Induction of Suppressor T Cells of Immunoglobulin Production by Alpha-foetoprotein in An *In Vitro* Human System*

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SUMMARY **Human T cells treated with alpha-foetoprotein (AFP) showed a suppressor effect on the generation of immunoglobulin-producing cells from lymphocytes stimulated with pokeweed mitogen (PWM). This effect seemed to be mediated by soluble factor(s), since the culture supernatant of AFP-treated T cells showed the same suppression. The suppressor factor(s) blocked the differentiation of B cells helped by T cells or mitomycin C-treated T cells, but not the B cells helped by soluble helper factor(s) from T cells. Therefore, the AFP-induced suppressor T cells seemed to exert their effect via helper T cells, but not directly on B cells. The induction of suppressor T cells might be one of the immunosuppressive mechanisms of AFP.**

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It is well known that alpha-foetoprotein (AFP) inhibits in *in vitro* proliferation of human lymphocytes in response to mitogens or in mixed lymphocyte culture reaction.[1-3] Similarly, AFP has been shown to exert an immunosuppressive effect on antibody synthesis in mice.[4] With regard to this immunosuppressive mechanism, suppressor T cells have proven to be induced by AFP in experimental animals.[5] It has been reported that the newborn T cells of newborn humans or cord blood T cells suppress the pokeweed mitogen (PWM)-driven T-cell-dependent differentiation of B cells to plasma cells.[6,7] These facts have suggested that AFP also induces suppressor T cells in human lymphocytes.

This paper demonstrates that AFP induces human T-cell activity which suppresses immunoglobulin (Ig) production by lymphocytes stimulated with PWM and that the suppression is mediated by soluble factor(s) secreted from suppressor T cells acting upon helper T cells.

MATERIALS AND METHODS

Peripheral blood lymphocytes

Heparinised venous blood drawn from healthy adults was gently layered on Ficoll-Hypaque density solutions (s.g., 1.074) and centrifuged at 400xg for 30 minutes. The mononuclear cells were collected from the interface and washed three times with phosphate buffered saline solution (pH 7.4) (PBS).

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

The mononuclear cells were mixed with neuraminidase-treated sheep erythrocytes in a ratio of about 1:100 in inactivated foetal calf serum (FCS) (Gibco), centrifuged at 200xg for 5 minutes to obtain tight cell contact. They were kept in ice water for one hour for the T cells to form rosettes with the sheep erythrocytes. The cells were then gently resuspended and applied to Ficoll-Hypaque density gradient. The cells that formed rosettes (T cells) were collected from the bottom; non-T cells (B-cell-rich fraction), from the interface. The contaminating erythrocytes were removed by lysing with tris-buffered 0.83% NH_4Cl.

The preparation of the human foetal α-foetoprotein

An enriched soluble preparation of human foetal α-foetoprotein was purchased from Calbiochem-Bering Co., Ltd. This solution was further fractioned by Sephadex-G 200 gel

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chromatography. The fractions with molecular weights ranging from 60,000 to 80,000 daltons were collected and concentrated by Amicon filtration. AFP was further purified from this fraction by immunoaffinity chromatography using rabbit anti-AFP antibody (Calbiochem-Bering Co., Ltd.) coupled to CNBr-activated Sepharose 4B. The concentration of AFP in this preparation was measured by immunoprecipitation.

Treatment of T cells with AFP

T cells ($5x10^5$) were incubated at 37°C with various concentrations of AFP (0.1, 1, 10, 20, 50, and 100 μg/ml) for various lengths of time ranging from 3 to 72 hours, in a humidified, 5% CO_2 incubator. The cells were then washed three times with PBS and resuspended in fresh culture medium (RPMI 1640 supplemented with 10% FCS (Gibco), 100 μg/ml gentamicin, and 100 units/ml penicillin-G).

Preparation of soluble suppressor factor(s)

T cells ($1x10^6$) in 1 ml of the medium containing various concentrations of AFP ranging from 10 to 100 μg/ml were cultured for 24 hours at 37°C in a humidified, 5% CO_2 incubator. After washing three times with PBS, they were then cultured in fresh medium for another four days before the cell-free supernatant was collected. The supernatant, after undergoing five-fold concentration by Amicon ultrafiltration, was used as a source of suppressor factor(s) in further experiments. The culture supernatants of untreated T cells were used as controls.

Preparation of soluble helper factor(s)

Non-T cells (B-cell-rich fraction) ($1x10^6$) in 1 ml of the medium were treated with 50 μg/ml mitomycin C (MMC) (Sigma) for 30 minutes at 37°C, in a humidified, 5% CO_2 incubator. A mixture of MMC-treated non-T cells ($5x10^5$) and allogenic T cells ($5x10^5$) in one millilitre of medium was cultured at 37°C for four days in a humidified, 5% CO_2 incubator. The cell-free supernatant was collected, concentrated five-fold using an Amicon filter, for use as a source of helper factor(s) in further experiments.

Assay for suppressor activity of T cells pretreated with AFP or of the culture supernatant from pretreated cells

The suppressor effect of T cells treated with AFP or of their culture supernatant (suppressor factor(s)) was assayed by the decrease of the immunoglobulin-producing cells (Ig-PCs) generated from the mixture of autologous non-T cells and T cells stimulated with PWM. T cells treated with AFP ($2x10^5$) or 0.2 ml of the five-fold concentrated culture supernatants from pretreated cells were added to the mixture of non-T cells ($2x10^5$) and T cells ($2x10^5$) and the cells were suspended in a culture tube (Falcon, 2054) in a total volume of 1 ml of medium containing 5 μg/ml of PWM. The cells were cultured at 37°C for seven days in a humidified, 5% CO_2 incubator. In some experiments, the T cells used to help the B cells were treated with 25 μg/ml of MMC at 37°C for 30 minutes to eliminate the effect of suppressor cell activity which is MMC-sensitive.[8] A count was made of the number of cells at the end of the culture. The cells were smeared on a glass slide, fixed with acetone for 10 minutes, stained with fluorescin-conjugated anti-human immunoglobulin antibody and observed under a UV-microscope. The percentage of cells showing cytoplasmic fluorescence (Ig-PCs) was then scored. The suppressor activity (i.e. the reduction in the number of Ig-PCs) was expressed in term of this number or as a ratio of this number in the presence of added T cells treated with AFP or their culture supernatants, relative to controls containing untreated T cells or their respective supernatants.

Effect of the suppressor factor(s) on the B cell differentiation exerted by the helper factor(s)

The helper factor(s) was added to non-T cells (B cell-rich fraction) ($4x10^5$) in a volume constituting 20 per cent of the entire culture medium and the cells were cultured in the presence of 5 μg/ml of PWM. These factors facilitated the B cell differentiation to Ig-PC, when tested by the aforementioned method. The suppressor factor (s) was added to this system as 20 per cent of the final culture volume and its effect was studied.

RESULTS

Suppressor activity of T cells treated with AFP

The T cells showed suppressor activity when they were treated with 50 μg/ml or more of AFP for longer than 12 hours (Fig. 1). The maximal suppressor activity was obtained by treatment with these concentrations for 24 hours or more.

Suppressor activity of the culture supernatant from T cells treated with AFP

The suppressor effect of the culture supernatant was demonstrated when T cells were treated with 50 μg/ml or more of AFP. The generation of Ig-PCs was suppressed by more than 50% (Fig. 2). The treatment with 20 μg/ml of AFP exerted some suppression but its effect was not sufficient.

Effect of the suppressor factor(s) on the differentiation of B cells co-cultured with T cells treated with mitomycin C

It is known that suppressor T cells are usually more sensitive to antimiotic drugs than helper T cells[9] and, therefore, most of the suppressor activity can be removed without affecting helper T cell function by using adequate doses of mitomycin C (MMC). The suppressor factor(s)

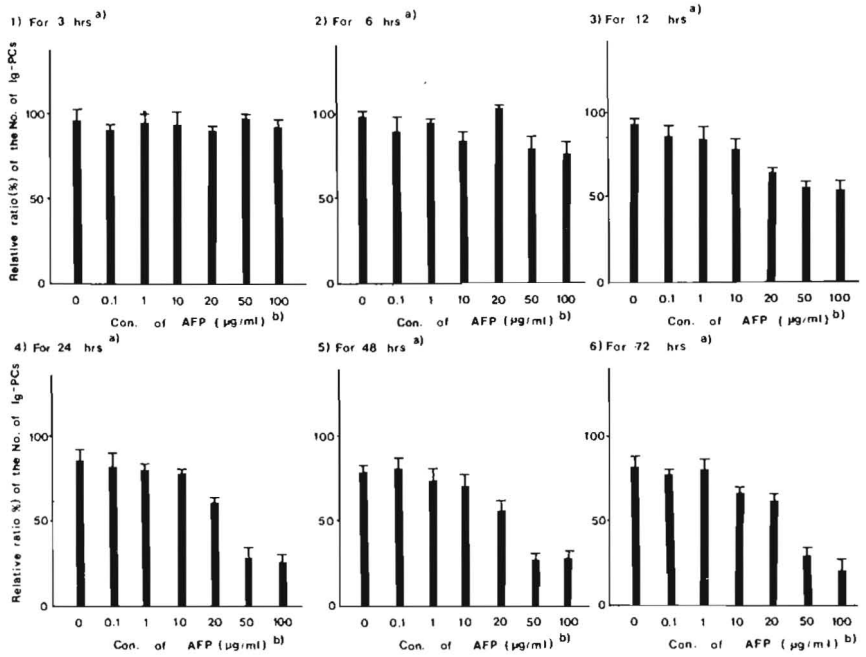

Fig. 1 Effect of AFP-treated T cells on the generation of immunoglobulin-producing cells (Ig-PCs) from the lymphocytes stimulated with PWM.
The percentages indicate the relative scores of the number of Ig-PCs from the combination of non-T cells and T cells added to T cells treated with AFP as compared with that of the combination of non-T cells and T cells alone.
a): Length of time for the treatment by AFP
b): The concentration of AFP used for the treatment of T cells.

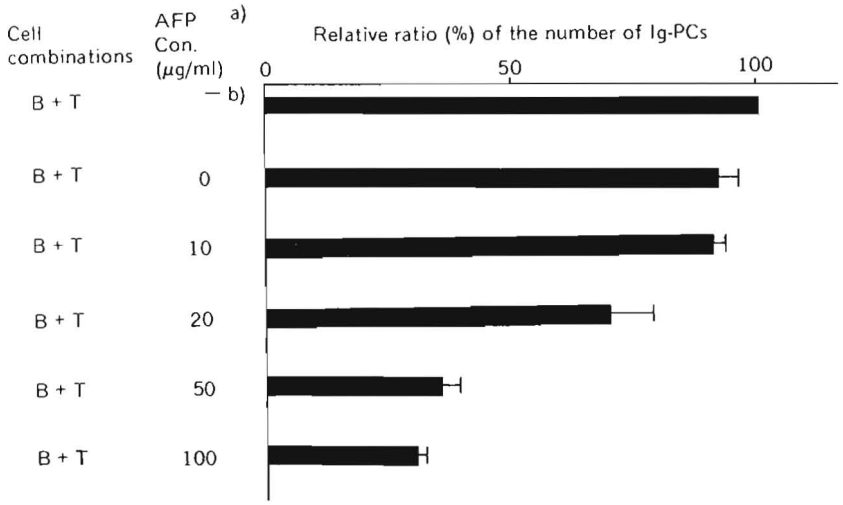

Fig. 2 Suppressor effect of the culture supernatant from T cells treated with AFP on the generation of Ig-PCs from the lymphocytes stimulated with PWM.
The percentages indicate the relative scores of the number of Ig-PCs from the combination of the non-T cells and T cells added to the supernatant as compared to that of the mixture of non-T cells and T cells alone.
B + T: Combination of non-T cells and T cells
a): The concentration of AFP used for the treatment of T cells from which the culture supernatant was obtained.
b): Control supernatant was added.

elicited their suppressor effect on B cell differentiation helped by MMC-treated T cells as well (Fig. 3). This indicates that the factor(s) exert their function, not by inducing MMC-sensitive suppressor T cells, but by directly working as a suppressor itself.

Effect of the suppressor factor(s) on B cell differentiation by helper factor(s)

The cell-free supernatant obtained from the mixed lymphocyte culture reaction facilitated the B cell differentiation on the Ig-PCs in response to PWM by functioning as helper factor(s). Suppressor factors from T cells treated with AFP did not inhibit the B cell differentiation helped by this factor(s) (Fig. 4). Therefore, the suppressor factors seemed to have no direct effect on B cells.

DISCUSSION

It has been shown that serum from pregnant women can suppress the mitotic response of lymphocytes.[10] Similarly, amniotic fluid inhibited the formation of antibody to sheep erythrocytes and AFP was found to be the causative agent for this suppression.[4,11] It was also reported that non-specific suppressor T cells induced by AFP seemed to be related to immunosuppression.[12] There were few reports with regard to the effect of AFP on the regulatory mechanisms of immunoglobulin production from peripheral blood lymphocytes in humans. T cells from cord blood in which AFP was present at a high concentration showed suppressor activity *in vitro*.[13,14]

This paper demonstrates that the T cells exerting suppressor activity in immunoglobulin formation are induced by concentrations of AFP physiologically present in the blood of newborn infants, and emphasises their role in the immunosuppressive mechanisms of AFP.

By studing various conditions, it was found that 50 μg/ml of AFP or

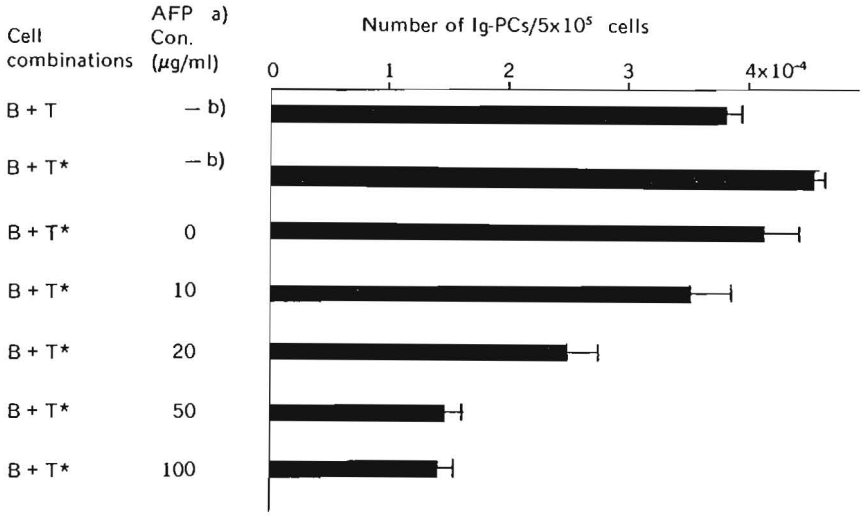

Fig. 3 Effect of the cell-free culture supernatant of AFP-treated T cells on the generation of Ig-PCs from non-T cells helped by T cells pretreated with mitomycin C (MMC).
B + T : The combination of non-T cells and T cells
B + T*: The combination of non-T cells and T cells treated with MMC.
a): The concentration of AFP used for treatment of T cells from which the culture supernatant was obtained.
b): Control supernatant was added.

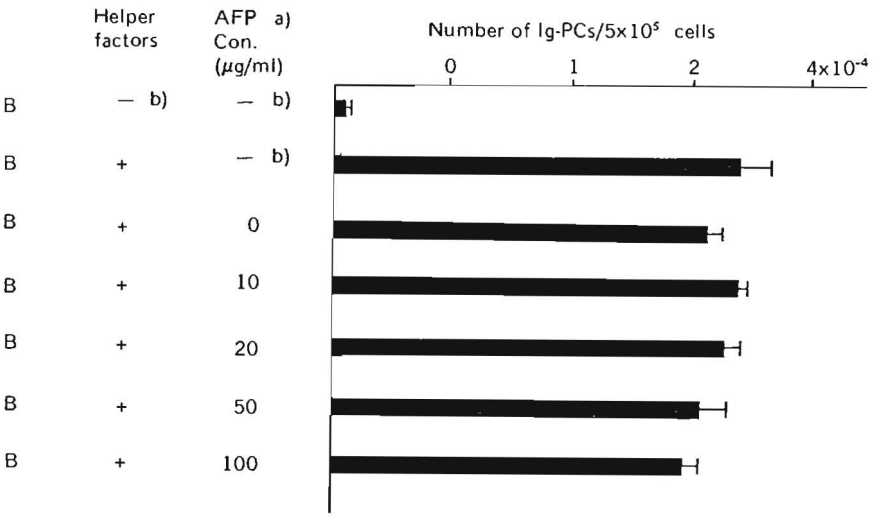

Fig. 4 Effect of the culture supernatant of AFP-treated T cells on B cell differentiation helped by soluble factor(s).
B : non-T cells (B-cell-rich fraction)
a): The concentration of AFP used for treatment of T cells from which the culture supernatant was obtained.
b): Control supernatant was added.

more, and a minimum of 12 hours of pre-culture were necessary to induce the suppressor T cells. The concentration of AFP which could induce suppressor T cells was approximately related to the mean concentration in the cord sera.[15] The cell-free supernatant from T cells pre-treated with AFP similarly suppressed the generation of Ig-PCs. Therefore, suppression by T cells treated with AFP seems to be mediated by soluble factor(s).

Our present data discloses that suppressor factor(s) from AFP-treated T cells suppressed B cell differentiation helped by MMC-treated T cells, but not that exerted by the helper factor(s) obtained from mixed lymphocyte culture reactions.[16] This suggests that the suppressor factor(s) inhibit helper T cells but have no direct effect on B cells. Other authors have reported that AFP does not exert a suppressive effect on B cells responding to thymus-independent antigens.[17] The factor(s) seemed to act as the suppressor(s) itself, and not through the induction of suppressor T cells, since it also suppressed the differentiation of B cells co-cultured with MMC-treated T cells, where the activation of most suppressor T cells was inhibited.[9]

These observations suggest that the immunosuppressive effect of AFP is due at least in part to the induction of suppressor T cells and that the activation of suppressor T cells observed in newborn infants might be caused by AFP which is present in high concentrations in the blood of foetuses and newborn in fants.

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