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Regulatory role of suppressor T cells in the expression of delayed-type hypersensitivity in mice

II. SOLUBLE FACTOR FROM THYMIC SUPPRESSOR CELLS STIMULATED WITH ANTIGEN *IN VITRO* AND ITS POSSIBLE INTERACTION WITH MACROPHAGES*

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Summary. Thymus cells from mice primed s.c. with a high dose (10 mg) of lysozyme (Lys) specifically suppressed delayed footpad reaction (FPR) in mice previously immunized with lipid-conjugated lysozyme (D.Lys), and also suppressed the transfer of FPR by D.Lys-immune spleen cells into normal mice. Furthermore, they inhibited antigen-stimulated DNA synthesis of D.Lys-immune spleen cells *in vitro*. If the suppressor thymus cells were cultured with Lys *in vitro*, they produced soluble factor which depressed the ability of D.Lys-immune spleen cells to transfer FPR. Both supernatant of culture without Lys and extract of suppressor thymus cells were inactive in suppression of FPR. The suppressor factor was antigen-specific because its suppressive activity was absorbed with Lys but not with an unrelated antigen, lactalbumin. The factor failed to depress the ability of D.Lys-immune spleen cells to transfer FPR when the spleen cells were depleted of glass-adherent cells. In addition, incubation of peritoneal exudate cells from normal mice with the factor rendered the cells suppressive for passive transfer of FPR. These results suggest that the suppressor factor depresses the effector function of T cells responsible for FPR possibly via macrophages.

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INTRODUCTION

Suppressor T cells have been shown to be important in the regulation of delayed-type hypersensitivity (DTH), such as contact sensitivity (Zembala & Asherson, 1973; Phanuphak, Moorhead & Claman, 1974) and delayed footpad reaction (FPR) (Ramshaw, Bretscher & Parish, 1976; Liew, 1977; Kojima & Egashira, 1979) in mice. These suppressor T cells seem to act on either the induction stage, the expression stage, or both, by inhibiting either cell proliferation after sensitization, effector function of T cells responsible for DTH, or both of them. Although the precise mechanisms of action of suppressor T cells are not completely understood, the production of soluble factor(s) would be more efficient than direct cell-to-cell contact to amplify the effect of a limited number of suppressor T cells. Until recently, only a few investigators have attempted to describe the properties of soluble suppressor factor(s) for DTH in mice (Zembala & Asherson, 1975; Liew & Liew, 1978).

In the preceding paper (Kojima & Egashira, 1979),

we investigated the properties of suppressor T cells involved in the regulation of the expression of FPR to lipid-conjugated lysozyme (D.Lys) in mice. The suppressor T cells were demonstrated to occur not only in the spleen but also in the thymus. In this paper, properties of soluble factor(s) from the thymic suppressor cells were investigated. Our results show that thymic suppressor cells, when stimulated with Lys *in vitro*, produce antigen-specific soluble factor(s) which suppress the transfer of FPR by D.Lys-immune cells into normal mice. The results also suggest that the target of the thymic suppressor factor(s) is possibly the macrophage.

MATERIALS AND METHODS

Animals

Female ddY/S mice from 6 to 8 weeks old were used in all experiments except those described in Table 8.

Antigens

Dodecanoic acid-conjugated lysozyme (D.Lys) and precipitated lysozyme (ppt-Lys) were prepared as described previously (Kojima, Sugimoto & Egashira, 1976) according to the method of Coon & Hunter (1973). D.Lys induced FPR against native lysozyme (Lys) without antibody responses in mice. Ppt-Lys had the same antigenicity as native Lys except that it was insoluble and easily spun down by low speed centrifugation. Preparation of alpha-lactalbumin (LA) (Sigma Co., St. Louis) was as described previously (Kojima & Egashira, 1979).

Sensitization

For the production of high levels of FPR, mice were injected intraperitoneally with 300 mg/kg of cyclophosphamide (CY) (Endoxan, Shionogi Co., Osaka) 3 days before subcutaneous immunization with 100 μg D.Lys in saline. These mice were used 7 days after immunization as donors of sensitized spleen cells for FPR and as recipients of suppressor cells. A state of unresponsiveness in mice was induced by a subcutaneous injection of 10 mg Lys or LA. These mice were used as donors of thymic suppressor cells 14 days later.

Cell suspensions

Spleen and thymus cell suspensions were prepared in balanced salt solution (BSS, pH 7·2) (Golub, Mishell, Weigle & Dutton, 1968) as described in Kojima & Egashira (1979). For the preparation of peritoneal exudate cells, 2·5 ml of a mixture of 5% starch (Wako, Ltd, Osaka) and 5% proteose peptone (Difco Lab., Detroit) was injected intraperitoneally in normal mice, and the peritoneal exudate cells were harvested 3 days later with BSS containing 1 unit/ml of heparin. Cells were passed through a stainless mesh and washed twice with minimum essential medium (MEM, Nissui Co., Tokyo). For the depletion of glass-adherent cells from spleen cell suspensions, spleen cells, suspended at a concentration of 1×10^8 cells/ml in RPMI 1640 (Nissui Co., Tokyo) supplemented with 10% foetal calf serum (FCS) (Gibco, Berkeley), were applied to a glass-wool column and incubated for 45 min at 37° in an atmosphere of 5% CO_2–95% air. The eluted cells were washed and resuspended in MEM.

Thymus cell extract

Thymus cells were washed three times with MEM and resuspended to 5×10^8 cells/ml in MEM. The cells were sonicated for 5 min in ice with a Kontes Sonicator (Berkeley). The lysate was then centrifuged at 40,000 ***g*** for 60 min at 4°. The supernatant was collected and assayed for its suppressive activity.

Culture supernatants of thymus cells

Thymus cells were cultured at 1×10^7 cells/ml with or without 10 μg/ml of ppt-Lys in RPMI 1640 supplemented with 10% FCS and 60 μg/ml of kanamycin in an atmosphere of 5% CO_2–95% air. The culture supernatants were harvested 48 h later, centrifuged at 3000 r.p.m. for 10 min to remove ppt-Lys and then assayed for their suppressive activity.

Antigen–Sepharose columns

Activated Sepharose was coupled according to the method of Porath, Axen & Eruback (1967). Sepharose 4B (Pharmacia Fine Chemicals, Inc., Uppsala) was activated with cyanogen bromide while keeping the solution at pH 10–11 with NaOH. Lys and LA were coupled to the activated Sepharose 4B at 4° with overnight continuous shaking.

Footpad test for DTH

DTH was determined as footpad swelling as described previously (Sugimoto, Kojima, Yaginuma & Egashira, 1975). Normal mice or mice injected with CY and immunized with D.Lys 7 days previously were given an eliciting injection into the footpad within 2 h after cell transfer. Footpad thickness was measured 24 h later.

Assay for thymic suppressor factor

The thymus cell extracts and supernatants from thymus cell culture were tested for their ability to inhibit the passive transfer of FPR by incubating sensitized spleen cells in them. Mice were injected with CY and 3 days later immunized with D.Lys. The spleen cells were taken 7 days after immunization. The sensitized spleen cells were suspended at 1×10^8 cells/ml in the extracts or culture supernatants and incubated for 60 min at 37°. The cells were washed twice with MEM and then transferred into normal mice. The recipient mice were given an eliciting injection with Lys into footpad within 2 h after cell transfer to see whether the incubation inhibited the ability of sensitized spleen cells to transfer FPR. Footpad swelling was determined 24 h later.

Assay for DNA synthesis

Triplicate cultures of spleen and thymus cells (5×10^6) with 10 μg ppt-Lys in 1 ml of RPMI 1640 containing 10% FCS and 60 μg/ml of kanamycin were pulsed for the final 18 h of a 72 h incubation period with 2 μCi/ml of tritiated thymidine ([6-^{3}H]-TdR, 4·5 Ci/mmol, the Radiochemical Centre, Amersham). Labelled cells were washed with BSS and precipitated with an equal volume of 10% trichloroacetic acid (TCA). Acid-insoluble materials were collected on glass fibre filters (Whatman GF/C, Whatman Ltd, England) and washed three times with 5% TCA then with ethanol and acetone. The filters were counted in a Beckman liquid scintillation counter.

Statistical analysis

Statistical analysis was carried out using Student's *t* test. Mean differences were considered significant when $P < 0.05$.

RESULTS

Antigen-specific suppression of the expression of FPR by thymus cells

Antigen specificity of thymic suppressor cells was investigated. Mice were injected with CY and 3 days later immunized with D.Lys (D.Lys-immune mice). Seven days after the immunization, these mice received the transfer of thymus cells (2×10^7) taken from mice primed 14 days previously with either 10 mg Lys or LA. The immune recipients were challenged for DTH into footpad immediately after the cell transfer, and their FPR was determined 24 h later (Table 1).

Table 1. Antigen-specific suppression of FPR in D.Lys-immune mice

Group	Thymus cells transferred (2×10^7)	Footpad swelling (±SE) (1/100 mm)
A	None	127·9 ± 11·1
B	Normal	113·1 ± 18·9
C	Lys-primed	52·0 ± 12·8*
D	LA-primed	126·0 ± 7·3
E	Negative control†	8·0 ± 6·7

Thymus cells from mice primed s.c. 14 days previously with 10 mg Lys or LA were transferred into mice treated with CY (300 mg/kg) 10 days previously and immunized 7 days previously with D.Lys. FPR was elicited immediately after the cell transfer in the immune recipients.

* Significantly different from Group A ($P < 0.001$).

† Normal mice received only eliciting injection into footpad.

D.Lys-immune mice developed high levels of FPR (Group A). This FPR was effectively suppressed by the transfer of Lys-primed thymus cells (Group C). FPR in D.Lys-immune mice, however, was not affected by the transfer of LA-primed thymus cells (Group D) although their donor mice were fully unresponsive to LA. Normal thymus cells failed to affect the levels of FPR in D.Lys-immune recipients (Group B).

The ability of thymic suppressor cells to depress the function of immune lymphoid cells responsible for FPR was further assessed in a passive transfer system of FPR. Spleen cells (1×10^8) taken from D.Lys-immune mice were mixed with thymus cells (2×10^7) from mice primed with 10 mg Lys and then transferred into normal mice. The normal recipients were immediately challenged into footpad (Table 2). D.Lys-immune spleen cells could transfer FPR to Lys into normal recipients (Group A). Their ability to transfer FPR, however, was depressed by the addition of Lys-primed thymus cells but not normal thymus cells (Groups B & C).

Inability of thymus cell extract to suppress the expression of FPR

The suppressive activity of thymus cell extract was tested in immune mice. Extracts equivalent to 8×10^7 normal or Lys-primed thymus cells were injected into D.Lys-immune mice immediately before elicitation of FPR (Table 3). The injection of extracts from normal thymus cells or Lys-primed thymus cells failed to affect the levels of FPR in D.Lys-immune mice

Table 2. Suppression of the passive transfer of FPR into normal mice

Group	Cells transferred: Spleen cells (1×10^8)	Cells transferred: Thymus cells (2×10^7)	Footpad swelling (±S.E.) (1/100 mm): Expt 1	Footpad swelling (±S.E.) (1/100 mm): Expt 2
A	D.Lys-immune	None	45·1 ± 8·1	43·7 ± 9·1
B	D.Lys-immune	Normal	42·9 ± 6·1	44·7 ± 13·5
C	D.Lys-immune	Lys-primed	16·7 ± 9·8*	12·3 ± 9·8*
D	None	None	—	5·4 ± 6·1†

Spleen cells taken from mice injected with CY 10 days previously and immunized with D.Lys 7 days previously, were mixed with thymus cells from mice primed s.c. 14 days previously with 10 mg Lys, and then transferred into normal mice. FPR was elicited immediately after the cell transfer.

* Significantly different from Group A ($P < 0.05$).

† Normal mice received only eliciting injection into footpad.

(Groups B & C). When the extract from Lys-primed spleen cells (9×10^7) was tested, it was also inactive in suppression of FPR (data not shown).

Inability of the thymus cell extracts to suppress FPR was further assessed in passive transfer experiments of FPR by immune lymphoid cells. Spleen cells (1×10^8) from D.Lys-immune mice were incubated in extracts equivalent to 1×10^8 normal thymus cells or lys-primed thymus cells at 37° for 60 min. The cells were washed and then transferred into normal recipients which were immediately challenged for FPR (Table 4). FPR could be transferred by D.Lys-immune spleen cells. Incubation of the immune spleen cells with normal or Lys-primed thymus cell extracts did not result in significant depression of their ability to transfer FPR (Groups B & C).

Table 3. Inability of thymus cell extract to suppress FPR in D.Lys-immune mice

Group	Injection with	Footpad swelling (±S.E.) (1/100 mm)
A	None	119·7 ± 12·7
B	Extract from normal thymus cells	108·7 ± 18·0
C	Extract from Lys-primed thymus cells	99·9 ± 17·6*
D	Negative control†	9·4 ± 6·2

Extracts equivalent to 8×10^7 normal thymus cells or thymus cells from mice primed s.c. 14 days previously with 10 mg Lys was injected i.v. into mice pre-treated with CY and immunized 7 days previously with D.Lys. FPR was elicited immediately after the i.v. injection.

* Not significantly different from Group A.

† Normal mice received only eliciting injection into footpad.

Suppressive activity in culture supernatant of thymus cells stimulated *in vitro*

The suppressive activity in culture supernatant of thymus cells was investigated. Normal or Lys-primed

Table 4. Inability of thymus cell extract to suppress the passive transfer of FPR into normal mice

Group	Treatment of immune spleen cells transferred	Footpad swelling (±S.E.) (1/100 mm)
A	None	36·0 ± 5·0
B	Extract from normal thymus cells	30·8 ± 5·0
C	Extract from Lys-primed thymus cells	27·7 ± 11·1*
D	Sup. from Lys-primed thymus cell culture	46·0 ± 10·5
E	Sup. from Lys-primed thymus cell culture stimulated *in vitro*	14·8 ± 6·6†

Extracts were prepared from thymus cells (1×10^8) of normal mice or mice primed s.c. 14 days previously with 10 mg Lys. Lys-primed thymus cells were cultured with or without 10 μg/ml of ppt-Lys and culture supernatants were harvested 48 h later. Spleen cells (1×10^8) from mice injected with CY and immunized with D.Lys 7 days previously, were incubated with the extracts or culture supernatants at 37° for 60 min and then transferred into normal mice. FPR was elicited immediately in the recipient mice.

* Not significantly different from Group A ($0.4 < P < 0.5$).

† Significantly different from Group A ($P < 0.05$).

thymus cells were cultured with or without a precipitated derivative of Lys, ppt-Lys, as an *in vitro* stimulating antigen. Culture supernatants were harvested 48 h later and centrifuged to remove ppt-Lys and cells. Spleen cells from D.Lys-immune mice were incubated with either culture medium, culture supernatant of normal thymus cells, or that of Lys-primed thymus cells, and then transferred into normal mice immediately before elicitation of FPR. D.Lys-immune cells incubated with culture medium or culture supernatant of normal thymus cells could transfer FPR into normal mice (Groups A & C in Table 5). They were also active in transfer of FPR even when incubated with culture supernatant of Lys-primed thymus cells without ppt-Lys *in vitro* (Group D in Table 4 & Group E in Table 5). In contrast, when Lys-primed thymus cells were stimulated with ppt-Lys *in vitro*, their culture supernatant depressed the ability of the immune spleen cells to transfer FPR (Group E in Table 4 & Group F in Table 5). Culture medium and culture supernatant of normal thymus cells in the presence of ppt-Lys, however, failed to depress the ability (Groups B & D in Table 5). These results show that thymic suppressor cells produce soluble suppressor factor(s) *in vitro* and antigenic stimulation is required for the production of suppressor factor(s).

Antigen specificity of suppressor factor

Antigen specificity of thymic suppressor factor was investigated in the following experiments. Suppressor supernatant was passed through either Lys-bound or LA-bound Sepharose column, and its residual suppressive activity in the effluent was tested by incubating D.Lys-immune spleen cells with it (Table 6). After absorption with Lys–Sepharose the suppressor supernatant virtually lost its activity, whereas it remained active after absorption with LA–Sepharose in depressing the ability of D.Lys-immune cells to transfer FPR (Expt 1). The suppressor supernatant was almost inactive when it was absorbed twice with Lys-Sepharose (Expt 2). These results suggest that the thymic suppressor factor has both affinity and specificity for Lys.

Possible interaction of thymic suppressor factor with macrophages

The susceptibility of immune spleen cells to suppression by thymic suppressor factor was compared before and after depletion of adherent cells (Table 7, Expt 1). Adherent cells were removed from the immune spleen cell suspension by passing it through a glass-wool column. Unseparated D.Lys-immune

Table 5. Suppression of passive transfer of FPR by culture supernatant of thymus cells stimulated with antigen *in vitro*

Group	Treatment of immune spleen cells transferred	Antigen in culture	Footpad swelling (±SE) (1/100 mm)
A	Culture medium	—	37·6 ± 5·5
B	Culture medium	ppt-Lys	36·7 ± 8·5
C	Sup. from culture of normal thymus cells	—	39·0 ± 7·3
D	Sup. from culture of normal thymus cells	ppt-Lys	36·7 ± 5·4
E	Sup. from culture of Lys-primed thymus cells	—	23·7 ± 8·1*
F	Sup. from culture of Lys-primed thymus cells	ppt-Lys	4·4 ± 4·0†

Thymus cells from normal mice or mice primed s.c. 14 days previously with 10 mg Lys were cultured with or without 10 μg/ml of ppt-Lys. The culture supernatants were harvested 48 h later. Spleen cells (1×10^8) from mice injected with CY and immunized with D.Lys 7 days previously were incubated at 37° for 60 min with culture medium or culture supernatants and then transferred into normal mice. FPR was elicited immediately in the recipient mice.

* Not significantly different from Group A ($0{\cdot}1 < P < 0{\cdot}2$).

† Significantly different from Group A ($P < 0{\cdot}001$).

Table. 6. Antigen specificity of suppressor factor

Group	Treatment of immune spleen cells transferred	Adsorption of supernatant	Footpad swelling (±S.E.) (1/100 mm) Expt 1	Expt 2
A	Control supernatant	—	33·1 ± 8·0	32·5 ± 5·7
B	Suppressor supernatant	Lys	24·9 ± 10·4*	39·0 ± 8·1
C	Suppressor supernatant	LA	6·5 ± 5·4†	—

Control supernatant and suppressor supernatant were prepared from cultures of normal or Lys-primed thymus cells in the presence of ppt-Lys. The suppressor supernatant was passed through either a Lys–Sepharose column or an LA–Sepharose column. In experiment 2, it was passed through a Lys–Sepharose column twice. Spleen cells (1×10^8) from CY-treated and D.Lys-immune mice were incubated with supernatants at 37° for 60 min and then transferred into normal mice. FPR was elicited immediately in the recipient mice.

* Not significantly different from Group A ($0{\cdot}5 < P < 0{\cdot}6$).

† Significantly different from Group A ($P < 0{\cdot}02$).

Table 7. Effect of suppressor factor on spleen cells depleted of adherent cells and on on peritoneal exudate cells

Group	Cells transferred	Treatment of cells	Footpad swelling (±SE) (1/100 mm)
Expt 1 A	Immune spleen cells	None	36·3 ± 8·3
B	Immune spleen cells	Control supernatant	36·0 ± 8·7
C	Immune spleen cells depleted of adherent cells	Control supernatant	42·2 ± 13·1
D	Immune spleen cells	Suppressor supernatant	10·0 ± 3·3*
E	Immune spleen cells depleted of adherent cells	Suppressor supernatant	37·0 ± 7·6
Expt 2 A	Immune spleen cells + Peritoneal exudate cells treated with	Control supernatant	42·1 ± 6·9
B		Suppressor supernatant	20·5 ± 4·6†

Spleen cells were taken from CY-treated and D.Lys-immune mice. For the depletion of adherent cells, the immune spleen cell suspension was passed through a glass-wool column.

Control or suppressor supernatant was harvested from cultures of normal or Lys-primed thymus cells in the presence of ppt-Lys. 1×10^8 immune spleen cells or the immune cells depleted of adherent cells were incubated with culture supernatant and then transferred into normal mice (Expt 1). In experiment 2, peritoneal exudate cells (1×10^7) incubated with culture supernatant were mixed with immune spleen cells (1×10^8) and then transferred into normal mice. FPR was elicited immediately in the recipient mice.

* Significantly different from Expt 1 Group A ($P < 0{\cdot}05$).

† Significantly different from Expt 2 Group A ($P < 0{\cdot}05$).

Table 8. Effect of suppressor thymus cells on DNA synthesis of D.Lys-immune spleen cells *in vitro*

Group	Cells cultured: Spleen cells	Cells cultured: Thymus cells	Antigen in culture	[^{3}H]-TdR incorporation (c.p.m./culture)
A	—	Normal	—	55 ± 1
B	—	Normal	ppt-Lys	61 ± 11
C	—	Lys-primed	—	65 ± 4
D	—	Lys-primed	ppt-Lys	69 ± 8
E	D.Lys-immune	—	ppt-Lys	1868 ± 72
F	D.Lys-immune	Normal	ppt-Lys	1726 ± 95
G	D.Lys-immune	Lys-primed	ppt-Lys	970 ± 79

Spleen cells were taken from C3H/He mice injected with CY and immunized with D.Lys 7 days previously. Thymus cells were taken from C3H/He mice primed s.c. with 10 mg Lys 14 days previously. 5×10^6 spleen cells, thymus cells, or both were cultured with or without ppt-Lys. Cultured cells were pulsed with [^{3}H]-TdR (2 μCi/ml) for the final 18 h of a 72 h culture period. Each value was expressed as mean c.p.m. ± SE of triplicate cultures.

spleen cells treated with control supernatant transferred significant levels of FPR into normal mice (Group B). The depletion of adherent cells from the immune spleen cells did not affect their ability to transfer FPR (Group C). On the other hand, unseparated D.Lys-immune cells were rendered inactive in transfer of FPR by the incubation with suppressor supernatant (Group D). When the immune spleen cells were depleted of adherent cells, however, their ability to transfer FPR was not inhibited by incubation with suppressor supernatant, and the recipient mice produced as high levels of FPR as control mice (Groups A & E). In experiment 2, peritoneal exudate cells (1×10^7) from normal mice were incubated with suppressor supernatant and then mixed with D.Lys-immune spleen cells (1×10^8). They were then transferred into normal mice. FPR in the recipient mice was suppressed by the addition of peritoneal exudate cells incubated with suppressor supernatant, but not by the addition of those incubated with supernatant from normal thymus cell culture (control supernatant) (Groups A & B). These results suggest that the target cells for suppressor factor are possibly macrophages.

Effect of thymic suppressor cells on DNA synthesis

Antigen-induced DNA synthesis of suppressor thymus cells and immune spleen cells was investigated *in vitro*. Thymus cells from normal mice and mice (C3H/He) primed *in vivo* 14 days previously with 10 mg Lys were cultured with or without ppt-Lys for 72 h. The cells were pulsed with [^{3}H]-TdR for 18 h before harvest (Table 8). DNA synthesis of normal and Lys-primed thymus cells without *in vitro* stimulation with ppt-Lys was little (Groups A & C). Even when stimulated *in vitro*, the thymus cells showed no significant increase of DNA synthesis (Groups B & D). Spleen cells from D.Lys-immune C3H/He mice, however, gave a significant response to the antigen ppt-Lys (Group E). The DNA synthesis of D.Lys-immune spleen cells was depressed by the addition of Lys-primed thymus cells (Group G) but not normal thymus cells (Group F). These results suggest that thymic suppressor cells inhibit proliferative response of immune lymphoid cells.

DISCUSSION

In the present experiments, we investigated the mechanism of suppression of the expression of FPR to D.Lys by thymus cells from unresponsive mice primed with a high dose of Lys. The results show that these thymus cells suppress the passive transfer of FPR by D.Lys-immune lymphoid cells and that they produce soluble suppressor factor(s) when cultured *in vitro* with ppt-Lys. Incubation of D.Lys-immune cells with the culture supernatant inhibits their ability to transfer FPR into normal mice.

The suppressor factor is antigen-specific, as its suppressive activity was adsorbed with Lys-Sepharose but

not with LA-Sepharose (Table 6). The suppressive activity seems, however, not to be due to antibody because it was detected in culture supernatant of thymus cells (Table 5) but not in the extract prepared from spleen cells of primed mice (data not shown). Furthermore, serum antibody failed to suppress FPR as shown in the preceding paper (Kojima & Egashira, 1979). These suggest that suppressor factor is a T-cell product, although a possiblity is not ruled out that it is a product of a small number of other cells in the thymus. Despite the affinity and specificity of suppressor factor for Lys, suppressor factor seems unlikely to be a complex with suppressor thymus cell product and antigen, since ppt-Lys, used as *in vitro* stimulating antigen, is an insoluble derivative of Lys and easily removed from culture supernatants by low speed centrifugation.

Suppressor factor was produced by the thymus cells from primed mice only if they were cultured with ppt-Lys (Table 5). Extract prepared from suppressor thymus cells and supernatant from their culture without ppt-Lys showed no detectable suppressive activity (Tables 3 and 4). These findings suggest that suppressor factor is made in response to antigen stimulation. A requirement for *in vivo* antigen stimulation for production of specific suppressor factor has been described by Zembala & Asherson (1974) and confirmed by Moorhead (1977) in contact sensitivity system in mice, where lymph node cells from tolerant mice did not produce suppressor factor *in vitro* without painting the donor mice with contact sensitizer 16–20 h before the lymph node cells were cultured. On the other hand, Liew & Liew (1978) have reported that suppressor factor for FPR to SRBC was produced by incubating the spleen or lymph node cells from tolerant mice without antigen stimulation. Although the nature of the interaction between the suppressor thymus cells and antigen is unknown, experiments on DNA synthesis *in vitro* (Table 8) suggest that proliferation of suppressor thymus cells is not required for the production of suppressor factor.

Incubation of D.Lys-immune spleen cells with suppressor factor depressed their ability to transfer FPR but failed to suppress the ability of the immune spleen cells when they were depleted of glass-adherent cells. Furthermore, peritoneal exudate cells from normal mice became suppressive for the transfer of FPR when incubated with suppressor factor (Table 7). These findings suggest that the target cells of the suppressor factor are macrophages. This is consistent with the earlier report by Zembala & Asherson (1974) who have shown that the suppressor factor for contact sensitivity was absorbed by peritoneal exudate cells which was then able to inhibit the passive transfer of contact sensitivity. Although our results basically agree with theirs, a major difference is concerning the source of cells which are served for the production of suppressor factor. We cultured thymus cells since suppressor cells in FPR occurred dominantly in the thymus of tolerant donors (Kojima & Egashira, 1979), whereas they used lymph node cells since suppressor cells in contact sensitivity occurred in the lymph nodes and to a lesser extent in the spleen and bone marrow but not in the thymus of tolerant donors (Asherson & Zembala, 1974b). Furthermore, they have shown that peritoneal exudate cells incubated with suppressor factor suppressed non-specifically the passive transfer of contact sensitivity when recipient mice were challenged with specific antigen, suggesting that macrophages armed for suppression release non-specific inhibitory agents when exposed to antigen (Asherson & Zembala, 1974a). We have not yet tested whether such is a case with our suppressor thymus cell factor in this FPR system.

Preliminary experiments on DNA synthesis of D.Lys-immune spleen cells cultured *in vitro* show that suppressor thymus cells inhibited the proliferative response of the immune spleen cells to antigen stimulation (Table 8). The *in vitro* proliferative response seems to be, at least in part, T-cell dependent, since D.Lys induced FPR in mice selectively without antibody response and donor mice were pretreated with a high dose of CY which acts severely on B cells (Poulter & Turk, 1972). It seems unlikely, however, that the suppressor thymus cells suppress the expression of FPR by inhibiting cell proliferation because of the following findings. First, proliferation of effector T cells for DTH was not required for expression of DTH (Feldman, 1968). Second, T cells which proliferated *in vitro* were a distinct subset of T cells from effector T cells for DTH (Moorhead, 1978). Furthermore, suppressor T cells which acted on the expression stage of contact sensitivity to PCl did not inhibit cell proliferation (Asherson & Zembala, 1974b). On the other hand, Moorhead (1976) has reported that suppressor T cells which acted on the induction stage of contact sensitivity to DNFB inhibited cell proliferation. Under these circumstances, suppression of the expression and induction may be mediated by functionally different subsets of suppressor cells, and our suppressor thymus cells may be a heterogenous population of these suppressor cells. Regulatory mechanisms of the

induction stage of FPR in mice are currently being investigated.

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