

SELECTIVE SUPPRESSION OF AN EARLY STEP IN HUMAN B CELL ACTIVATION BY CYCLOSPORIN A

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The interaction of antigen with the surface membrane immunoglobulin (sIg)¹ of B lymphocyte, or cross-linkage of the sIg receptor by anti-Ig initiates a complex process whereby a resting B cell proceeds to an Ig-secreting cell via sequential activation, proliferation, and differentiation steps. Several groups have proposed models for B cell activation and differentiation that are similar in many respects, but with certain variations (1–10). We have been involved in attempting to delineate the optimal signals required for induction of a resting human B cell to proliferate, as well as the signals for the induction of the activated B cells to differentiate. We have shown that *Staphylococcus aureus* Cowan strain I (SAC) directly induces proliferation of human B cells, while low dose anti- μ stimulates B cells to express receptors for B cell growth factor (BCGF); BCGF, in turn, drives activated B cells to proliferate. These proliferating B cells can then be induced to differentiate into immunoglobulin-secreting cells (ISC) by B cell differentiation factor (BCDF), alternatively referred to as T cell-replacing factor (TRF) (8, 9, 11–13). Within this context, the present study was designed to determine the effect, if any, of cyclosporin A (CsA) on the proliferation and differentiation of human B cells at various states of activation and to delineate the mechanisms of action whereby CsA inhibits proliferative and/or differentiative responses of B cells to anti- μ and SAC.

With regard to the usage of CsA, this drug was first isolated from the fungi *Trichoderma polysporum* and *Cylindrocarpon lucidum booth* (14). CsA has a molecular weight of 1,202 with an unusual cyclic endecapeptide composed of 11 amino acids, several of which are N-methylated, making the molecule highly lipophilic and hydrophobic. Subsequent studies have revealed that this drug selectively acts on lymphocytes to suppress immune function by noncytotoxic mechanisms (15, 16), and it has been shown to prolong allograft survival in many species including man (17, 18). CsA has been reported to inhibit not only T cell proliferative response to mitogens or alloantigens but also the induction of cytotoxic cells in the mixed leukocyte reaction (15, 16, 19–24). Although it has been thought that CsA acted preferentially on T cells with considerably less effect on B cells,

¹ Abbreviations used in this paper: AET, 2-aminoethylisothiuronium bromide; BCDF, B cell differentiation factor; BCE, B cell enriched; BCGF, B cell growth factor; CsA, cyclosporin A; E, erythrocytes; ISC, immunoglobulin-secreting cell; PFC, plaque-forming cells; SAC, *Staphylococcus aureus* Cowan strain I; sIg, surface immunoglobulin; TCGF, T cell growth factor; TRF T cell replacing factor.

Paavonnen et al. (25, 26) first claimed that CsA interfered with the ability of both human T and B cells to generate Ig-producing cells. More recent studies in the mouse model showed that a functional B cell subset, namely B cells responsive to thymus independent-2 antigens, are exquisitely sensitive to the suppressive effect of CsA (27, 28).

In the present study, we have examined the direct effects of CsA on purified human B cells and have demonstrated a selective drug-induced suppression of the activation phase vs. the proliferative and differentiative phases of the human B cell cycle.

Materials and Methods

Reagents. Anti- μ antibody is the F(ab')₂ fragment of goat anti-human heavy chain-specific IgM (N. L. Cappel Laboratories, Cochranville, PA). SAC is the same preparation as that used previously (11). CsA was obtained from Sandoz (Basel, Switzerland) and dissolved at 10 mg/ml in absolute alcohol. This was diluted to appropriate concentrations with medium before use.

Preparations of Factors. Monoclonal BCGF used in the present study is the same preparation as previously described in detail (8). Briefly, monoclonal BCGF was obtained from a human T-T hybridoma cell line, 2B₁₁, which was established in our laboratory (29). In this monoclonal BCGF preparation, there was no interleukin-2 activity as determined by measurement of [³H]thymidine incorporation by an interleukin-2-dependent cell line as described by Farrar et al. (30). In addition, there was no BCDF activity as determined by measurement of the induction of plaque-forming cells (PFC) from SAC-stimulated normal B cells described by Falkoff et al. (11) and from an Epstein-Barr virus-transformed B blastoid cell line, CESS, as described previously (31). BCDF-containing supernatants were prepared by coculture of sheep erythrocyte (E) rosette-positive cells of multiple donors with irradiated E rosette-negative cells in the presence of 2 μ g/ml of phytohemagglutinin in serum-free medium as formulated by Mosier et al. (33). Following 72 h of culture, the supernatants were harvested and assayed for BCDF activity in the SAC system (11) and CESS system (31) as described above. These assays gave concordant results. The supernatants were stored at -20°C until used.

Cell Preparations. Human tonsils were obtained at tonsillectomy from 12- to 22-yr-old patients with chronic tonsillitis and were dispersed into single cell suspensions (8). Tonsillar mononuclear cells were separated by the standard Hypaque-Ficoll gradient method. B cell-enriched populations (BCE) were obtained by depletion of T cells and monocytes (8). Briefly, T cells were depleted by twice rosetting with 2-aminoethylisothiuronium bromide (AET)-treated sheep E. Monocytes were depleted by adhering 50×10^6 T cell-depleted cells to plastic flasks (100 mm \times 10 mm style, Falcon 3003; Becton-Dickinson and Co., Oxnard, CA) containing RPMI 1640 with 1% fetal calf serum for 45 min at 37°C. In these BCE populations, there were >90% sIg+ cells determined by staining with a fluorescein-conjugated F(ab')₂ fragment goat anti-human Ig (Cappel Laboratories); <0.1% sheep E receptor-positive cells as enumerated by rosetting the cells with AET-treated sheep E; and <0.1% monocytes as determined by nonspecific esterase staining (8). Tonsillar BCE suspensions were further separated into fractions according to cell-volume by using counterflow centrifugation-elutriation (Beckman, Model J-6B, Beckman Instruments, Palo Alto, CA) by the method previously described in detail (28) with certain modifications (T. L. Gerrard and A. S. Fauci, unpublished observations). Briefly, 500×10^6 cells in 3 ml elutriator buffer (phosphate-buffered saline containing 1.25% human albumin and 50 μ g/ml of gentamicin) were injected into the elutriator chamber and spun with the J-6B elutriator at a constant speed of $2,020 \pm 10$ rpm with the countercurrent flow rate started at 7.5 ml/min. and increased every 200 ml by 0.5 ml/min increments. Each 50-ml aliquot was collected from the exit part of the elutriator centrifuge. Cellular volume was quantitated with a Coulter Channelyzer (Coulter Electronics, Inc., Hialeah, FL). Mean volume of the small B cells used in the following experiments was 170 ± 10

μm^3 . Characterization of the surface markers of fractionated cells has been described elsewhere (8).

Cell Cultures. In order to study the effect of stimulation of B cells with anti- μ on cell volume, cells were cultured in a serum-free medium (standard medium) described by Mosier (33). Standard medium contains 50% Isocove's modified Dulbecco's medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) and 50% Ham's F-12 medium (Gibco Laboratories) added with insulin (5 $\mu\text{g}/\text{ml}$; Sigma Chemical Co., St. Louis, MO), progesterone (3×10^{-8} M; Upjohn Laboratories, Kalamazoo, MI), 2-mercaptoethanol (5×10^{-5} M), gentamycin (50 $\mu\text{g}/\text{ml}$), and trace elements. Standard medium is 0.281 osmolal, which is comparable to that of RPMI 1640 medium as determined by a DigiMatic Osmometer (Advanced Instruments, Inc., Needham Heights, MA). 2×10^6 cells were cultured with goat anti- μ antibody (15 $\mu\text{g}/\text{ml}$) in the presence or absence of various concentrations of CsA, as indicated, in 1 ml of serum-free standard medium on 16-mm diameter flat-bottomed tissue culture wells (3524; Costar, Data Packaging, Cambridge, MA). Control cultures were pulsed with 0.1% of ethanol, which is the solvent for the drug. Cells were harvested from cultures at 30–36 h as indicated, and recovered cells were layered on a Hypaque-Ficoll gradient and then centrifuged at 2,500 rpm for 20 min at 4°C. Viable cells (>90%) were removed from the interphase layer and washed with Hanks' Balanced Salt Solution (M. A. Bioproducts, Walkersville, MD), and cell volume was quantitated with a Coulter Channelyzer.

For determining incorporation of [^3H]thymidine or [^3H]uridine, fresh B cells or precultured B cells in serum-free medium (5×10^4 /well) were cultured with anti- μ (15 $\mu\text{g}/\text{ml}$), BCGF (20% vol/vol), or anti- μ plus BCGF in the presence or absence of CsA, as indicated, in 0.2 ml standard medium containing 10% fetal calf serum (M. A. Bioproducts) in flat-bottomed microtiter plates (Costar 3596). Cells cultured in the absence of CsA had 0.1% ethanol added as previously mentioned. All cultures were incubated in 100% humidity in 5% CO_2 in air at 37°C. Cultures were pulsed with 1 μCi of [^3H]thymidine (6.7 Ci/mM; New England Nuclear, Boston, MA) or 1 μCi or [^3H]uridine (42 Ci/mM; New England Nuclear) over various periods as indicated before harvesting, and incorporation of [^3H]thymidine or [^3H]uridine was measured by standard liquid scintillation counting techniques after harvesting with a Titertek cell harvester (Flow Laboratories, Inc., Rockville, MD).

Assay for B cell Differentiation. Small tonsillar B cells purified by the method described above were cultured for 3 d with SAC ($10^{-3}\%$ vol/vol) in 0.2 ml standard medium containing 10% fetal calf serum in flat-bottomed microtiter plates to activate B cells to a state of responsiveness to BCDF (11). BCDF-containing supernatants (10% vol/vol) with or without CsA at various concentrations were added to the cultures, and the number of ISC per well was determined in reverse hemolytic plaque assay following an additional 3 d of culture (11).

Results

CsA Inhibits B Cell Proliferative Responses to Either SAC or Anti- μ Plus BCGF. In order to study the effect of CsA on B cell proliferative responses, small B cells obtained from tonsillar mononuclear cells were stimulated with either SAC or anti- μ plus BCGF, and CsA was added to the cultures at the time of their initiation at various concentrations (0.1–10,000 ng/ml). In control cultures, 0.1% of ethanol was added. As shown in Fig. 1, addition of CsA (5–1,000 ng/ml) completely inhibited B cell proliferative responses to costimulation with anti- μ and BCGF, while the same concentration of CsA showed considerably less inhibitory effects on SAC-induced B cell proliferation. 50% inhibition (I_{50}) of B cell proliferation induced by anti- μ plus BCGF was obtained by the addition of 1–2 ng/ml of CsA, whereas the I_{50} of SAC-induced B cell proliferation was obtained with 200–300 ng/ml of CsA. Cell recovery and viability of the cultures

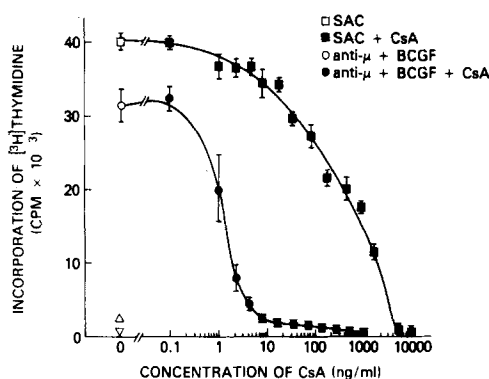


FIGURE 1. Effect of CsA on the proliferative response of small B cells to SAC and costimulation of anti- μ plus BCGF. Small B cells (5×10^4 /well) separated from tonsillar mononuclear cells were cultured with 15 $\mu\text{g/ml}$ of anti- μ alone (Δ); 25% vol/vol of BCGF alone (∇); anti- μ and BCGF with 0.1% ethanol (\circ); $10^{-5}\%$ vol/vol of SAC with 0.1% ethanol (\square); anti- μ plus BCGF and CsA (\blacktriangle); SAC and CsA (\blacksquare). Cultures were incubated for 3 d with the addition of [^3H]thymidine (1 μCi /well) over the last 16 h. Data represent the mean (\pm SEM) of triplicate cultures.

to which CsA (1–1,000 ng/ml) had been added for 36 h were comparable to those of the control culture. Addition of more than 10 $\mu\text{g/ml}$ of CsA to the cultures resulted in cell death (data not shown). These data demonstrate that CsA inhibits the B cell proliferative response to costimulation with anti- μ and BCGF in a noncytotoxic manner at 50- to 100-fold lower concentrations than are required for suppression of SAC-induced B cell proliferation. It is unclear whether the suppressive effects of CsA are irreversible. Preincubation of resting B cells with the drug for 24 h followed by washing and stimulation with anti- μ plus BCGF always resulted in responses significantly lower than those seen in cultures preincubated with medium alone (data not shown). However, it is still possible that the cells exposed to CsA might have recovered fully if cultured for variable periods of time in medium before stimulation with anti- μ and BCGF. These experiments proved difficult and inconclusive since B cells left in culture without stimulation for 24–48 h generally manifest poor responses to anti- μ plus BCGF.

CsA Acts on B Cells at an Early Step in the Inhibition of B Cell Proliferative Responses to Costimulation with Anti- μ Plus BCGF. In order to examine at which stages in the activation-proliferation cascade CsA acts to inhibit the proliferative response of B cells to costimulation with anti- μ plus BCGF, CsA (200 ng/ml) was added to the cultures at various times after initiation of culture in which small B cells had been incubated with anti- μ and BCGF. As shown in Fig. 2, CsA-mediated inhibition was found only when CsA was added to the cultures during the first 24 h of incubation. The addition of CsA after 40 h failed to inhibit B cell proliferation. These results indicate that CsA acts on B cells at an early step in the inhibition of proliferative responses to costimulation with anti- μ plus BCGF.

CsA Inhibits the Anti- μ -Induced Cell Enlargement of Small B Cells. We have recently shown that in the costimulation system of anti- μ and BCGF, stimulation of small B cells with low doses of anti- μ alone activates the cells and induces an

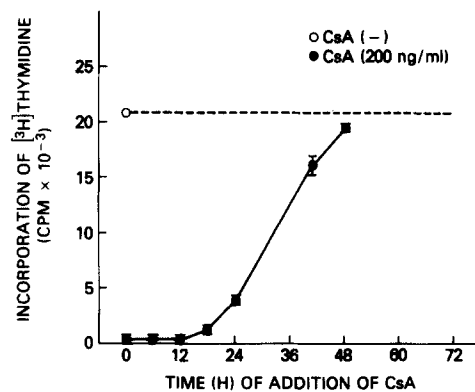


FIGURE 2. Time dependence of CsA-mediated inhibition of B cell proliferative responses to costimulation with anti- μ plus BCGF. Small B cells separated from tonsillar mononuclear cells were cultured with anti- μ (15 μ g/ml) plus BCGF (25% vol/vol). CsA (200 ng/ml) was added to the cultures at various times, and incorporation of [3 H]thymidine was measured after 72 h of incubation. Cultures were pulsed with 1 μ Ci of [3 H]thymidine over the last 16 h of incubation. Data represent the mean (\pm SEM) of triplicate cultures.

increase in cell size (9). Cells stimulated by anti- μ started to enlarge within 8 h and achieved their maximal volume at 24–36 h (9). In order to examine whether CsA inhibits enlargement of B cells induced by low concentrations of anti- μ , small B cells prepared from tonsillar BCE fractions by elutriation were stimulated by anti- μ for 36 h with or without various concentrations of CsA, and cell sizes of cultured cells were determined by a Coulter Channelyzer. Although cells cultured in medium alone enlarged to some extent, a significant increase in cell size and generation of large cells was observed in cultures stimulated with 15 μ g/ml anti- μ (Table I). When CsA was added to the cultures at a concentration of 10–1,000 ng/ml, substantial reduction of both mean volume of cultured cells and the number of large cells in the culture were observed (Table I). These data demonstrate that CsA interferes with the ability of small B cells to increase in size in response to anti- μ stimulation.

CsA Inhibits RNA Synthesis As Well As DNA Synthesis of Small B Cells Stimulated by Anti- μ With or Without BCGF. In order to study the effect of CsA on RNA synthesis vs. DNA synthesis of B cells activated by anti- μ with or without BCGF, small B cells were stimulated by anti- μ , BCGF, or anti- μ plus BCGF in the absence or presence of 200 ng/ml CsA, and incorporation of [3 H]uridine and [3 H]thymidine was measured at various times after initial incubation (Fig. 3). In the absence of BCGF, incorporation of [3 H]uridine by anti- μ -stimulated B cells began within 8 h, reached maximal response at 16–30 h, and then the response declined. Addition of CsA (200 ng/ml) inhibited these responses by >60%. No incorporation of [3 H]thymidine by anti- μ -stimulated B cells was observed during 72-h culture with or without CsA (Fig. 3A). In the presence of BCGF, incorporation of [3 H]uridine by anti- μ -stimulated B cells also started within 8 h and increased until the termination of the incubation. CsA reduced the response by >50%. Incorporation of [3 H]thymidine by the same B cells started \sim 36–40 h after initial incubation and increased up to the end of the culture. CsA completely suppressed this response (Fig. 3C). Stimulation of small B cells by BCGF with or

TABLE I
Effect of CsA on Cell Enlargement of Resting B Cells Stimulated by Low-Concentration Anti- μ

Culture	Exp. 1		Exp. 2	
	Mean vol*	Large cells [‡]	Mean vol*	Large cells [‡]
	μm^3	%	μm^3	%
Before culture	175	<10	170	<10
After 36 h culture				
Control	224	26	226	28
Anti- μ [§]	315	60	275	48
Anti- μ + CsA (1,000) [‡]	182	10	202	28
Anti- μ + CsA (100)	221	25	201	23
Anti- μ + CsA (10)	236	23	210	28
Anti- μ + CsA (1)	253	45	237	36
Anti- μ + CsA (0.1)	281	54	280	51

* Cell volume of viable cells recovered from the cultures or before culture were determined by Coulter channelizer.

[‡] Percentage of large cells (cells larger than mean volume + 1 SD of cells before culture) were enumerated by a Coulter Channelyzer.

[§] Cultures were pulsed by 0.1% of ethanol alcohol with or without anti- μ (15 $\mu\text{g}/\text{ml}$).

[‡] Various concentrations of CsA (0.1–1,000 ng/ml) were added to the culture from initial incubation.

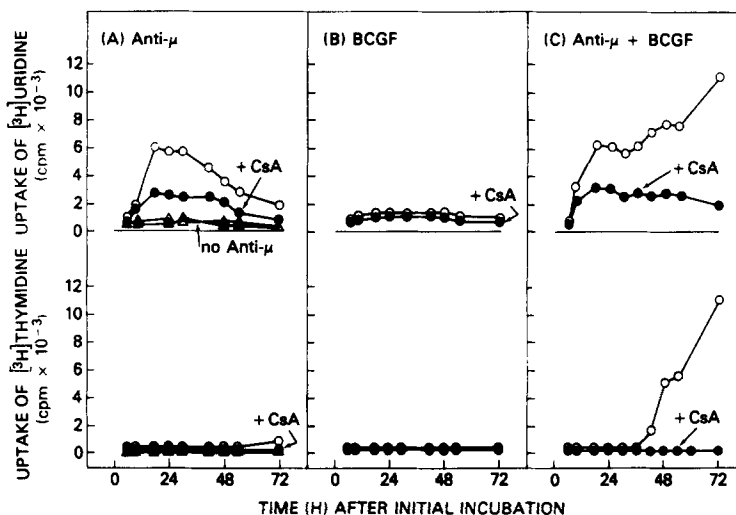


FIGURE 3. Effect of CsA on RNA synthesis and DNA synthesis of small B cells stimulated by anti- μ with or without BCGF. Small B cells separated from tonsillar mononuclear cells were stimulated by anti- μ (15 $\mu\text{g}/\text{ml}$), BCGF (25% vol/vol) or anti- μ plus BCGF in the culture containing 0.02% ethanol (O) or containing 200 ng/ml of CsA (●). As a control, small B cells were also cultured with 0.02% ethanol alone (Δ) or with 200 ng/ml CsA alone (\blacktriangle). Cultures were pulsed with 1 $\mu\text{Ci}/\text{well}$ of [³H]uridine or 1 $\mu\text{Ci}/\text{well}$ of [³H]thymidine for 6 h before harvesting.

without CsA induced no incorporation of [³H]uridine or [³H]thymidine (Fig. 3B). These results demonstrate that (a) although low concentrations of anti- μ can induce RNA synthesis in small B cells, it cannot induce DNA synthesis unless BCGF is present; (b) CsA inhibits RNA synthesis of small B cells in response to

anti- μ in the presence or absence of BCGF; and (c) although BCGF by itself does not directly stimulate B cells to initiate RNA synthesis or DNA synthesis, it does synergize with anti- μ and stimulates B cells to initiate DNA synthesis. The critical time for the effect of BCGF may be 24–36 h after anti- μ stimulation. These data suggest that CsA inhibits DNA synthesis of small B cells in response to anti- μ plus BCGF because the drug inhibits RNA synthesis by which small B cells can be induced to acquire the responsiveness to BCGF.

Differential Sensitivity of Resting B Cells and Activated B Cells to CsA. In order to examine whether CsA can inhibit BCGF-induced B cell proliferation, large, activated B cells (mean size of $280 \mu\text{m}^3$) that had been incubated with low concentrations of anti- μ for 30 h were stimulated with BCGF for an additional 42 h with or without CsA. Addition of CsA at a concentration of 5–500 ng/ml did not significantly suppress the proliferative response of activated B cells to BCGF, although this range of concentrations of CsA completely suppressed the B cell proliferative response of resting B cells to anti- μ and BCGF (Fig. 4). These data indicate that once resting B cells were activated by triggering signals such as anti- μ , CsA did not interfere with the ability of these activated B cells to respond to proliferating signals such as BCGF.

CsA Does Not Interfere With the Ability of Proliferating Cells to Respond to BCDF. It has been shown that SAC causes B cell proliferation in a T cell-independent fashion, and T cells or T cell-derived factors such as BCDF stimulate SAC-activated proliferating B cells to differentiate into ISC (11). In order to study the effect of CsA on BCDF-induced cell differentiation, activated B cells which had been precultured with SAC for 3 d were cultured with BCDF in the presence or absence of CsA, and PFC were enumerated after an additional 3-d culture

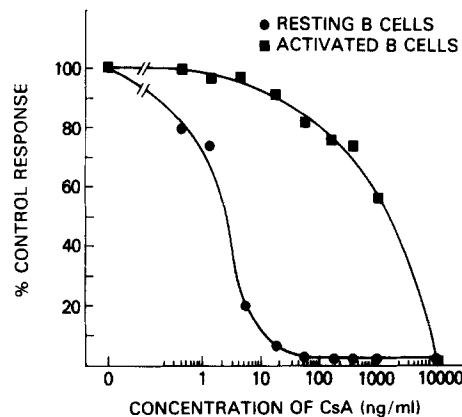


FIGURE 4. Differential sensitivity of resting B cells and activated B cells to CsA. Activated B cells, whose mean size was $280 \mu\text{m}^3$, were prepared by incubation of small B cells with anti- μ for 30 h. These activated B cells were recultured with BCGF for an additional 42 h. 0.1% ethanol or various concentrations of CsA were added to the second culture. Resting B cells, whose mean size was $170 \mu\text{m}^3$, were cultured with anti- μ (15 $\mu\text{g}/\text{ml}$) plus BCGF (25% vol/vol) for 72 h. 0.1% of ethanol or various concentrations of CsA were added to the cultures. All cultures were pulsed by 1 $\mu\text{Ci}/\text{well}$ of [^3H]thymidine over the last 6 h of incubation. Control response of activated B cells to BCGF was $8,320 \pm 560$ cpm; background response was 630 ± 110 cpm. Control response of resting B cells to anti- μ plus BCGF was $12,020 \pm 450$ cpm; background response to anti- μ alone was 820 ± 50 ; background response to BCGF alone was 820 ± 210 .

(Table II). BCDF induced significant numbers of PFC, and the addition of CsA (1–1,000 ng/ml) into the cultures showed little or no inhibitory effect on the induction of PFC. These data show that CsA does not interfere with the ability of activated B cells to differentiate into ISC in response to T cell-derived differentiation factors.

Discussion

The present study has demonstrated that CsA has selective suppressive effects on the activation vs. proliferation and differentiation phases of the B cell cycle. It has been clearly shown that CsA interferes with the ability of the small resting B cells to respond to an initial activation signal delivered via its sIg by anti- μ . In contrast, CsA does not interfere with the ability of a B cell that has been activated by anti- μ stimulation to progress to DNA synthesis in response to a proliferative signal delivered by BCGF. Furthermore, it has been demonstrated that once B cells are activated and are proliferating, they can then respond to BCDF by differentiating into ISC. This response of activated B cells to differentiation factors is resistant to the suppressive effects of CsA.

Previous studies on the immunosuppressive effects of CsA indicated that the drug acted selectively on T cells with little if any direct effect on B cell function. It was shown that CsA inhibited T cell proliferative responses and the induction of cytotoxic cells in mixed lymphocyte reactions (19–24). Studies on the binding of CsA to lymphocyte populations also suggested that a receptor for CsA analogues in both mouse and man was present in greater quantities on T cells than on B cells (34, 35). Paavonnen et al. (25, 26) first claimed that CsA interfered with the ability of both human T and B cells to generate Ig-secreting cells. Recently, selective effects of CsA on functional B cell subsets have been reported in the mouse (27, 28). It has also been well known that CsA favors proliferation of Epstein-Barr virus-positive lymphoblastoid human B cells (36, 37). Although recent studies in man (38, 39) suggest that CsA modulates T cell-independent B cell responses to SAC and/or anti- μ antibody, the role of CsA on human B cell

TABLE II
Effect of CsA on BCDF-Induced B Cell Differentiation

Culture with:*	PFC/well [‡]	
	No BCDF	With BCDF
Nil	0 (0)	120 (12)
CsA 1 ng/ml	0 (0)	108 (14)
10	0.6 (0.6)	112 (5.2)
100	3.4 (2.4)	99.4 (9.4)
1,000	2.6 (1.4)	100 (4.2)
10,000	0 (0)	0 (0)

* Small tonsillar B cells were precultured with SAC for 3 d, harvested, washed, and recultured (3×10^4 /well) in the presence or absence of BCDF (10% vol/vol) with various concentrations of CsA (1 ng/ml to 10 μ g/ml).

[‡] PFC were enumerated after 3 d incubation. The data represent the mean number (SEM) of PFC/well of triplicate cultures.

function has not been clearly determined. In the present study, we examined the direct effects of CsA on purified human B cells and demonstrated that CsA inhibited human B cell proliferative responses to either SAC or costimulation with anti- μ and BCGF. CsA suppressed the response of B cells to costimulation with anti- μ plus BCGF at 50- to 100-fold lower concentrations than are required for the suppression of SAC-induced B cell proliferation (Fig. 1). This finding is similar to that of Dongworth and Klaus (28), in the murine system. In that study, the authors demonstrated that CsA suppressed the proliferative responses of murine B cells to anti- μ antibody at 300- to 400-fold lower concentrations than are required to inhibit B cell proliferation induced by lipopolysaccharide. One possible interpretation drawn from these data is that there are two distinct B cell subsets: one responds to an anti- μ signal and is more sensitive to CsA, and the other responds to SAC in man or LPS in mouse and is more resistant to CsA. However, this does not rule out the possibility that anti- μ and SAC (in man) or lipopolysaccharide (in mouse) stimulate the same B cells to proliferate via two biochemically distinct triggering mechanisms, one of which is CsA sensitive and the other CsA resistant.

A point of particular interest that emerged from the present study is the observation that B cells were differentially sensitive to CsA on the basis of sequential activation steps induced by anti- μ and BCGF: the early phase of B cell activation triggered by anti- μ stimulation was sensitive to CsA, while the late phase in B cell activation, namely, the proliferation triggered by BCGF, was resistant to the drug. In the present study, it has been shown that (a) CsA inhibited human B cell proliferative responses to costimulation with anti- μ plus BCGF, (b) the inhibition by CsA was found only when CsA was added to the culture within 24 h from initial incubation, (c) CsA inhibited cell enlargement of small B cells stimulated by anti- μ , and (d) CsA inhibited the increase of RNA synthesis induced by anti- μ . These data substantiate the concept that CsA exerts its suppressive effects on B cell proliferation by selectively inhibiting a well-defined phase of the B cell cycle in that it interferes with the ability of small B cells to become activated, whereas B cells preactivated by anti- μ stimulation were resistant to the drug in the initiation of DNA synthesis induced by BCGF (Fig. 4).

We have presumed that stimulation of human B cells by SAC or anti-Ig induces BCGF receptors on the cell membrane, and BCGF delivers a signal for proliferation to these activated, BCGF-responsive B cells (8, 9, 12). Thus, it is possible that CsA inhibits the induction of functionally active BCGF receptors although direct evidence must await binding assays employing radiolabeled BCGF. In this regard, analogous studies employing CsA have been performed delineating the various phases of the cell cycle in mouse and human T cells after binding of ligands such as antigens or lectins to the T cell membrane (23, 24). These reports suggest that lectins or alloantigens can activate resting T cells to express functional receptors for T cell growth factor (TCGF), and that CsA interferes with the ability of resting T cells to become activated, while preactivated T cells or TCGF-dependent T cells are resistant to CsA in the induction of proliferation triggered by TCGF.

Another point that emerged from the present study is that although low doses

of anti- μ stimulate small B cells to initiate RNA synthesis, they cannot induce DNA synthesis unless BCGF is added. Kinetic studies have revealed that RNA synthesis by anti- μ -stimulated B cells in the absence of BCGF started within 8 h, reached maximal response by 24 h, and the response declined at 36 h or later. On the other hand, in the presence of BCGF, these anti- μ -activated B cells initiated additional RNA synthesis at 24–36 h that was followed by the initiation of DNA synthesis (Fig. 3). This finding is relevant in light of our previous observations that human resting B cells showed sequential requirements for cell cycle progression following activation by anti-Ig (9). We have shown that small resting B cells increased in cell size within 36 h of anti- μ stimulation without incorporation of [3 H]thymidine, and these anti- μ -activated B cells then acquired responsiveness to the proliferative signal delivered by BCGF (9). Thus, it seems likely that anti- μ stimulates small resting (G_0 phase) B cells to initiate RNA synthesis concomitant with cell enlargement within 24 to 36 h, thereby driving these cells to a G_{1A} phase. BCGF may push these activated G_{1A} phase B cells through G_{1B} phase into S phase. These findings substantiate the observations of DeFranco et al. (4) in the murine system who demonstrated that all murine B cells were stimulated by low dose anti- μ to become larger (i.e., to proceed from G_0 phase to G_1 phase of the cell cycle) and that a subpopulation of these cells entered S phase in response to high concentrations of anti- μ or lipopolysaccharide.

Finally, the present study demonstrated that differentiative responses of activated human B cells to BCDF were not inhibited by CsA. We showed that SAC-activated B cells could differentiate normally into ISC in response to BCDF in the presence of CsA (Table II). These findings are compatible with those of Paavonen et al. (26). These authors showed that CsA suppressed pokeweed mitogen- or SAC-induced PFC response of human peripheral blood mononuclear cells when CsA was present throughout the 6-d culture. However, CsA had no effect on PFC responses when added to the culture at day 4 or later. These findings suggest that CsA preferentially inhibited blastogenesis of human B cells with minimal effect on the differentiative phase.

Thus, the present study has employed a pharmacologic approach to dissect the distinct stages of human B cell proliferation and has confirmed our model of the clear-cut dichotomy between the activation and proliferation phases of the B cell cycle. Furthermore, since CsA is being used with greatly increasing frequency as an immunosuppressive agent in man, particularly in the prevention and treatment of transplanted allografts, it is critical to appreciate the full scope of the immunoregulatory potential of this important therapeutic agent.

Summary

The effect of cyclosporin A (CsA), a fungal metabolite with immunosuppressive properties, on the induction of human B cell proliferation and differentiation, has been described. CsA had a selective inhibitory effect on the activation phase of the cell cycle vs. the proliferation phase following preactivation of the cells. Cell enlargement and RNA synthesis of small resting B cells triggered by anti- μ were inhibited by addition of CsA (5–500 ng/ml). The inhibitory effect of CsA was found only when the drug was added within 24 h of initiation of culture. In

marked contrast, once small B cells were activated by anti- μ , the resulting large, activated B cells could be induced to initiate DNA synthesis by incubation with B cell growth factor (BCGF), and addition of CsA (1–1,000 ng/ml) to the culture did not suppress this BCGF-induced B cell proliferation. Addition of CsA to cultures of B cells which had been preactivated with *Staphylococcus aureus* Cowan strain I (SAC) and were already proliferating did not suppress B cell differentiation factor (BCDF)-induced differentiation of these cells.

Thus, these data indicate that CsA can be used as a pharmacologic tool to dissect out human B cell responses into two distinct steps: (a) the initial activation step induced by anti-Ig, which is characterized by cell enlargement, RNA synthesis, and expression of receptors for BCGF; and (b) the proliferative step induced by BCGF in these preactivated B cells that undergo DNA synthesis and can then go on to differentiate in the presence of BCDF. In this regard, CsA selectively suppresses an early step of human B cell activation and has little inhibitory effect on the subsequent factor-dependent proliferation and differentiation.

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