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Immunomodulatory Effect of Fosfomycin on Human B-Lymphocyte Function

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Fosfomycin (FOM) is an unique antibiotic which is chemically unrelated to any other known antimicrobial agent. Recent investigations have demonstrated that FOM inhibits histamine release from basophils. In this study, we examined the effect of FOM on human B-cell functions. FOM inhibited the proliferative response of resting B cells induced by *Staphylococcus aureus* Cowan 1 in a dose-dependent manner. FOM interfered with the transition from the G0 to the G1 phase of the cell cycle, leading to cell arrest. The proliferative response of in vivo-activated B cells and lymphokine-induced B-cell proliferation were also affected by FOM. In addition, FOM suppressed immunoglobulin secretion by antibody-producing B cells. Interestingly, FOM did not affect the expression of activation antigens such as the CD25 (interleukin-2 receptor) and CD71 (transferrin receptor) antigens. Moreover, FOM sustained the increased Ia expression on B-cell membranes induced by *S. aureus* Cowan 1 stimulation, which suggests that FOM may not block the role of B cells in antigen presentation in T-cell–B-cell interaction.

A number of investigations undertaken to elucidate the immunological mechanisms involved in the pathogenesis of infectious diseases suggest that a significant role is played by not only the pathogens themselves but also the immune response to them. This occurs as a result of the formation and deposition of a microbe's antigen-antibody immune complex, the lytic activity of cytotoxic lymphocytes, and the generation of cytokines. Although these effector mechanisms are required for protection and function in most infections, as part of the host's defenses, they may also be responsible for the pathogenic consequences of these infections. An exaggerated reaction to or escape from normal control mechanisms can lead to the development of autoimmune diseases. Thus, besides protecting the host against microbial infections and playing a cardinal role in clearing infectious agents, the immune response can also lead to the injury of host cells and tissues during acute and persistent infections. On the basis of these facts, it is necessary to evaluate antibiotics not only for their degrees of bactericidal activity but also for their effects on host immunity. Fosfomycin (FOM), L-*cis*-1,2-epoxypropylphosphoric acid (molecular weight, 138), is an unique antibiotic which is chemically unrelated to any other known antimicrobial agent. FOM is bactericidal, because it selectively inactivates the bacterial enzyme *N*-acetylglucosamine-3-*O*-enolpyruvyl transferase, which is essential for the synthesis of bacterial wall peptidoglycans (3). FOM is also actively transported and accumulates in human polymorphonuclear cells, while most beta-lactams seem to have difficulty penetrating the cell membrane (3). FOM can increase cyclic AMP levels in rat peritoneal mast cells (13). Recent investigations have demonstrated that FOM inhibits immunoglobulin E (IgE)-mediated histamine release from peripheral blood basophils (5) and pulmonary histamine release from lungs isolated from guinea pigs (12). In clinics, FOM is used in combination with steroids to reduce the dose of steroids for patients with severe bronchial asthma (9). However, it is unknown whether FOM may compensate for the immunoregulatory action of steroids. Although T lymphocytes are considered to be of critical importance in microbial infections, B lymphocytes also play a cardinal role in preventing microbial infections and in altering the course of infection once it has begun. In this study, we examined the effect of FOM on the function of human B lymphocytes.

MATERIALS AND METHODS

Cell preparation. Human tonsils were obtained by tonsillectomies from juvenile patients with chronic tonsillitis and were dispersed into single-cell suspensions. Mononuclear cells were isolated on Ficoll-Hypaque gradients. Monocytes and natural killer cells were depleted by incubation with 5 mM L-leucine methyl ester (Sigma, St. Louis, Mo.) in serum-free medium (7). T cells were removed by rosetting twice with 2-aminoethylisothiouronium bromide (Sigma)-treated sheep erythrocytes. Nonrosetted (E^-) cells were further purified by isolating small, dense B cells from the 60%/70% interface of a discontinuous Percoll density gradient (Pharmacia, Uppsala, Sweden). The isolated cell population contained 90 to 95% surface Ig^+ and $CD3^+$ cells; $CD13^+$ and $CD16^+$ cells were less than 1%, as determined by flow cytometric analysis.

Reagents. FOM sodium and ampicillin sodium were supplied by Meiji Seika, Ltd., Tokyo, Japan. FK506 was provided by Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan. These drugs were dissolved in ethanol and further diluted in medium before being used. *Staphylococcus aureus* Cowan 1 (SAC), a polyclonal B-cell mitogen, was purchased from Calbiochem-Behring, La Jolla, Calif. Human recombinant interleukin-2 (rhIL-2) and low-molecular-weight B-cell growth factor were obtained from Shionogi Pharmaceutical Co., Ltd. (Osaka, Japan) and Cellular Products (Buffalo, N.Y.), respectively. Mixed-lymphocyte culture supernatants (MLR-SN) were prepared as described previously (7). The monoclonal antibodies, fluorescein isothiocyanate (FITC)-conjugated anti-transferrin receptor antibody (LO1.1; anti-CD71), FITC-conjugated anti-IL-2 antibody (IL-2R1; anti-CD25), and anti-Ia antibody (OKIa1) were purchased from Becton Dickinson (Mountain View, Calif.),

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Coulter Immunology (Hialeah, Fla.), and Ortho Diagnostic Systems (Raritan, N.J.), respectively.

Measurement of lymphocyte proliferation. Triplicate lymphocyte cultures were incubated in 96-well, flat-bottom microplates. The culture medium consisted of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories, McLean, Va.), streptomycin (100 μg/ml), penicillin (100 U/ml), and 2-mercaptoethanol (0.005 mM). One hundred thousand B cells were cultured in 200 μl of medium in the presence or absence of stimulators for 3 days at 37°C in 5% CO_2. The cells were pulsed with 1 μCi of [^{3}H]thymidine per well during the last 18 h of culture, unless otherwise stated, and were harvested with a multiple-cell harvester. To examine the RNA synthesis, cells were cultured under the same conditions, except that they were pulsed with 1 μCi of [^{3}H]uridine per well for the last 18 h.

Cell viability. The viability of B cells cultured in microwells in the presence or absence of FOM was determined by the trypan blue dye-exclusion method.

Detection of Ig production. High-density B cells were stimulated with SAC (1:10^5, vol/vol) for 2 days. Then, viable cells were collected by Ficoll-Hypaque density gradient and cultured at a ratio of 2 × 10^5 cells in 200 μl of medium in RPMI 1640 containing 10% fetal calf serum in the presence or absence of IL-2 (100 U/ml) or MLR-SN (25%, vol/vol) preparations for 7 days. The amounts of IgM and IgG secreted in the culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (7).

Cell cycle analysis with propidium iodide. Total DNA content was determined by flow cytometry as described elsewhere (8). Briefly, B cells were washed twice with phosphate-buffered saline (PBS) and resuspended in 70% ethanol. After being mixed vigorously, the cells were dispersed, centrifuged, and washed with PBS, and then they were resuspended in RNase A and incubated for 1 h at 37°C. The cells were then centrifuged and resuspended in PBS containing 50 μg of propidium iodide per ml. Fluorescence analyses were performed 20 min later with a FACScan (Becton Dickinson). The percentages of cells in the G1-G0, S, and G2-M compartments were obtained by using the program Ver C Polynomical Model (FACScan).

Immunofluorescence. To detect cell surface activation antigens, the cells were examined by direct immunofluorescence with FITC-conjugated monoclonal antibodies and by indirect immunofluorescence with FITC-labeled F(ab)$_2$ fragment of goat anti-mouse Ig reagent and purified monoclonal antibody. Cell surface immunofluorescence was analyzed with a FACScan (Becton Dickinson). The cell size was measured by forward light angle scatter with the FACScan.

RESULTS

Effect of FOM on resting B lymphocytes stimulated by SAC. The effect of FOM on the proliferative response of resting B cells to stimulation with SAC was examined. A known immunosuppressant, FK506, and an antibiotic, ampicillin sodium, were used as the positive and negative controls, respectively. High-density B cells were cultured in microtiter plates for 3 days with SAC (1:10^5, vol/vol). At concentrations of 60 μg/ml to 7.5 mg/ml, FOM suppressed DNA synthesis of SAC-stimulated B cells in a dose-dependent manner; the effectivity of FK506 was more than fivefold that of FOM, but ampicillin sodium had no inhibitory effect (Fig. 1). When B cells were cultured in the presence of different concentrations of SAC, with or without FOM at the final concentration of 1.5 mg/ml, FOM inhibited the proliferative responses of B cells regardless of the concentrations of SAC added to the cultures (Fig. 2). We confirmed that FOM had no cytotoxic effect on human B cells at any concentration employed by the trypan blue dye-exclusion test when the B cells were cultured for 3 days in the presence of FOM.

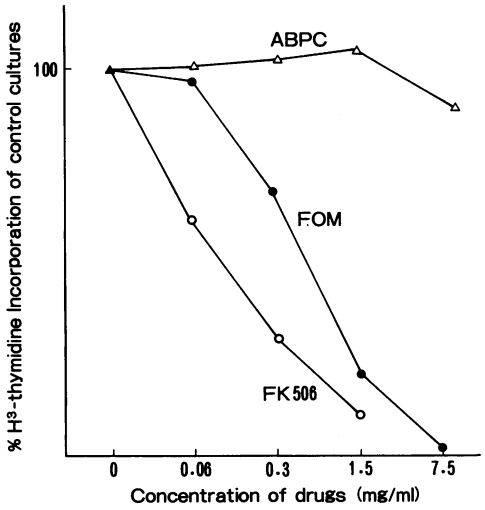

FIG. 1. High-density B cells from tonsillar samples were cultured for 3 days with SAC (1:10^5, vol/vol) in the presence of various concentrations of FOM (●), FK506 (○), or ampicillin sodium (△). The cells were pulsed with [^{3}H]thymidine during the last 18 h of culture.

Kinetic studies on DNA and RNA syntheses by B lymphocytes. DNA and RNA syntheses by B cells were examined by pulsing B cells with [^{3}H]thymidine or [^{3}H]uridine for the last 18 h of each day during 72 h of culture (Fig. 3). The cells were collected on days 1, 2 and 3, and radioincorporation was measured. Twenty-four hours (day 1) after the start of the cultures, DNA and RNA syntheses by B cells cultured in

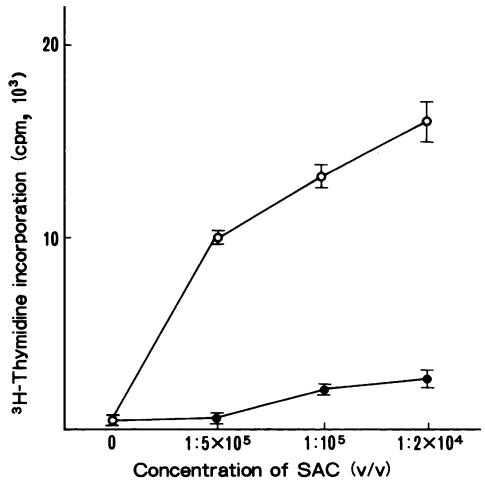

FIG. 2. Tonsillar B cells were cultured for 3 days with different concentrations of SAC alone (○) or in combination with FOM (1.5 mg/ml) (●). The B-cell response was measured by the incorporation of [^{3}H]thymidine over the last 18 h of culture. The values represent the means ± standard errors of the means (represented by bars) of triplicate cultures.

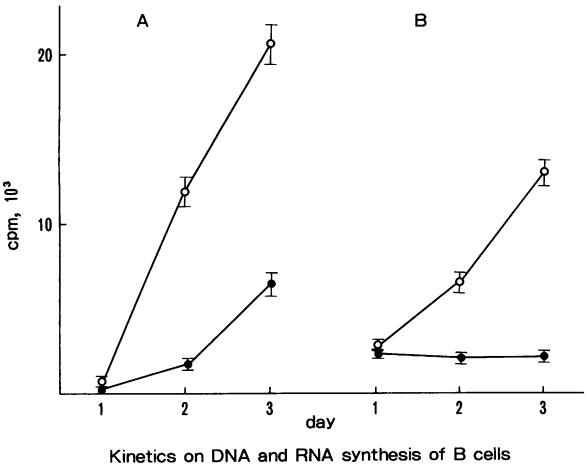

FIG. 3. Kinetics of the effect of FOM on DNA and RNA syntheses by B cells. The cells were cultured in the presence of SAC (1:10^5, vol/vol) with or without FOM (1.5 mg/ml). Pulsing of [^{3}H]thymidine in DNA synthesis or [^{3}H]uridine in RNA synthesis was carried out daily for 18 h. Cells were collected on days 1, 2, and 3, and radioincorporation was measured. Incorporation of [^{3}H]thymidine (A) and [^{3}H]uridine (B) is shown. ○, SAC; ●, SAC plus FOM. Bars indicate standard errors.

the presence of FOM were similar to those by nontreated B cells. The incorporation of [^{3}H]uridine and [^{3}H]thymidine was markedly accelerated in SAC-stimulated B cells on days 2 and 3, but it did not change in FOM-treated B cells on day 2; RNA and DNA syntheses were suppressed in FOM-treated B cells on day 3.

Effect of preincubation with FOM. We examined whether pretreatment with FOM would have a similar effect on the proliferative response of B cells to SAC stimulation. We incubated high-density B cells for 48 h in the presence or absence of FOM (1.5 mg/ml). These B cells were then washed twice with medium and stimulated with SAC at a cell density of 10^5 cells per ml for 3 days. Though preincubation of resting B cells with FOM for 48 h had no cytotoxic effect, it resulted in a response to subsequent stimulation with SAC remarkably lower than that seen in cultures preincubated with medium alone (Table 1). These results suggested that the inhibitory effect of FOM is irreversible.

DNA content of the B cells. When B cells were cultured for 72 h, 30 or 25% of these cells entered into the S phase in SAC-stimulated cultures, while 7 or 6% entered into this phase in nonstimulated cultures. In the presence of FOM, 25 or 14% of the B cells reached the S phase, confirming that the drug inhibits DNA synthesis by B cells. The results of one of two experiments are shown in Table 2. The inhibitory level determined by the number of DNA-stained cells suggested a reduction in the number of cultured cells entering the S phase in the presence of FOM.

TABLE 1. Effect of pretreatment of B cells with FOM[a]

Type of pretreatment	Culture	[^{3}H]thymidine incorporation (cpm)
Medium alone	Medium	512 ± 21
	SAC	1,1050 ± 636
	FOM	596 ± 50
	SAC + FOM	3,712 ± 528
Medium + FOM	Medium	373 ± 29
	SAC	2,400 ± 174
	FOM	463 ± 242
	SAC + FOM	1,724 ± 518

[a] High-density B cells were treated with or without FOM (1.5 mg/ml) for 48 h and then washed thoroughly before being stimulated for another 3 days with SAC (1:10^5, vol/vol) or FOM (1.5 mg/ml). Eighteen hours before being harvested, the cultures were pulsed with 1 μCi of thymidine per well. The values represent the means ± standard errors of triplicate cultures.

TABLE 2. DNA content of B cells after stimulation with SAC in the presence or absence of FOM[a]

Phase of cell cycle	% Cells after the following type of stimulation		
	None	SAC	SAC + FOM
G1 (G0)	93	72	84
S	6	25	14
G2-M	1	3	2

[a] High-density B cells were cultured in the presence or absence of FOM (1.5 mg/ml) for 72 h prior to cell cycle analysis. Cells were washed and stained with propidium iodide, and their DNA contents were analyzed with a FACScan. Data are expressed as the percentages of cells in each phase of the cell cycle, which was calculated with the program Ver C Polynomical Model.

Effects of FOM on the expression of activation antigens, Ia antigen, and cell size. B cells were stimulated with SAC in the presence or absence of FOM. After 72 h of culture, the cells were stained with FITC-labeled anti-CD25, anti-CD71, and anti-Ia monoclonal antibodies, and then they were analyzed by flow cytometry. SAC stimulation induced a remarkable increase in the expression of CD25 and CD71, and FOM had no effect on SAC-induced expression of these antigens (Fig. 4B). Although resting B cells expressed Ia on their cell surfaces, SAC activation increased the membrane Ia expression on these cells. In contrast, in Ia density increases during the transition of cells from the G0 to the G1 phase of the cell cycle, there is a decrease in membrane Ia expression through the S, G2, and M phases (6). The addition of FOM to SAC-stimulated B cells sustained the increased Ia expression (Fig. 4B), which indicates that FOM exerts its effect after the increase in Ia expression induced by SAC stimulation during the transition from the G0 to the G1 phase takes place. In the same experiment, sizes of B cells were measured by forward light angle scatter with nonstimulated, SAC-treated, and SAC-plus-FOM-treated cells. FOM decreased the cell enlargement induced by SAC stimulation (Fig. 4A).

Effect of FOM on B cells of different buoyant densities. High-density B-cell subpopulations from the 60%/70% interface, designated resting B cells (10), were stimulated to a greater degree by SAC than were intermediate- or low-density fractions from the 50%/60% or the 40%/50% interface, respectively. The low-density B cells (40%/50% interface), which are regarded as in vivo-activated B cells (6), showed higher spontaneous [^{3}H]thymidine incorporation and a worse response to SAC stimulation than the higher-density B cells (Fig. 5). However, FOM consistently inhibited SAC-stimulated B-cell cultures regardless of the B-cell activation state.

Influence of FOM on in vivo-activated B cells. High-density B cells were stimulated with SAC for 3 days, and then they were thoroughly washed and cultured in the presence or absence of FOM for another 3 days. Although B cells prestimulated with SAC showed significant levels of spontaneous DNA synthesis without lymphokines, the presence of rhIL-2 (100 U/ml) or low-molecular-weight B-cell growth

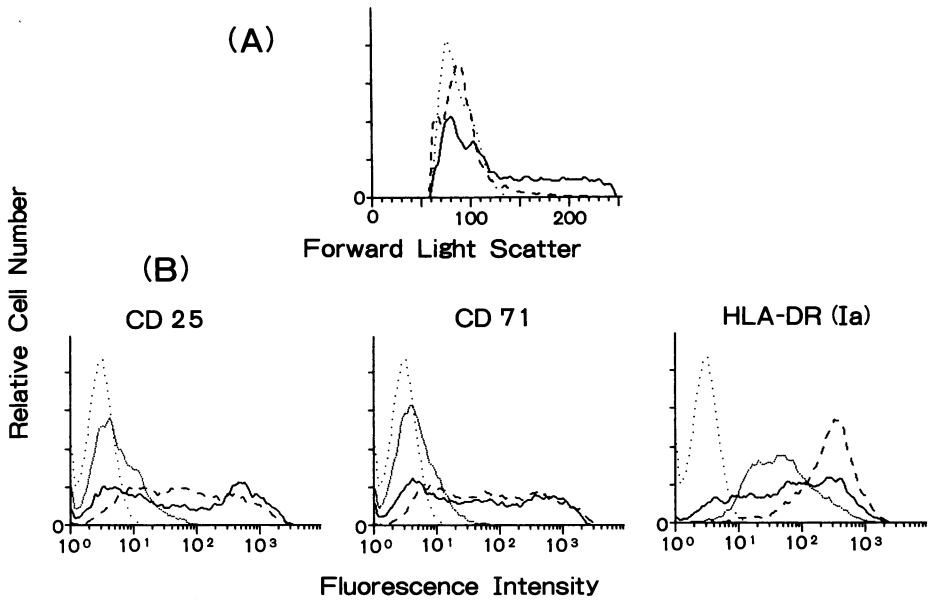

FIG. 4. Flow cytometric analysis of the effect of FOM on the expression of activation antigens (B) and cell size (A) of B cells stimulated with SAC. The percentages of $CD25^+$, $CD71^+$, and Ia^+ cells in each group were as follows: 23.9, 15.3, and 92.3% in nonstimulated B cells (·····); 68.5, 65.8, and 80.7% in SAC-stimulated B cells (———); and 81.4, 78.8, and 98.5% in B cells cultured with SAC plus FOM (— —). The cell size was measured by forward light angle scatter. Three different experiments were carried out, and representative results are presented.

factor (10%, vol/vol) increased DNA synthesis in these preactivated B cells. The addition of FOM markedly inhibited DNA synthesis of B cells preactivated with SAC in the presence and absence of growth factor (Fig. 6).

Effect of FOM on Ig generation by B lymphocytes. High-density B cells were first activated with SAC for 3 days. When B cells preactivated with SAC were cultured further for 7 days in the presence of rhIL-2 (100 U/ml) or MLR-SN (25%, vol/vol), these lymphokines induced Ig production in B cells. The addition of FOM to the cultures suppressed Ig secretion by B cells in a dose-dependent fashion at concentrations of 60 μg/ml to 7.5 mg/ml (Table 3). These results suggested that FOM had an inhibitory effect on the differentiation of B cells into antibody-forming cells.

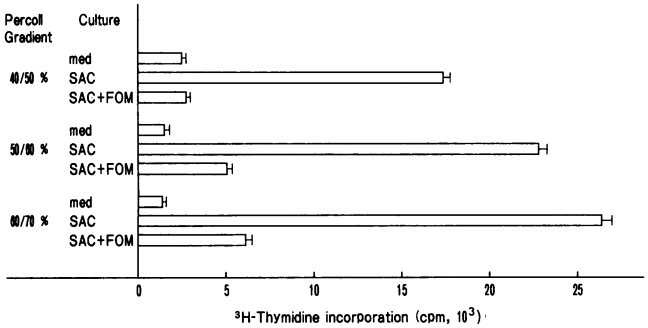

FIG. 5. Effect of FOM on the proliferation of B cells of different densities. Tonsillar B cells were fractionated by Percoll buoyant density gradient. Each B-cell fraction was cultured for 3 days with SAC in the presence or absence of FOM (1.5 mg/ml). med, medium. Bars indicate standard errors.

DISCUSSION

The maturation process of resting B cells into Ig-secreting cells involves three distinct stages: activation, proliferation, and differentiation. Resting B cells are activated by polyclonal B-cell mitogens so that they enter the S phase of the cell cycle from the G0 phase and proliferate. The early G1 phase is characterized by RNA synthesis, cell enlargement,

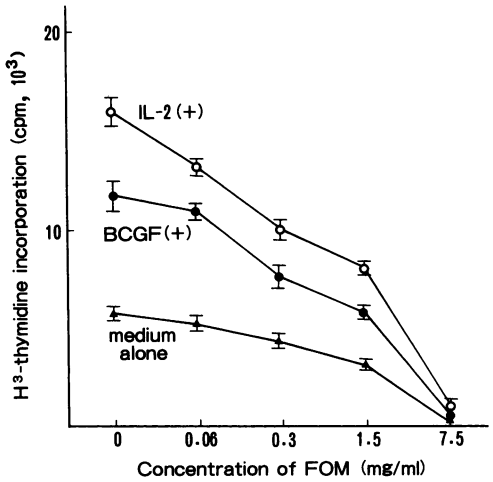

FIG. 6. Effect of FOM on in vitro-activated B cells. High-density B cells were prestimulated with SAC for 3 days and then washed and cultured for another 3 days in the absence (▲) or presence of IL-2 (○) (100 U/ml) or B-cell growth factor (●) (10%, vol/vol). Different concentrations of FOM were added at the initiation of the second culture. During the last 18 h of culture, [^{3}H]thymidine was added. Bars indicate standard errors.

TABLE 3. Effect of FOM on Ig generation by activated B lymphocytes[a]

Additive and concn of FOM (mg/ml)	Ig generation (ng/ml)	
	IgM	IgG
MLR-SN		
0	3,052	2,121
0.06	2,734	1,900
0.3	1,075	789
1.5	693	537
7.5	219	160
rhIL-2		
0	1,732	1,310
0.06	1,304	1,236
0.3	941	883
1.5	340	130
7.5	20	20

[a] Highly purified B lymphocytes were prestimulated with SAC (1:10^5, vol/vol) for 2 days and then washed thoroughly and cultured in the presence of MLR-SN (25%, vol/vol) or rhIL-2 (100 U/ml) for 7 days. Various concentrations of FOM were added to the second cultures from the beginning. Ig generation was determined by ELISA. Representative data from three different experiments are the means of triplicate cultures.

and increased CD25 (IL-2 receptor) expression, whereas in the late G1 phase, CD71 (transferrin receptor) increases and there is a second increase in RNA synthesis (10). SAC, a polyclonal B-cell mitogen, cross-links with the surface membrane Ig and results in cellular activation, as shown by increased cellular volume, enhanced RNA synthesis, and increased DNA content (10). Furthermore, B cells preactivated with SAC can produce Ig when subsequently cultured in the presence of the T-cell factor (7). They receive the signal to differentiate during the G1 phase, before they enter the S phase (11). By using these systems, the present study was undertaken to examine at which stage, activation, proliferation, or differentiation, FOM exhibits its effect on human B lymphocytes. Here, we showed that FOM regulates human B-cell functions. FOM inhibited the proliferative response of resting B cells stimulated by the polyclonal B-cell mitogen SAC in a dose-dependent manner (Fig. 1), regardless of the concentration of SAC (Fig. 2). From the data on DNA content, cell size, and RNA synthesis in FOM-treated B cells, it is suggested that FOM acts at the time at which cells go from the G0 to the G1 phase (Table 2; Fig. 3 and 4). Furthermore, B cells progressing from the G1 to the S phase are also sensitive to the inhibitory effect of FOM, because the DNA synthesis of in vivo- as well as in vitro-activated B cells was suppressed in the presence of FOM (Fig. 5 and 6). Pretreatment of B cells with FOM also inhibited subsequent activation by SAC (Table 1). These data indicate that FOM suppresses the proliferative response regardless of the state of B-cell activation. Furthermore, FOM inhibited Ig generation by activated B lymphocytes in a dose-dependent manner (Table 3). FOM did not affect the expression of activation antigens such as CD25 and CD71 on human B lymphocytes. It has been reported previously that the sequential induction of CD25 and CD71 on the same cells is essential for DNA synthesis in T cells (11). The expression of CD25 and CD71 on B cells correlates with the stage of activation of B lymphocytes (4), but the functional significance of these activation antigens on human B cells remains unknown. We have previously found that FK506, a new immunosuppressive drug, does not interfere with CD25 or CD71 expression on human B cells, although it has a strong inhibitory effect on their proliferative response (8). It is interesting that FOM sustained the enhanced levels of Ia expression in B cells activated with SAC. It has been suggested previously that the increased expression of Ia might play an important role in directing Ia-restricted, antigen-specific T-cell help for thymus-dependent, antigen-driven B-cell activation (6). Our results suggest that FOM may not interfere with the role of B lymphocytes regarding antigen presentation in T-cell–B-cell interaction (1). It is unknown whether the mechanism of immunoregulatory action of FOM is related to the known action of FOM as an antibiotic. Further studies are necessary to clarify this issue. The results obtained in this study may reflect one aspect of the steroid-saving effect of FOM in asthma patients. Taken together, these data demonstrate that FOM inhibits the proliferative response and differentiation capacity of human B cells but does not inhibit their Ia or activation antigen expression. This unique effect of FOM on human B lymphocytes would not be totally disadvantageous for the host, considering the evidence that polyclonal B-cell activation against microbial agents participates in the pathophysiological events that follow chronic inflammation and lead to the development of autoimmune diseases (2). In our present experiment, rather high concentrations of FOM were employed. Such high concentrations may not be attainable at the local site of inflammation when the drug is administered intravenously. Nevertheless, the results of the present study may support the view that some kinds of antibiotics can get involved in immune reactions in vitro. They also suggest that this drug can be used advantageously in the treatment of certain bacterial infections in patients with accompanying autoimmune and chronic inflammatory pathologies.

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