In Vitro Study of T-cell Subsets Regulating Anti-ovalbumin Antibody Formation in Humans*

Noriaki Shinomiya, M.D.
Junichi Yata, M.D.

SUMMARY **Regulatory mechanisms of T cells in anti-ovalbumin (OVA) antibody formation in human lymphocytes were studied. The antibody formation was T-cell dependent and helper activity was present in the T-cell subset with a low ability to bind OVA and bearing OKT_4 antigen. The addition of T cells with high binding ability of OVA suppressed antibody formation. Both OKT_4^+ and OKT_8^+ subsets seemed to be needed to induce the suppressor activity. T cells stimulated by high doses of OVA antigen produced antigen-specific humoral suppressor factors(s) with an allogenic barrier in the early phase and an antigen non-specific one in the late phase.**

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The production of antibodies to most antigens is regulated by helper and suppressor T cells.[1] *In vitro* studies of antibody production by human blood lymphocytes have usually been performed using polyclonal activators, such as pokeweed mitogen (PWM). Although PWM-driven immunoglobulin production is T-cell-dependent[2] and useful for the analysis of T-B-cell interactions,[3] it does not necessarily reflect the specific antibody response correctly. Therefore, the *in vitro* studies on the T cells' regulation of specific antibody response triggered by a specific antigen would be important to disclose the mechanisms of *in vivo* antibody production in humans. Several *in vivo* systems to estimate specific antibody response of human blood lymphocytes to particles and soluble antigens have been reported.[4-6] However, there existed few reports analysing the interactions of T-cell subsets in the regulation of specific antibody production.[7] In the present work we have studied the role of T-cell subsets distinguished by the differences of cell surface antigens in anti-ovalbumin (OVA) antibody production from lymphocytes stimulated with OVA.

MATERIALS AND METHODS

Peripheral blood lymphocytes

Heparinised peripheral blood drawn from healthy adult donors was layered on Ficoll-Hypaque solution (s.g., 1074), centrifuged at 400xg for 30 minutes. The mononuclear cells were collected from the interface. The cells were resuspended in the medium and incubated at 37°C in dishes coated with foetal calf serum (FCS) (Gibco) for two hours in a humidified incubator to remove the adherent cells. The non-adherent cells thus obtained contained about 3-5% non-specific esterase-positive cells.

Separation of non-T cells (B-cell-rich fraction) and T cells

The non-adherent mononuclear cells containing about 3-5% non-specific esterase-positive cells were mixed with sheep erythrocytes treated with 2-isothiouronium bromide hydrobromide (AET) at a ratio of about 1:100 in heat-inactivated FCS and centrifuged at 200xg for five minutes. The cells were incubated in ice water for one hour to obtain tight cell contact and for the T cells to form

*From the Department of Paediatrics, Tokyo Medical and Dental University, 5-45, Yushima 1 chome, Bunkyo-Ku, Tokyo, Japan.

rosettes with sheep erythrocytes. Rosette-forming cells (T cells) were separated from non-rosette-forming cells (non-T cells, B-cell-rich fraction) using the same Ficoll-Hypaque density gradient. T cells were collected from the bottom and the non-T cells from the interface. The contaminating erythrocytes were removed by lysing with 0.83% Tris-buffered NH_4Cl.

Complement-dependent lysis of T cells with monoclonal antibodies

T cells ($1x10^7$) were incubated with 1 ml of 1/125 diluted monoclonal OKT_4 or OKT_8 antibodies (Orth. Pharmatceutical Co., Ritan, NJ) for 1.5 hours at room temperature. Fresh rabbit serum (0.2 ml) (CL Co., 3441) was added to the mixture as complement. The mixture was incubated for another 1.5 hours in a 37°C shaking water-bath and the cells were washed three times with medium. Thus, T-cell-depleted OKT_4^+ or OKT_8^+ cells were prepared.

Preparation of OVA-binding and non-binding T cells

OVA solution (1 mg/ml) (Sigma) was added to the plastic dishes (Nunc, 15x30 mm) with poly-L-lysine (50 μg/ml) (Sigma) and incubated for three hours in a 37°C humidified incubator. T-cell suspension ($1x10^7$ /ml) was added to these dishes and incubated for six hours in a 37°C 5% CO_2 humidified incubator. Non-adherent cells (OVA-non-binding T cells, OVA^-T cells) were collected. Adherent cells (OVA-binding T cells, OVA^+T cells) were detached with cold medium and vigorous pipetting and thereafter collected. Both OVA^-T cells and OVA^+T cells were washed three times with fresh medium.

Preparation of the culture supernatant from T cells

The peripheral blood lymphocytes depleted of adherent cells ($2x10^6$) were suspended in 1 ml of the culture medium with 200 μg/ml of OVA antigen and cultured for four days in a 37°C humidified incubator. At the completion of the culture, the cells were washed with medium. The cells were resuspended in fresh culture medium and incubated for two hours in dishes (Nunc, 15x30 mm) coated with rabbit antihuman immunoglobulin antibody (1 mg/ml) to remove B cells. The non-adherent cells ($1x10^6$) were resuspended in fresh culture medium and cultured for another two or four days, and the culture supernatants were collected. Each supernatant was concentrated five times using an Amicon filter.

***In vitro* anti-ovalbumin (OVA) antibody production**

The mixture of B-cell-rich fraction ($4x10^5$) and T cells ($6x10^5$) was suspended in a total volume of 250 μl of medium RPMI 1640 supplemented with 10% FCS (Gibco), 20 mM HEPES (Sigma) 5 mM L-glutamine (Sigma), 100 μg/ml gentamicin, 100 units/ml penicillin G, $5x10^{-5}$ 2-mercaptoethanol (2ME) and cultured for six days in a 37°C humidified 5% CO_2 incubator under the stimulation of various concentrations of OVA antigen (from 3 μg/ml to 100 μg/ml). At the completion of the culture, the culture cells were washed three times by medium and resuspended in 200 μl of fresh culture medium and cultured for another three days. The culture cell suspensions were frozen and thawed three times, and centrifuged at 500xg; the culture supernatant was then separated and its anti-OVA antibody titre measured.

Estimation of the titre of anti-OVA antibody of the supernatant

An enzyme-linked immunosorbent assay (ELISA) was used. Added to each well of the microculture plates coated with poly-L-lysine was 50 μl of the 1 mg/ml OVA solution. The plates were then incubated overnight at 4°C to coat the surface of the wells with OVA. The wells were washed three times with phosphate-buffered saline containing 0.02% Tween 20 (PBS-Tween) and 100 μl of the culture supernatant was added to each well. After incubation at 4°C for 16 hours, the wells were washed three times with PBS-Tween, filled with 50 μl of biotin-binding anti-human IgG (10 μg/ml), incubated for one hour at room temperature, washed with PBS-Tween and added to 50 μl of peroxidase-conjugated avidin (1 μg/ml) diluted with PBS. After washing three times with PBS-Tween, azino-bis (3-ethylbenzothiazoline-6-6 sulfonic acid) (ABTS) containing H_2O_2 was added to each well. Extinction in the fluid of each well was estimated at 414 nm with a spectrophotometer.

T-cell subset showing helper activity in the generation of anti – OVA antibody

B-cell-rich fraction ($4x10^5$) was mixed with autologous OVA^-T cells ($6x10^5$) or OVA^-T cells ($6x10^5$) depleted of OKT_4^+ cells or OKT_8^+ cells and cultured under the stimulation of 5 μg/ml OVA antigen. The culture supernatants from each culture were estimated for the titre of anti-OVA antibody.

Suppression of the production of anti-OVA antibody by T-cell subset or its products

A mixture of B-cell-rich fraction ($4x10^5$) and OVA^-T cells ($6x10^5$), which usually produce a certain amount of anti-OVA antibody, was added to various numbers of OVA^+T cells, OVA^+T cells depleted of OKT_4^+ cells or OKT_8^+ cells ($1x10^5$) or their culture supernatant (20% to total culture medium) at the beginning of the culture. The suppressor activity was expressed as the reduction rate of the titre of anti-OVA antibody by the addition of the T cells or their products.

Suppression of anti-tetanus-toxoid antibody formation by the supernatant

Peripheral blood lymphocytes depleted of the adherent cells

($1x10^6$ per well) from normal individuals immunised with tetanus toxoid (TT) antigen two weeks previously were cultured in a total volume of 200 μl of culture medium using a 96-well microculture plate for six days at 37°C in a humidified 5% CO_2 incubator under the stimulation of TT antigen (5 Lf units/ml). Added to the wells at the beginning of the culture were 50 μl of each test supernatant or simple medium. At the completion of the culture, the cells were washed three times with medium. The cells were resuspended in the new medium and cultured for another three days. The culture supernatant was collected and its anti-TT antibody titre was estimated with ELISA.

RESULTS

In vitro anti-ovalbumin (OVA) antibody formation from peripheral blood lymphocytes

The peripheral blood lymphocytes depleted of T cells did not produce anti-OVA antibody, but they did in the presence of autologous T cells in some individuals (Fig. 1). There were high and low responders with regard to anti-OVA response. In the high-responder group, 5 μg/ml of OVA antigen was found to be the optimal concentration for antibody formation.

T-cell subset showing helper activity in the production of anti-OVA antibody

B cells from peripheral blood produced more antibody in the presence of T cells with low OVA-binding ability (OVA^-T cells) than total T cells (Fig. 2). Even the B cells from low responders, which did not produce much antibody in the presence of unseparated T cells, produced antibody when they were helped by OVA^-T cells (Fig. 2). These observations indicate that anti-OVA antibody formation was helped by OVA^-T cells and that the low response was caused by OVA^+T cells.

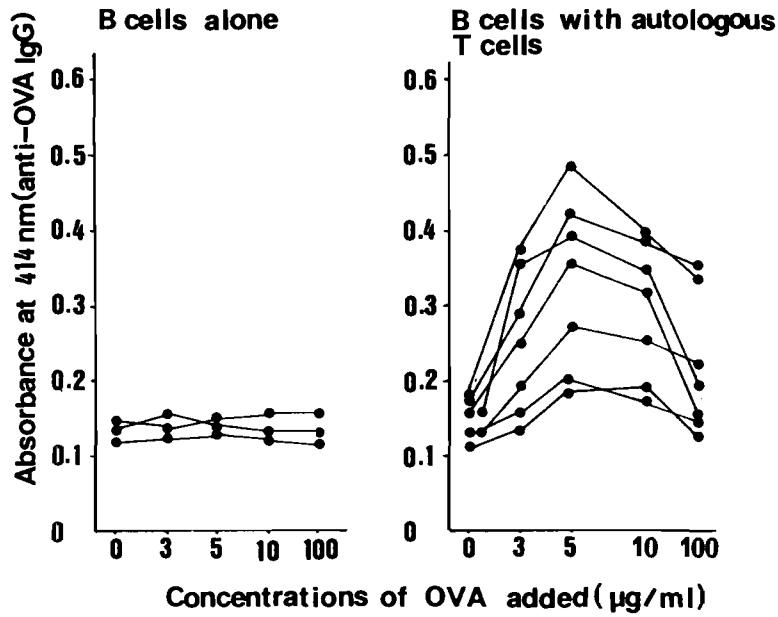

Fig. 1 Anti-ovalbumin (OVA) antibody production from B cells with or without the combination of autologous T cells stimulated with OVA antigen. Each line indicates different individuals.

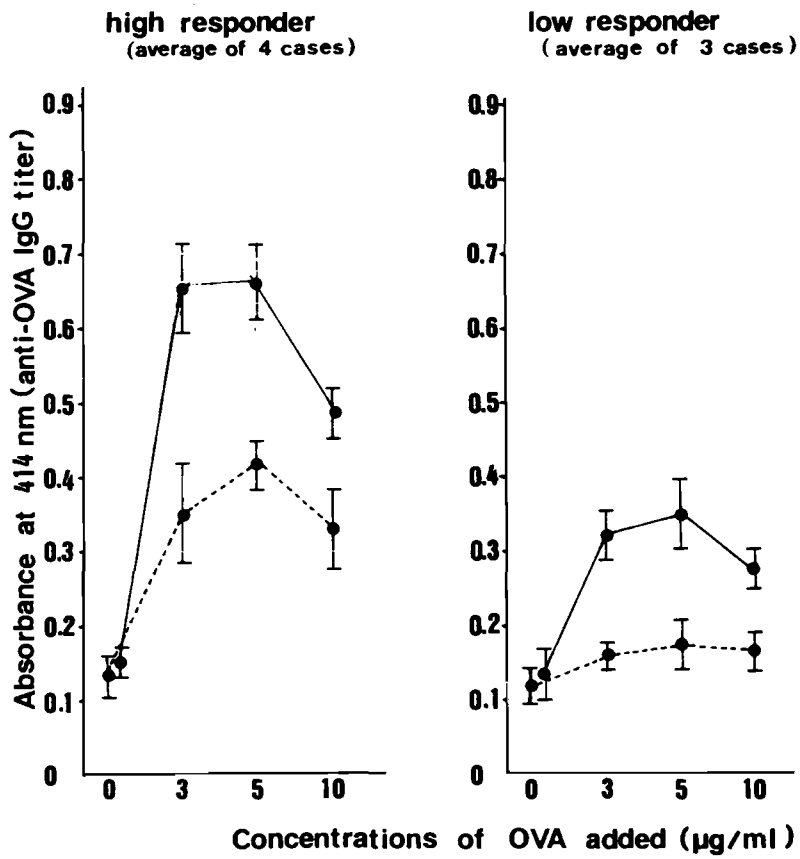

Fig. 2 Helper activity of OVA-non-binding T cells in anti-OVA antibody production.
●--● : the combination of B cells and autologous unseparated T cells.
●—● : the combination of B cells and autologous OVA non-binding T cells.

The helper activity of OVA^-T cells was lost by depleting OKT_4^+ cells but not OKT_8^+ cells (Fig. 3). Therefore, the helper T cells of anti-OVA antibody formation seemed to belong to the OKT_4^+ population.

T-cell subset showing suppressor activity in anti-OVA antibody formation

The formation of anti-OVA antibody from a mixture of B cells and OVA^-T cells was reduced by the addition of the autologous OVA^+T cells (Fig. 4). The suppressor activity of OVA^+T cells was significant when more than $1x10^5$ of OVA^+T cells were added. The suppressor activity of OVA^+T cells was cancelled by depleting either OKT_4^+ or OKT_8^+ cells (Fig. 5). This indicated that both OKT_4^+ and OKT_8^+ cells were needed for the induction of the suppressor activity.

Suppression of anti-OVA antibody formation by the culture supernatant from the T cells stimulated with high doses of OVA antigen

Culture supernatant from T cells stimulated with high doses of OVA antigen was added to the combinations of autologous B and OVA^-T cells or allogenic B and OVA^-T cells. The supernatant obtained from both the sixth-and eighth-day cultures suppressed anti-OVA antibody formation in autologous lymphocytes. The supernatant from the eighth-day, but not the sixth-day culture, suppressed antibody formation from allogenic lymphocytes (Fig. 6). This indicates that an allogenic-barrier exists between the suppressor factor on the sixth day and the lymphocytes receiving the action.

Antigen specificity of the suppressor factors

Culture supernatant obtained on the sixth day or the eighth day, which had shown a suppressive effect on anti-OVA antibody formation, were studied for their

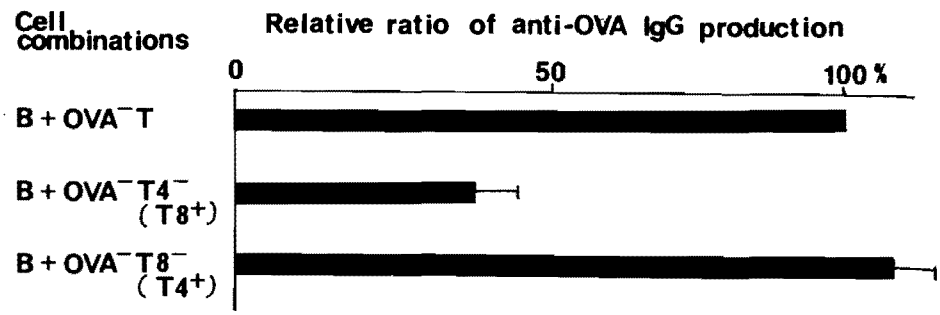

Fig. 3 Effect of the depletion of $T4^+$ or $T8^+$ population on the helper activity of OVA-non-binding T cells in anti-OVA antibody production stimulated with OVA antigen (5 μg/ml).
B : non-T cells (B-cell-rich fraction)
OVA^- : T cells which did not bind to OVA antigen.
$T4^-$: T cells depleted of OKT_4^+ cells.
$T8^-$: T cells depleted of OKT_8^+ cells.

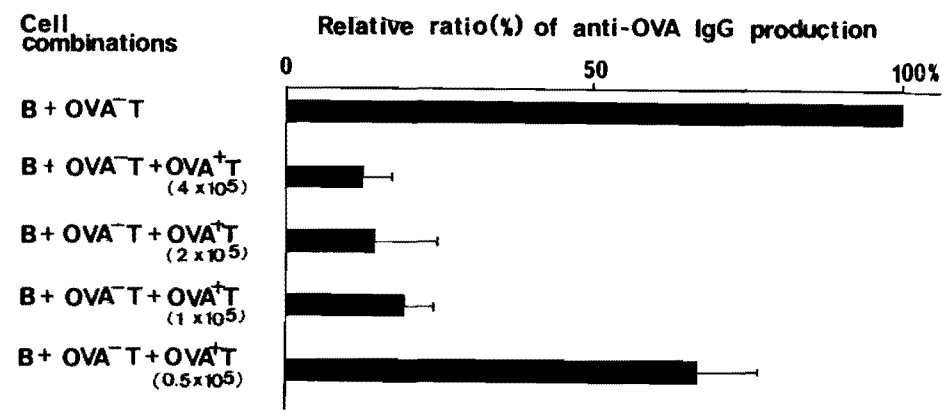

Fig. 4 Suppressor activity of OVA-binding T cells on the anti-OVA antibody production.
$B + OVA^-T$: the combination of non-T cells ($4x10^5$) and autologous OVA-non-binding T cells ($6x10^5$).
OVA^+T : autologous OVA-binding T cells.

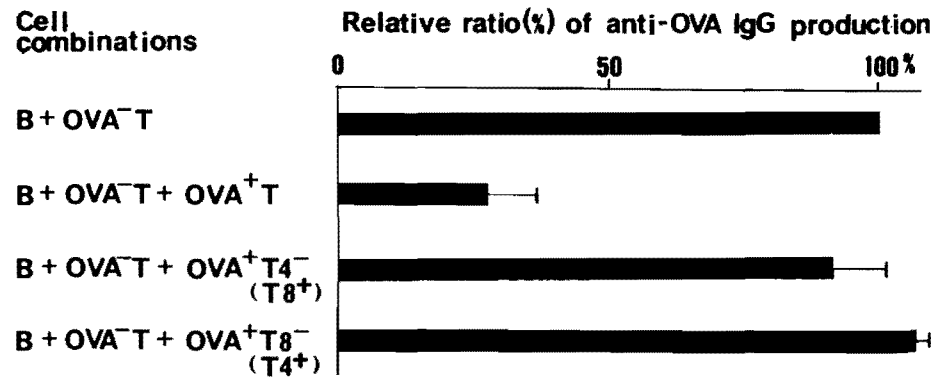

Fig. 5 Effect of depletion of T_4^+ or T_8^+ cells on the suppressor activity of OVA-binding T cells in anti-OVA antibody production stimulated with OVA (5 μg/ml).
$B+ OVA^-T$: the combination of B cells ($4x10^5$) and autologous OVA-non-binding T cells ($6x10^5$).
OVA^+T : OVA-binding T cells ($1x10^5$)
$OVA^+T_4^-$: OVA-binding T cells depleted of OKT_4^+ cells ($1x10^5$).
$OVA^+T_8^-$: OVA-binding T cells depleted of OKT_8^+ cells ($1x10^5$).

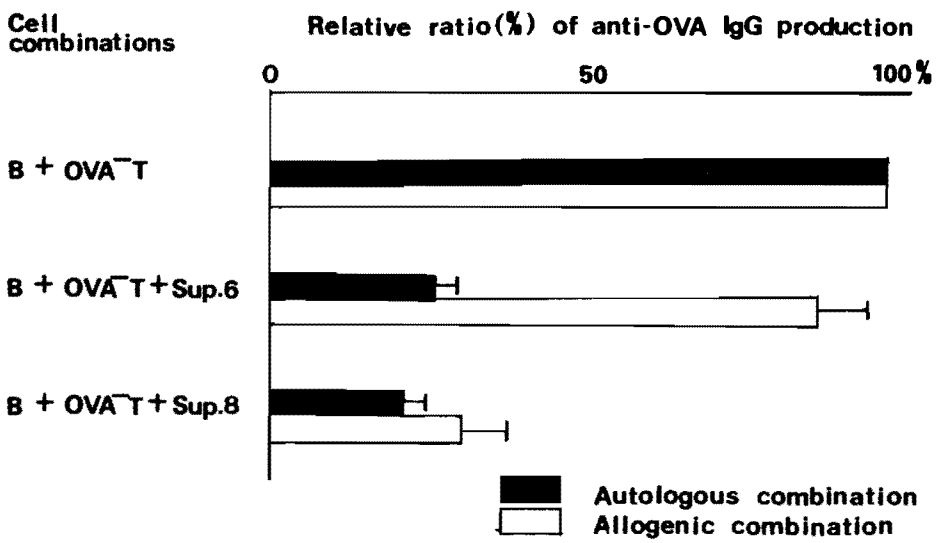

Fig. 6 Effect of the culture supernatants from T cells stimulated with high doses of OVA antigen on anti-OVA antibody production from the combination of autologous or allogenic B cells and OVA-non-binding T cells stimulated with OVA antigen (5 μg/ml).

B + OVA⁻T : the combination of non-T cells ($4x10^5$) and autologous OVA-non-binding T cells ($6x10^5$).
Sup 6 : culture supernatant on the sixth day.
Sup 8 : culture supernatant on the eighth day.

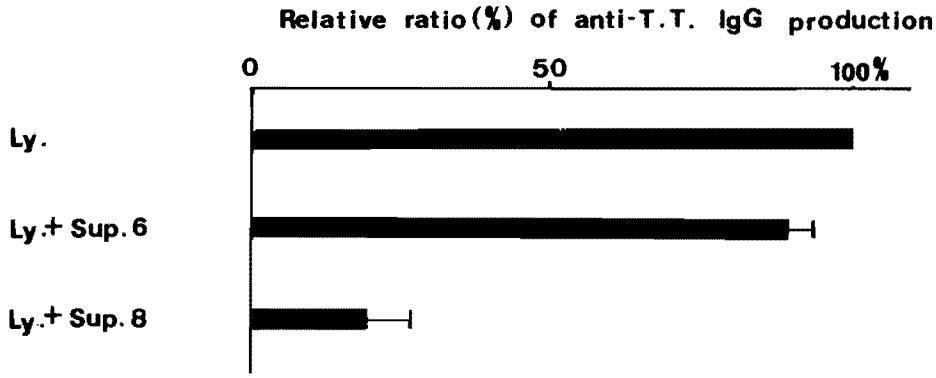

Fig. 7 Effect of the culture supernatant from T cells stimulated with high doses of OVA antigen on anti-tetanus-toxoid (TT) antibody production from the combination of autologous B cells and T cells stimulated with tetanus toxoid antigen.

Ly. : lymphocytes from individuals immunised with TT antigen.
Sup 6 : culture supernatant on the sixth day.
Sup 8 : culture supernatant on the eighth day.

suppressor activity on the anti-TT antibody formation in autologous lymphocytes. Suppression was observed in the supernatant on the eighth but not on the sixth day (Fig. 7).

This indicates that the suppressor factor(s) in the supernatant of the sixth-day cultures was (were) OVA-antigen specific and the suppressor factor(s) of the eighth-day cultures was (were) antigen-non-specific.

DISCUSSION

Most of the work concerning the analysis of *in vitro* antibody formation from human peripheral blood lymphocytes has been performed by activating the lymphocytes with pokeweed mitogen (PWM).[8] However, this response is less satisfactory for mimicking the antibody response in several points, these points include such factors as the lack of a major histocompatibility complex (MHC) restriction between T cells and B cells or macrophages, which complex is usually observed in cases of antigen-driven antibody formation.[9] It is now well accepted that MHC compatibility for some T-B or T-T cell interactions exists in specific antibody responses in humans as well as in mice.[10,11] Therefore, *in vitro* study of specific antibody response is really needed to clarify the regulatory mechanism of T cells of *in vivo* antibody production in humans stimulated with a specific antigen.

In the present work, we have studied the T-cell subsets regulating anti-OVA antibody production.

T cells may be divided into several subsets according to differences among cell surface differentiation antigens. And it has been clarified that functional subsets of T cells regulating antibody production are related to the subsets defined by surface antigen.[12] Human OKT_4^+ cells function as helper or inducer cells, while OKT_8^+ cells function as suppressor or killer cells.[13,14] Another phenotypic difference of T-cell subsets is that suppressor T cells have a stronger ability to bind to antigens than other subsets.[15] It was revealed in the present study that anti-OVA antibody was produced by B cells in the presence of T cells and stronger antibody formation was observed when OVA non-binding (OVA^-) T cells were used as helpers. Moreover, the helper activity of OVA^-T cells was negated by the depletion of OKT_4^+ cells. These observations indicate that the helper T cells in this system belong to the OKT_4^+-cell subset in OVA^-T cells.

Anti-OVA antibody production was suppressed by the addition of

OVA-binding (OVA^+) T cells. The suppressor activity of OVA^+ T cells was negated by depleting either OKT_4^+ cells or OKT_8^+ cells. This suggests that the suppressor T cells belonged to OVA^+ T cells and interaction between OKT_4^+ cells and OKT_8^+ cells was needed for the induction of the suppressor activity. It was reported in other systems that the interaction of different T-cell subsets was required for the induction of suppressor T cells[16] and that the suppressor activity of mouse T cells is usually mediated by humoral factors produced by the T cells.[17] Coincident to this, the culture supernatant produced from the T cells stimulated with high doses of OVA antigen suppressed the formation of anti-OVA antibody.[18]

We further studied the nature of the suppressor factor(s) in the supernatant. The suppressor activity was detected in the culture supernatant obtained from the sixth-day or eithth-day cultures of T cells. The eighth-day culture supernatant suppressed both anti-OVA antibody and anti-TT antibody production, while that of the sixth-day suppressed anti-OVA antibody production, but not anti-TT antibody production. Therefore, suppressor factor(s) in the culture supernatant on the sixth day seemed to be OVA-antigen specific; that (those) on the eighth day, antigen-non-specific. Suppressor factors on the sixth day had no effect on anti-OVA antibody formation from allogenic lymphocytes, suggesting the presence of some allogenic barrier. It was reported that antigen-specific suppressor factor(s) produced from mouse T cells had MHC barrier[19] and our factor(s) seem(s) to correspond with that finding. Our results suggested that antigen-specific and MHC-restricted suppressor factor(s) is (are) produced first (on approximately the sixth day) and antigen non-specific factors(s) is (are) generated later (on approximately the eighth day) under stimulation with high doses of OVA antigens. It is known that various species of regulatory factors, such as antigen-specific and non-specific suppressor T-cell factor(s) and anti-idiotype antibody, are produced from lymphocytes under stimulation with antigens. These factors correlate and regulate each other and form a complex network.[20-22]

Further analysis of these regulatory factors would be needed to disclose the precise mechansisms of antibody formation in humans.

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