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Activation of human B lymphocytes

XII. DIFFERENTIAL EFFECTS OF *IN VITRO* CYCLOPHOSPHAMIDE ON HUMAN LYMPHOCYTE SUBPOPULATIONS INVOLVED IN B-CELL ACTIVATION

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Summary. The differential effects of *in vitro* cyclophosphamide (CY) on subpopulations of normal human peripheral blood lymphocytes involved in the pokeweed mitogen-induced plaque-forming cell (PFC) response against sheep red blood cells were examined. It was found that the plaque-forming B cells in this system are sensitive to CY over a wide concentration range including concentrations which have a minimal effect on overall cell viability. Kinetic experiments revealed that CY exerts its inhibitory effect on the PFC response only if added very early in culture. Thus, it appears that *in vitro* CY must exert its inhibitory influence on an early phase of polyclonal B-cell activation. When T-cell enriched (TCE) populations were incubated overnight with high concentration CY and then added back in co-culture to fresh autologous B cells, significant enhancement of PFC responses was observed suggesting a selective inhibition or elimination of a regulatory suppressor cell population found in TCE lymphocyte preparations. Helper T cells are relatively resistant to the inhibitory actions of CY. Thus, human B cells appear to be most sensitive to CY, followed in sensitivity by the suppressor cell populations in the T-cell fraction with relative resistance of the helper T cells. These observations have direct relevance in understanding the mechanisms of selective action of CY on normal human lymphocyte subpopulations with possible application to disease states in man.

Abbreviations: CY, cyclophosphamide; PFC, plaque-forming cell; PWM, pokeweed mitogen; SRBC, sheep red blood cell; TCE, T-cell enriched.

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INTRODUCTION

Cyclophosphamide (CY) is an alkylating agent which has been utilized extensively in recent years not only in the treatment of neoplastic diseases, but also as an effective agent in numerous non-malignant, immunologically mediated diseases (Gershwin, Goetzl & Steinberg, 1974). The successful use of CY in certain well defined inflammatory diseases has been striking (Fauci & Wolff, 1973; Fauci, Doppman & Wolff, 1978). Because of the difficulty in fully appreciating the pathogenic mechanisms of many of these diseases, therapy with CY has been for the most part empiric in nature. There have been several animal models of CY regimens (Gershwin *et al.*, 1974; Balow, Hurley & Fauci, 1975; Noble, Parker, Scheper & Turk, 1977), but their exact correlation to suppression of human immunological diseases is uncertain. Although numerous studies of the human immune response have

been performed in patients receiving CY for a wide variety of disease entities (Fauci, Wolff & Johnson, 1971; Fauci, Dale & Wolff, 1974; Curtis, Sharp, Lidsky & Hersh, 1973) extrapolations of these results to the mechanisms of CY action on the normal human immune system have been difficult because of conflicting data and due to the fact that many of these diseases manifest underlying immunological abnormalities.

The present study investigated the effect of *in vitro* CY on one aspect of the normal human immune response utilizing a haemolysis-in-gel plaque-forming cell (PFC) assay to measure pokeweed mitogen (PWM)-induced antibody production against sheep red blood cells (SRBC) by human peripheral blood lymphocytes (Fauci & Pratt, 1976a). This system measures antibody production by individual human B lymphocytes (Fauci & Pratt, 1976b) and is a T-cell dependent process modulated by a balance between naturally occurring helper and suppressor factors in normal individuals (Fauci, Pratt & Whalen, 1976; Haynes & Fauci, 1978). Using this *in vitro* assay, we were able to demonstrate selective effects of CY directly on human B-cell function and on the various subpopulations of lymphocytes involved in the immunoregulation of human B-cell function.

MATERIALS AND METHODS

Cell suspensions

Heparinized venous blood was obtained from normal adult donors and mononuclear cell suspensions were obtained by standard Hypaque-Ficoll density centrifugation. Cells were counted on a Model Fn Coulter Counter (Coulter Electronics, Hialeah, FL) and differential counts were performed on cytocentrifuge preparations stained with Wright's stain. T-cell enriched (TCE) cell suspensions were obtained by sheep erythrocyte (E) rosetting of unfractionated lymphocytes followed by a separation of rosetted and non-rosetted cells by centrifugation over Hypaque-Ficoll gradients as previously described in detail (Fauci & Pratt, 1976b). TCE suspensions contained 95–100% rosette-positive cells. Cell viability was determined by the trypan blue dye exclusion method.

Cell cultures

Culture conditions for the generation of anti-SRBC PFC responses following polyclonal activation of human peripheral blood lymphocytes with PWM have been prevously described in detail (Fauci & Pratt, 1976a,b). Briefly, cells were cultured in RPMI-1640 containing 1% trypticase soy broth, 2 mM L-glutamine, penicillin 100 units/ml, streptomycin sulphate 100 μg/ml and 10% heat-inactivated pooled human AB serum absorbed twice with SRBC. All cultures were incubated on a rocker platform (7 cycles/min) at 37° in 5% CO_2 in air, and cultures were stimulated either with PWM (Grand Island Biological Company, Grand Island, NY) at final concentrations ranging from 1:100 through 1:2000 of stock solution or with medium alone as a control (background PFC). Cultures were carried out in 12 × 75 mm plastic tubes (Falcon Plastics, Oxnard, CA) at a density of 2×10^6 cells in 1 ml.

Activated CY solution

Since CY as such is biologically inactive and must be activated *in vivo* by oxidation via hepatic enzymes (Cohen & Jao, 1970), 'activated' cyclophosphamide was obtained from the serum of rhesus monkeys following intravenous injection of CY according to the following protocol. A 9·8 kg male rhesus monkey was injected intravenously with a bolus of 100 mg/kg CY (Cytoxan®, Mead Johnson Laboratories, Evansville, IN). Peripheral venous blood samples were drawn 10–25 min following the intravenous injection. The blood was allowed to clot at room temperature, serum was obtained, pooled and divided into 1 ml specimens and frozen at −20° until used. Untreated control monkey serum was harvested in the same fashion from the same monkey several days prior to the administration of CY. When 50 μl of the full strength serum was added to the usual culture media volume (0·95 ml), this created a 1:20 dilution of the CY. When further dilutions were required (ranging as high as 1:200,000), RPMI-1640 was used as the diluent.

Assay for PFC

At the end of the 6 day culture period, cells were harvested and assayed for direct PFC against SRBC by an ultrathin layer haemolysis-in-gel technique previously described in detail (Fauci & Pratt, 1976a,b).

Blastogenic responses

Blastogenic responses of lymphocytes to stimulation with PWM were determined in microtitre plates in the presence and absence of CY by the incorporation of tritiated thymidine as previously described (Fauci, 1975).

Dose–response curves

The effect of activated CY-containing monkey serum on lymphocyte viability and PFC responses was per-

formed by adding CY in dilutions ranging from 1:20 to 1:200,000 to unfractionated lymphocytes cultured in the manner described above. RPMI alone or untreated monkey serum was added to separate cell cultures as control. At 6 days, cell viabilities and anti-SRBC PFC responses were determined as described above.

Kinetics of CY effect

To determine the time interval necessary for CY to be present in culture in order to modulate PFC responses, unfractionated lymphocytes were cultured as described above and 1:100 CY or 50 μl of RPMI were added to separate cultures on days 0 through 6. All PFC responses were then assayed on day 6.

TCE co-culture experiments

TCE lymphocytes were obtained as described above and pre-incubated for 24 h with CY serum in final concentrations of 1:20 or 1:200,000. Other TCE suspensions were irradiated with 2000 rad from a Phillips 250 kVp dual head X-ray system and then cultured separately overnight. TCE lymphocytes that received no treatment were also cultured separately for 24 h as controls. The next day, all lymphocyte preparations were washed three times with RPMI and subsequently 1×10^6 cells of each suspension were co-cultured with 1×10^6 fresh autologous unfractionated lymphocytes. PFC responses were determined on day 6. Data are expressed as expected PFC response per 10^6 cells compared to observed PFC response per 10^6 cells. Expected PFC responses are based on the individual responses of each fraction of the co-culture when cultured alone.

Statistical analysis

Data were compared by using the two-tailed Student's *t* test or where indicated by using the two-tailed *t* test of the mean log of paired sample differences.

RESULTS

Dose-response curves of CY effect on PFC

The effect of activated CY on the PFC response of unfractionated lymphocytes when CY is added directly to cultures on day 0 is shown in Fig. 1. The PFC responses are virtually abolished when CY is present in culture in the range of a 1:20 dilution (full strength serum) to a 1:100 dilution. At greater dilutions of CY, less suppression of PFC responses is seen. By the

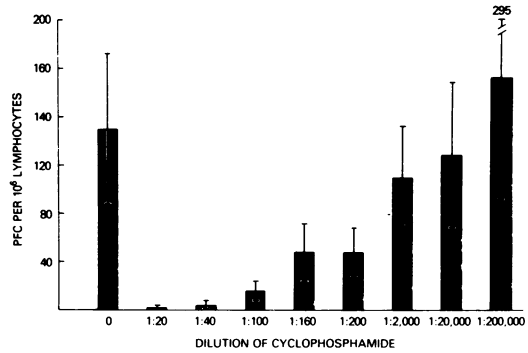

Figure 1. Effect of various dilutions of activated CY on PWM-induced PFC responses in human peripheral blood mononuclear cells. Unfractionated mononuclear cells were cultured with medium alone or CY in dilutions ranging from 1:20 to 1:200,000 in the presence of PWM. After 6 days, cultures were harvested and assayed for PFC responses against SRBC. Data represent the mean (±SEM) of ten separate experiments.

1:2000 dilution of CY, PFC responses are normal, and at a 1:200.000 dilution there is a suggestion of increased PFC responses above baseline, but this is not statistically significant ($P > 0{\cdot}20$). It should be noted that adding full strength (1:20) untreated control monkey sera to culture had no observable effect on PFC responses.

Effect of CY on cell viability

The effect of activated CY on the viability of lymphocytes cultured under the same conditions as those cultured for PFC determination is shown in Fig. 2. CY in a 1:20 dilution caused cell viability to decrease from 90 to 31%. Of note is the fact that at the 1:100 dilution of CY, viability had returned to 61% whereas PFC response was only 7% of baseline. At further dilutions of CY, cell viability continued to increase slightly towares baseline, while PFC responses which had been markedly depressed returned towards normal more dramatically. Thus, we chose the 1:100 dilution of CY as an optimal dose of CY for subsequent studies since it provided the optimal balance of inhibition of the PFC response together with preserved viability of the cultured cells. Non-treated control monkey serum in a 1:20 dilution caused no significant change in cell viability ($P > 0{\cdot}20$).

Kinetics of CY effect

To evaluate the amount of time required for CY to be

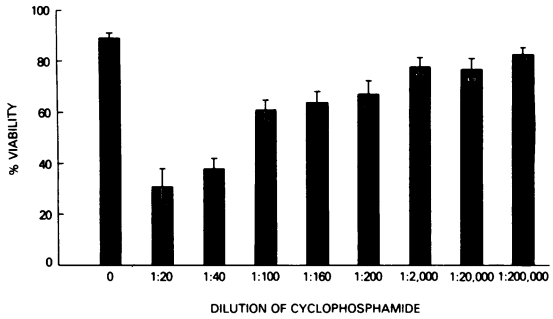

Figure 2. Effect of various dilutions of activated CY on viability of human peripheral blood mononuclear cells. Unfractionated mononuclear cells were cultured with medium alone or with CY in dilutions ranging from 1 : 20 to 1 : 200,000 in the presence of PWM. After 6 days, viability was assayed by the 0·4% trypan blue dye exclusion method. Data represent the mean (±SEM) of ten separate experiments.

present in culture in order to modulate the PFC response, four separate experiments were performed utilizing the standard unfractionated lymphocyte culture conditions while adding CY to the system on one of the 6 days of incubation. On the appropriate day (from 0 to 6), CY in a 1:100 dilution or 50 μl of RPMI as a control was added to separate cultures on days 0 through 6. The day 0 cells treated with RPMI have a mean PFC response of 217 (±80) per 10^6 cells whereas the CY-treated cells have a mean PFC value of 17 per 10^6 cells (±13) ($P<0.05$). When CY was added to cultures subsequent to day 0 (day 1 through 6) there was no significant effect on PFC responses ($P>0.20$). This suggests that CY inhibits events associated with the early activation process of polyclonally induced B-lymphocyte differentiation.

Effect of CY on lymphocyte blastogenic responses

The effects of various dilutions of CY on PWM-induced lymphocyte blastogenic responses are shown in Fig. 3. There was a marked suppression of blastogenic responses when CY was added to cultures at a 1:20 dilution ($P<0.01$). At the highest dilution of CY, the blastogenic response was not significantly affected ($P>0.20$). In some experiments, untreated monkey serum in a 1:20 dilution caused a decrease in PWM-induced blastogenic responses ($P<0.05$). However, the marked suppression of blastogenesis caused by CY serum was always significantly greater than that caused by control monkey serum ($P<0.01$).

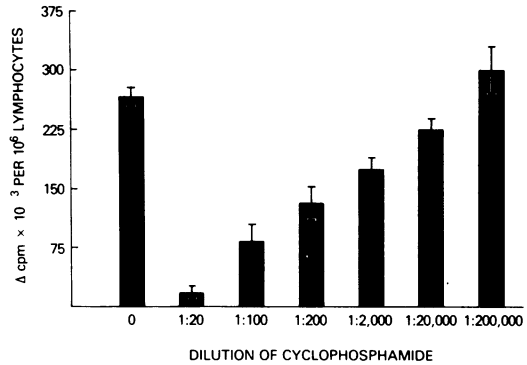

Figure 3. Effect of various dilutions of CY on the PWM-induced blastogenic response of human peripheral blood mononuclear cells. Unfractionated mononuclear cells were incubated with medium alone or with CY in dilutions ranging from 1 : 20 to 1 : 200,000 in the presence of PWM. Blastogenic responses were measured by the incorporation of tritiated thymidine at day 5. Δc.p.m. equals the c.p.m. of stimulated cultures minus the c.p.m. of unstimulated cultures. Data represent the mean (±SEM) of four separate experiments.

Effect of CY on T cells

In order to determine if CY acts directly on B cells to suppress their mitogen-induced PFC responses and/or has effects on some regulatory accessory cell populations, a series of co-culture experiments were performed. Figure 4 demonstrates that T lymphocytes that had been pre-incubated with RPMI for 24 h (control) did not significantly modify the expected

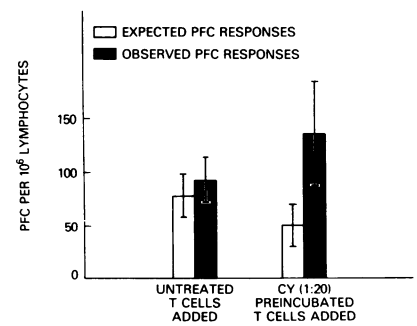

Figure 4. Effect of co-culture of CY (1 : 20) pre-incubated T cells with autologous unfractionated mononuclear cells. CY-treated T cells and untreated unfractionated mononuclear cells were co-cultured at a ratio of 1 : 1 in the presence of PWM. After 6 days of co-culture, the cells were harvested and assayed for anti-SRBC PFC responses. Data are expressed as the number of PFC per 10^6 viable fresh cells placed into culture. Data represent the mean (±SEM) of seven separate experiments.

PFC responses when co-cultured with fresh mononuclear cells in the presence of PWM ($P < 0{\cdot}20$). However, when T lymphocyte populations were pre-incubated with high concentrations of CY (1:20), significant enhancement of the expected PFC response was seen when these cells were subsequently co-cultured with fresh mononuclear cells in the presence of PWM ($P < 0{\cdot}01$). Pre-incubation with 1:200,000 CY had no significant effect on expected PFC. It should be pointed out that untreated TCE fractions usually give as many if not more PFC than do unfractionated lymphocytes. The mechanism for this phenomenon has been detailed in a separate paper (Dimitriu, Haynes & Fauci, 1979). In brief, the B-cell precursors of the anti-SRBC PFC have surface immunoglobulin which binds to SRBC to form SRBC rosettes. They therefore segregate with the E-rosetting T cells during the E-rosette fractionation procedure used to obtain TCE suspensions. For this reason, the expected PFC responses in the CY pre-incubation column of Fig. 4 are somewhat lower than control since CY pre-incubation lowered the PFC response of TCE fractions alone. In addition, consistent enhancement of PFC responses was also seen in separate experiments when lymphocytes were pre-treated with 2000 rad and co-cultured with fresh unfractionated lymphocytes confirming the prevous reports from our laboratory (Fauci, Pratt & Whalen, 1978) and others (Siegal & Siegal, 1977) of the relative sensitivity of suppressor T cells and resistance of helper T cells to X-irradiation. This degree of enhancement was somewhat greater than the amount seen with CY pre-treatment. Since it is quite unlikely that high-dose CY pre-incubation might cause a direct amplification of helper regulatory T cells, these results suggest that 1:20 dilution CY inhibits or eliminates a regulatory suppressor cell population in human peripheral blood, creating a net enhancing effect on PFC response.

DISCUSSION

CY is a potent cytotoxic agent which has been shown to have numerous effects on various parameters of the immune system and clinically it has been used effectively to treat various animal models of human immunologically mediated diseases (Gershwin *et al.*, 1974). CY has also been utilized in several animal systems to delineate the mechanisms of regulation of cell function, particularly the etiology of immune tolerance (Polak & Turk, 1974). An area of recent intense interest has been the assessment of differential sensitivity of various lymphocyte subpopulations to CY. B cells appear to be more sensitive to the cytotoxic effects of CY than do T cells (Turk & Poulter, 1972; Revell, 1974). Of interest is a recent report of CY administration to mice, where it was found that in contrast to other alkylating agents, CY blocks the re-expression of surface immunoglobulin on cells after modulation of B-cell membranes with $F(ab')_2$ fragments, perhaps explaining why CY lowers the B-cell tolerance threshold to thymus-independent antigens (Shand & Howard, 1978). In addition, while many B-cell subpopulations are depressed with CY pre-treatment, it is becoming clear that other B-cell subpopulations have functions which are relatively resistant to CY (Askenase, Hayden & Gershon, 1975; Noble *et al.*, 1977). Likewise, T-cell subpopulations have been shown to have selective susceptibility to the effects of CY. The suppressor cells found in certain animal systems have been shown to be sensitive to CY (Sy, Miller & Claman, 1977; Ferguson & Simmons, 1978), while other suppressor cell populations appear to be CY resistant (Bonavida, 1977). In addition, a CY-sensitive T cell has been described in mice which suppresses the reaction of B cells to a T-independent antigen (Duclos, Galanaud, Devinsky, Maillot & Dormont, 1977).

Studies on the functional capabilities of normal human lymphocytes during *in vivo* CY therapy have been difficult to design. Patients receiving CY for immunologically mediated diseases probably do not have a normally functioning immune system. In addition, it is possible that lymphocytes express a distinctly different type of abnormality for each disease in question. This leads to obvious difficulties in the extrapolation of data generated in this abnormal setting of disease activity to the normal state. Furthermore, many of the malignancies treated with CY are associated with demonstrable defects in the immune response (Broder, Humphrey, Durm, Blackman, Meade, Goldman, & Waldman, 1975; Hanock, Bruce, Sugden, Ward & Richmond, 1977), and these patients are usually receiving other chemotherapeutic agents in addition to CY. Most of the above cited problems can be avoided with an assay of the *in vitro* effects of CY on normal human PB lymphocytes as presently described.

The present study has demonstrated that *in vitro* CY is capable of suppressing PFC responses when added to cell suspensions that contain plaque-generating B cells. In addition *in vitro* activated CY is capable of suppressing blastogenic responses of unfractionated

lymphocytes. This correlates with the finding that T lymphocytes pre-incubated with CY produced significant enhancement of the expected polyclonally triggered PFC response when co-cultured with unfractionated PB cells.

It is well demonstrated that PWM-induced PFC responses of human B lymphocytes represent a balance of naturally occurring helper and suppressor influences (Fauci & Pratt, 1976a; Fauci *et al.*, 1976). Furthermore, other studies have shown that a variety of cytotoxic agents are capable of enhancing B-cell function in a mitogen-triggered system when T cells are pre-treated and subsequently cocultured with fresh B cells (Dimitriu & Fauci, 1978; Fauci *et al.*, 1978). When T cells have been treated with each of the agents thus far studied (mitomycin C, azathioprine and irradiation), an increase in net helper effect has been consistently demonstrated. The differential inhibitory effects of a number of agents (all of which inhibit blastogenic responses) such as corticosteroids (Fauci, Pratt & Whalen, 1977), azathioprine (Dimitriu & Fauci, 1978) and irradiation (Siegal & Siegal, 1977; Fauci *et al.*, 1978), on suppressor T-cell function with relative sparing of helper T-cell function strongly suggest that suppressor cells themselves are dependent on cell division to express suppressor function, or alternatively suppressor cells require another cell which must divide. The same situation is likely to be true with CY, although metabolic requirements of suppressor cells other than actual cell division may be the critical factors suppressed by CY as well as the other agents discussed above.

Thus, the results described in this paper may help to further our understanding of the effects of CY on antibody production and on the subsets of lymphocytes involved in regulating B-cell function. The totally *in vitro* format of this study may be of further use in additional investigations of the effects of activated CY on other limbs of the normal human immune response, hopefully leading to greater insight into the *in vivo* effects of this cytotoxic agent which is used clinically in diseases of aberrant immunological reactivity.

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