal dose of SRBC is drastically impaired if splenectomy is performed before sensitization (28) or before challenge (29). Such is not the case in DH to MHSA (Tables I and V), and our findings are consistent with other reports dealing with DH induced by sensitization with the help of CFA (30, 31). In the case of the DH to SRBC, CY treatment of mice, as well as splenectomy, is considered to result in a markedly decreased capacity of sensitization. As the result, if the actual processed antigen dose is taken into account, administration of high dose SRBC in such treated mice may be equivalent to that of low dose SRBC in intact mice, hence induction of feedback regulation by B cells (18) or suppressor T cells (26) being indeed minimum.

Finally, suppressor T cells which are generated without corresponding antigen pretreatment are reportedly involved in in vivo generation of antigen-specific cytotoxic T cells to allogeneic or hapten-conjugated syngeneic lymphocytes (32). A similar regulation to that involved in DH to MHSA reported herein may play a role in this case.

Suppressor T cells in the regulation of experimental autoimmune disease (33) may be another example of the implication of the physiological role of these cells.

Summary

Effect of treatment of mice with cyclophosphamide (CY) on the delayed hypersensitivity (DH) response was investigated in C57BL/6 mice. DH to methylated human serum albumin (MHSA) could be enhanced with CY in young mice but not in aged ones. DH enhancement with CY appeared to be due to elimination of suppressor T cells involved in DH. Effector T cells were also sensitive to CY, the damaging effect of CY on these latter cells was, however, transient suggesting the rapid recovery of effector T cells. The overshooting recovery of the effector T cells required the presence of the thymus.

It is more probably that there are at least two distinct subpopulations of T cells in DH, effector T cells, and suppressor T cells. The distinction is already apparent in the thymus stage. The suppressor T cells, categorized as a central regulator, seem to be antigen nonspecific and regulate more effectively the DH in young mice, thus physiological role of these cells in age-associated immune alterations is implicated.

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