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Monocyte Suppression of *Fusobacterium nucleatum*-Induced Human Polyclonal B-Lymphocyte Activation

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Previous studies reported that *Fusobacterium nucleatum* induced polyclonal B-lymphocyte activation (PBA) as determined by immunoglobulin M production in cultures of human peripheral blood mononuclear cells. However, the PBA response was greatly enhanced when the cells were depleted of esterase-positive, adherent cells (i.e., monocytes). The purpose of this study was to confirm and further examine the suppression of *F. nucleatum*-induced PBA (*F. nucleatum*-PBA) by blood monocytes. For comparison, PBA induced by pokeweed mitogen (PWM-PBA), which is enhanced by monocytes, was assessed in some experiments. We found the removal of monocytes from unfractionated cells by (i) Sephadex G-10, (ii) anti-monocyte specific OM-1 monoclonal antibody plus complement, or (iii) L-leucine methyl ester, a compound which selectively kills lysosome-rich cells, resulted in a population of cells responsive to *F. nucleatum*-PBA and unresponsive to PWM-PBA. The addition of double adherence-purified monocytes (>85% esterase-positive cells), particularly in concentrations of >10%, to lymphocytes depleted of monocytes by G-10, OM-1, or L-leucine methyl ester treatments, suppressed *F. nucleatum*-PBA and enhanced PWM-PBA. Monocytes also suppressed a mixture of isolated T and B cells combined in a T/B cell ratio of 3:1, which is an optimal ratio for *F. nucleatum*-PBA. Allogeneic monocytes suppressed *F. nucleatum*-PBA, although at low numbers these cells were not as suppressive as autologous monocytes. Heating at 56°C for 15 min, sonicating, or freeze-thawing the monocyte preparations resulted in an abrogation of monocyte-induced suppression of *F. nucleatum*-PBA. Kinetic studies in which fresh monocytes were added daily to lymphocytes stimulated with *F. nucleatum* or PWM showed that the monocytes must be added within the first 2 days of culture to suppress *F. nucleatum*-PBA or enhance PWM-PBA. Monocytes incubated with *F. nucleatum* for 48 h released into the culture medium a soluble factor that suppressed *F. nucleatum*-PBA. The results from this study demonstrate a potent mechanism by which the host might prevent exaggerated nonspecific immunoglobulin responses when exposed to PBA-inducing concentrations of *F. nucleatum*. On the other hand, the induction of suppressive monocytes (or monocyte-mediated suppressive factors) by interaction with *F. nucleatum* might result in the inhibition of host protective immune reactions.

Periodontitis is a chronic inflammation of the tissues that surround the teeth. The disease is initiated and perpetuated by subgingival plaque, which is composed of an accumulation of predominantly anaerobic gram-negative bacteria. The plaque acts as an irritant to the adjacent tissue, and inflammation subsequently develops. The inflammatory cell infiltrate consists of macrophage, T lymphocytes (T cells), and a characteristically large percentage of immunoglobulin-secreting B lymphocytes (plasma cells) (21, 30). The unusually high number of plasma cells in these lesions suggests that B lymphocytes (B cells) may be activated in the local gingival tissue, perhaps by factors originating in the subgingival plaque. Many bacteria isolated from subgingival plaque have the capacity to nonspecifically activate human B cells to differentiate into plasma cells (3, 22, 23). Only a small proportion of the immunoglobulins produced during polyclonal B-cell activation (PBA) react with the stimulating bacteria (24).

Fusobacterium nucleatum is a gram-negative, anaerobic fusiform bacterium that is frequently isolated from the subgingival plaque of patients with chronic periodontitis. Previous studies reported that the insoluble fraction from sonicated *F. nucleatum* is a potent activator of PBA in vitro (23). *F. nucleatum*-induced PBA (*F. nucleatum*-PBA) is greatly enhanced in lymphocyte cultures depleted of adherent cells by passage through a Sephadex G-10 column (23). This provides strong evidence that blood monocytes suppress *F. nucleatum*-PBA. Monocyte inhibition of lymphocyte functions, including B-cell activation and differentiation (4, 8, 10–12, 18, 20, 29), is recognized as an important form of immunoregulation. Suppressor monocytes are associated with a number of diseases, such as systemic lupus erythematosus (19), sarcoidosis (16), multiple myeloma (6), tuberculosis (9, 17), and fungal diseases (31). As reported in a recent study by Carpenter et al. (7) and in a previous study from our laboratory (22), the removal of adherent cells from unfractionated cell populations is necessary to obtain PBA responses to other microorganisms associated with periodontal diseases. In contrast, pokeweed mitogen (PWM)-induced PBA (PWM-PBA) is optimal when monocytes are present in moderate concentrations (5 to 30%) and is abrogated by thorough monocyte depletion (<4%) or when excess (>30%) monocytes are present in lymphocyte cultures (10, 18, 29).

In this study of *F. nucleatum*-PBA, the in vitro effects of monocytes on PBA were examined in more detail than previously reported in the literature. Monocytes were removed from unfractionated cells by three different methods, all of which resulted in an enhanced PBA response to *F. nucleatum* and a decreased PBA response to PWM. When added back to monocyte-depleted lymphocytes, highly purified monocytes (>85% esterase positive cells) induced a

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dose-dependent suppression of *F. nucleatum*-PBA and enhancement of PWM-PBA. Evidence presented in this report suggests that suppression of *F. nucleatum*-PBA is mediated by a soluble factor(s) produced during interaction of monocytes with the *F. nucleatum* stimulant.

MATERIALS AND METHODS

Stimulants. *F. nucleatum* strain Per-1, isolated from the subgingival plaque of a patient with chronic periodontitis, was cultured in Schaedler broth (Oxoid USA Inc., Columbia, Md.) under anaerobic conditions and processed as reported previously (23). The bacteria were centrifuged, washed, and suspended in water. The intact cells were disrupted by sonication, and the suspensions were centrifuged at 12,000 × *g*. The resulting pellet material was washed in water, lyophilized, and stored at −20°C. The pellet material was suspended in culture medium (see below) and added to cultures on a weight-to-volume basis. For each preparation of *F. nucleatum*, the optimal PBA-inducing concentration was used in these studies. PWM was purchased from GIBCO Laboratories (Grand Island, N.Y.).

Mononuclear cell isolation. Healthy donors between the ages of 20 and 35 years were used in this study. Mononuclear leukocytes from heparinized peripheral venous blood were isolated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) density centrifugation as described previously (22, 23). The cells were suspended in RPMI 1640 culture medium (M. A. Bioproducts, Walkersville, Md.) supplemented with 30 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer, 2 mM L-glutamine, and 50 μg of gentamicin (GIBCO) per ml. This supplemented culture medium will be referred to as RPMI in this paper.

Sephadex G-10 monocyte depletion. Unfractionated mononuclear cell suspensions in RPMI supplemented with 20% newborn calf serum (NCS; GIBCO) were depleted of monocytes by adherence on Sephadex G-10 (Pharmacia) columns prepared in a 20-ml syringe barrel plugged with nylon wool (2, 14). The cells were incubated on the columns at 37°C under 5% CO_2 for a total of 30 min. The nonadherent cells were eluted with 20 ml of 20% NCS-RPMI and washed once in RPMI. The nonadherent cells, referred to as lymphocytes in this paper, contained <1% nonspecific esterase positive monocytes (see below). In some experiments the lymphocytes were further depleted of monocytes by a second adherence procedure. Lymphocytes were suspended to 15 × 10^6/ml in 20% NCS-RPMI. Samples of 2 ml of this cell suspension were added to empty 60- by 15-mm plastic petri dishes that were pretreated with 3 ml of 20% NCS-RPMI at 37°C for 30 min. The plates were then incubated at 37°C in 5% CO_2 for 90 min. After the plates were gently swirled, the nonadherent cells were aspirated and washed once in RPMI. These double adherence-purified lymphocytes contained <0.25% esterase positive cells.

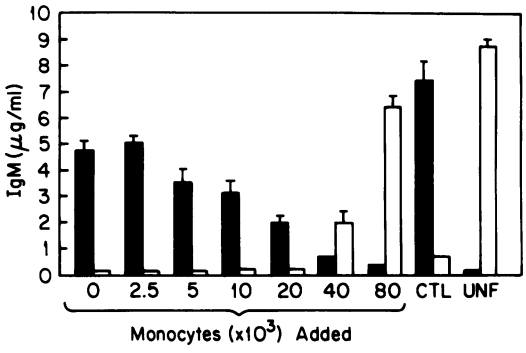

FIG. 1. Double adherence-purified monocytes (91% esterase-positive cells) were added to 1.6 × 10^5 double-adherence, monocyte-depleted (<0.25% esterase positive cells) lymphocytes. Cultures were stimulated with *F. nucleatum* (5 μg/ml) (■) or PWM (1%) (□). Unstimulated (medium) control cultures contained <0.39 μg of IgM per ml and are not shown. CTL, Cell density control cultures containing 2.4 × 10^5 lymphocytes; UNF, 2 × 10^5 unfractionated cells containing 26% esterase-positive cells. The means (± standard errors of the mean) of four replicate cultures are shown. The results are qualitatively representative of at least six similar experiments with different donors.

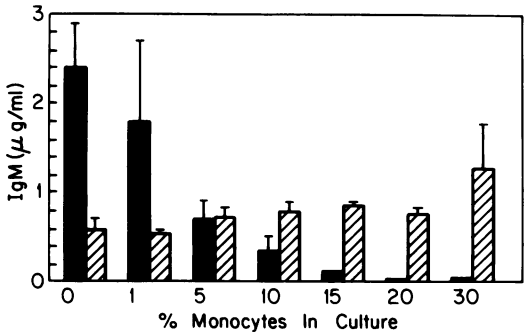

FIG. 2. Monocytes and lymphocytes were mixed in various ratios to obtain the indicated percentage of monocytes in a total cell concentration of 2 × 10^5 cells per well. Cultures were stimulated with *F. nucleatum* (1 μg/ml; ■) or PWM (1%; ▨). Unstimulated (medium) control cultures produced no measurable IgM. The means (± standard errors of the mean) of four replicate cultures are shown. The results are representative of two similar experiments with different donors.

Monocyte isolation. Monocytes were recovered by washing the G-10 columns with 20 ml of RPMI and then incubating with 15 ml of a 0.5% lidocaine solution in RPMI (14, 27). The columns were incubated at 22°C for 15 min, and the cells were then eluted with 15 ml of lidocaine solution. A second incubation and elution was performed, the eluates were pooled, and the cells were washed twice in cold RPMI. In some experiments, the monocyte suspension was further purified by adherence on plastic. Washed cells eluted from the G-10 column were adjusted to 5 × 10^6/ml in 20% NCS-RPMI and added to empty 60-by-15-mm plastic petri dishes pretreated with 3 ml of 20% NCS-RPMI. After a 90-min incubation in 5% CO_2 at 37°C, the nonadherent cells were removed by washing the plates twice with RPMI. The adherent cells were detached by adding 2 ml of ice-cold phosphate-buffered (0.05 M PO_4) saline (0.15 M NaCl) supplemented with EDTA (500 mg/liter) and incubating the plates on ice for 5 min. The plates were then thoroughly scraped with a rubber policeman, and the cell suspension was collected. This procedure was repeated two to three more times. The pooled cell suspensions were washed three times in cold RPMI. These monocyte preparations contained >85% esterase-positive cells.

T- and B-lymphocyte isolation. T cells were separated from unfractionated mononuclear cells by rosetting overnight with 2-aminoethylisothiuronium bromide-treated sheep erythrocytes at 5°C (15). The rosetted cells (T cells) were then separated from nonrosetted cells (B cells, monocytes, and other non-T cells) by centrifugation on Ficoll-Hypaque. The nonrosetted cells at the gradient interface were washed twice in RPMI and suspended in 20% NCS-RPMI, and the

monocytes were removed by adherence on Sephadex G-10 columns as described above. The cells that eluted from the columns were considered B cells and contained <3.2% esterase-positive cells. The T cells were isolated by lysis of the sheep erythrocytes with 0.16 M NH_4Cl and then washed in RPMI. The T cells contained <1% esterase-positive cells.

The percentage of T and B cells in unfractionated and G-10-fractionated cells was determined in some experiments. T cells were identified as sheep erythrocyte rosette-positive cells (15). B cells were identified as nonphagocytic, surface immunoglobulin-positive cells, by using Immunobead rosettes (Bio-Rad Laboratories, Richmond, Calif.).

Nonspecific esterase stain. A rapid esterase stain was performed with reagents purchased from Technicon Instrument Corp., Tarrytown, N.Y. (34). In a 12- by 75-mm polypropylene tube, 0.3 ml of 1% sodium nitrite solution, 0.3 ml of dye (pararosaniline-hydrochloride), 0.9 ml of buffer, and 0.15 ml of substrate (diethylene glycol alpha naphthyl butyrate) were mixed. In a second tube, 0.1 ml of cells (2×10^5), 0.1 ml of serum, and 0.2 ml of fixative were mixed for 40 s. The contents of the first tube were transferred to the second tube, mixed, and incubated in a 37°C water bath for 15 min. The tubes were centrifuged at 250 × *g* at 5°C, and the cell pellet was suspended in a small amount of residual supernatant. The cell suspensions were held on ice until counted.

In vitro PBA cultures. Cell suspensions were cultured in RPMI supplemented with 10% heat-inactivated (56°C, 60 min) fetal bovine serum (Hyclone lot 100185; Sterile Systems Inc., Logan, Utah) in 0.2-ml microcultures in 96-well, round-bottom tissue culture plates (Costar, Cambridge, Mass.). Multiple replicate cultures were stimulated with medium (unstimulated controls), *F. nucleatum* (1 or 5 μg/ml, optimal predetermined PBA concentrations), or PWM (1%), and incubated at 37°C in 5% CO_2 for 9 days. Details of PBA by *F. nucleatum* have been published (23).

IgM assay. Immunoglobulin M (IgM) was measured in culture supernatant by a micro-enzyme-linked immunosorbent assay as described previously (23).

OM-1 monoclonal antibody treatment. Samples (1 ml) of unfractionated mononuclear cells (20×10^6) were added to three tubes. The first tube represented untreated control cells and received no antibody or complement. The second tube was a control for complement effects and received 0.2 ml of rabbit complement (Low Tox; Cedarlane Laboratories, Ltd., Hornby, Ontario, Canada). The third tube received both OM-1 anti-monocyte monoclonal antibody (5; Ortho Pharmaceutical Corp., Raritan, N.J.) (1 μg of antibody per 10^6 cells) and complement. All three tubes were incubated at 5°C for 45 min during the OM-1 binding step and at 37°C for 60 min during the complement cytolysis step. The cells were centrifuged at 250 × *g* for 10 min, suspended in 1 ml of RPMI, and treated two more times by the same protocol. After the last treatment, the cells were washed twice and suspended in RPMI to yield 2×10^5 viable cells per culture.

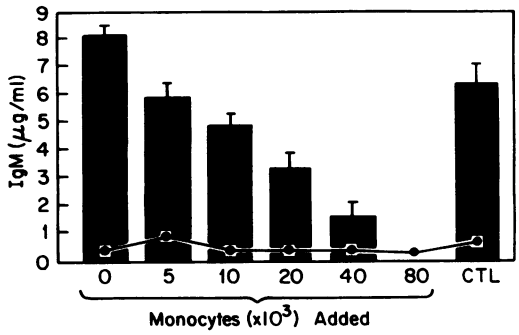

FIG. 3. T and B cells, separated from unfractionated cells by the sheep erythrocyte rosette technique, were combined in a T/B cell ratio of 3:1. Various numbers of monocytes were added to 1.6×10^5 mixed B and T cells. Cultures received either medium alone (unstimulated control; ●———●) or *F. nucleatum* (5 μg/ml; ■). CTL, Cell density control cultures containing 2.4×10^5 mixed B and T cells. The means (± standard errors of the mean) of four replicate cultures are shown. The results are representative of three similar experiments with different donors.

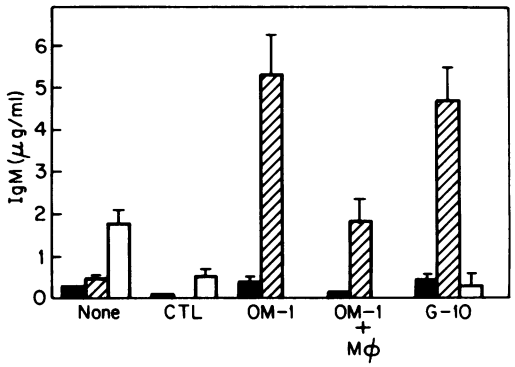

FIG. 4. Unfractionated cells were untreated (none), treated three times with complement (control, CTL), or treated three times with OM-1 antibody (1 μg/10^6 cells) plus complement (OM-1). For comparison, unfractionated cells were depleted of monocytes by adherence on Sephadex G-10 (G-10). All cultures contained 2×10^5 viable cells. OM-1 + Mϕ cultures contained 1.4×10^5 OM-1-treated cells and 0.6×10^5 fresh monocytes. Cultures were stimulated with medium (unstimulated controls, ■), *F. nucleatum* (1 μg/ml; ▨), or PWM (1%; □). The means (± standard errors of the mean) of six to eight replicate cultures are shown. The results are from one experiment.

Leu-O-Me treatment. Monocytes were depleted from unfractionated cells by treatment with L-leucine methyl ester (Leu-O-Me; Sigma Chemical Co., St. Louis, Mo.) as described recently by Thiele et al. (32). Leu-O-Me was prepared immediately before use in RPMI and sterilized by filtration (0.22-μm Acrodisc; Gelman Filters, Ann Arbor, Mich.). Cell suspensions were adjusted to 5×10^6 cells per ml in RPMI and incubated with Leu-O-Me (2.5 or 5 mM) at 22°C for 40 min. The cells were then washed twice and suspended in RPMI.

Physical treatments of monocytes. Double adherence-purified monocytes were adjusted to 1.2×10^6 cells per ml in 10% fetal bovine serum-RPMI, and samples were treated as follows: (i) untreated controls held on ice, (ii) heat in a 56°C water bath for 15 min, (iii) sonicated on high intensity for 10 s while incubated in an ice water bath (>95% disruption), and (iv) freeze-thawed twice.

Monocyte supernatants. Monocytes (>70% esterase-positive cells) were cultured at 0.4×10^5 cells per round-bottom well in 0.2 ml of 10% fetal bovine serum-RPMI. Control cultures contained 0.2 ml of 10% fetal bovine serum-RPMI. The cultures received either culture media (unstimulated controls) or *F. nucleatum* (5 μg/ml) and were incubated for 48 h. After pooling, the respective cultures were centrifuged at 500 × *g* for 15 min, and the cell free-supernatant was carefully collected. Supernatants were used the same day as collected or were stored at −20°C. The supernatants were

TABLE 1. Removal of monocytes by Leu-O-Me treatment: effect on IgM production[a]

Cells	Leu-O-Me treated	Monocytes[b]	IgM production [μg/ml (SEM)] with the following stimulant:					
			Medium		*F. nucleatum*		PWM	
			Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
Unfractionated	−	−	0.51 (0.06)	0.08 (0.02)	0.07 (0.04)	0.21 (0.07)	5.96 (0.69)	26.74 (1.63)
	+	−	0.18 (0.07)	0.13 (0.08)	7.52 (0.33)	2.31 (0.31)	3.69 (0.99)	3.85 (0.60)
	+	+	0.23 (0.11)	ND[c]	1.18 (0.41)	ND	7.66 (0.72)	ND
Lymphocytes	−	−	ND	0.24 (0.05)	ND	33.60 (2.40)	ND	18.37 (2.17)
	+	−	ND	0.26 (0.07)	ND	25.23 (3.49)	ND	11.66 (2.29)

[a] Unfractionated mononuclear cells or Sephadex G-10 monocyte-depleted lymphocytes from two donors (experiments 1 and 2) were treated with 2.5 mM (experiment 1) or 5 mM (experiment 2) Leu-O-Me or with medium. The cells were then cultured at a concentration of 2×10^5 per well with medium alone, *F. nucleatum* (1 μg/ml), or PWM (1%) and incubated for 9 days. For experiment 1, $n = 4$; for experiment 2, $n = 8$.

[b] +, Monocytes (2×10^4) added to culture; −, no monocytes added.

[c] ND, Not determined.

added to a final concentration of 50% volume in fresh lymphocyte cultures.

Statistics. The differences between test and control groups were statistically calculated by using the Student *t* test with a limit of $P \leq 0.05$.

RESULTS

Removal of monocytes by Sephadex G-10. Unfractionated mononuclear cells, containing 13 to 35% esterase-positive cells, were separated into adherent and nonadherent fractions by passage through a Sephadex G-10 column. Adherent cells, isolated from the G-10 columns by lidocaine treatment, contained from 40 to 75% esterase-positive monocytes. In some experiments these cells were further isolated from coeluting nonadherent cells by a second adherence separation on serum-coated plastic dishes. The cells obtained by this protocol were >85% monocytes. The nonadherent cells that eluted from the column contained <1% monocytes; after a second depletion of adherent cells on plastic plates, the eluate contained <0.25% monocytes.

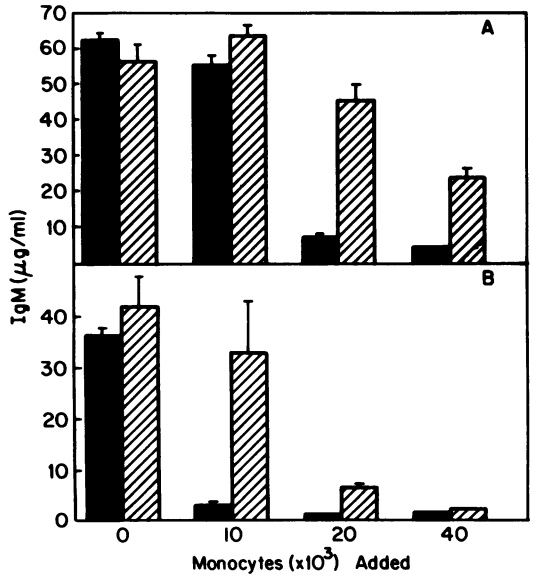

FIG. 5. Monocytes and lymphocytes were isolated from two donors. Autologous (■) or allogeneic (▨) monocytes were added in various numbers to 1.7×10^5 lymphocytes. The lymphocyte cultures from donor A are shown in graph A, and those from donor B are shown in graph B. Cultures were stimulated with *F. nucleatum* (5 μg/ml). The means (± standard errors of the mean) of three replicate cultures are shown.

To confirm their suppressive role on *F. nucleatum*-PBA, purified monocytes were added to thoroughly monocyte depleted lymphocytes (Fig. 1). With total IgM production as the measure of PBA, *F. nucleatum*-PBA was found to be optimal in cultures thoroughly depleted of monocytes. The addition of monocytes to these cultures resulted in a dose-dependent decrease in *F. nucleatum*-induced IgM production. In contrast, when PWM was used as the PBA stimulant, IgM production was abrogated in monocyte-depleted cultures and was enhanced when monocytes were added to these cultures. In general, the suppression (*F. nucleatum*-PBA) or enhancement (PWM-PBA) occurred when monocytes were added in excess of 10% of the total cells. However, it must be noted that the magnitude of the monocyte effects and the number of monocytes that induce these effects varied from donor to donor. For example with some donors' cells, adding as little as 2.5% monocytes caused complete suppression of *F. nucleatum*-PBA and maximal enhancement of PWM-PBA. Therefore, throughout this study, the magnitude of the monocyte effect induced by a particular concentration of monocytes varied from donor to donor. Nevertheless, the qualitative effects of monocytes on PBA were consistent with all donors tested: monocytes (at physiological concentrations of 10 to 30%) suppressed *F. nucleatum* and enhanced PWM-PBA.

In these add-back experiments, monocytes were added to a constant number of lymphocytes. Therefore, the total cell density in each culture changed depending on the number of monocytes added. We previously reported that cell density modulates the magnitude of *F. nucleatum*-PBA (23), and we were concerned that the monocyte suppression might reflect inhibitory cell densities in the cultures. Therefore, in the add-back experiments, a suboptimal number of lymphocytes were cultured so that the addition of monocytes would yield a more optimal total cell density, thereby favoring PBA. Nevertheless, the addition of monocytes suppressed *F. nucleatum*-PBA (Fig. 1). Furthermore, *F. nucleatum*-PBA was not suppressed in control cultures, which contained the same number of monocyte-depleted lymphocytes as the cultures with added monocytes (CTL, Fig. 1). Finally, in experiments in which the total cell concentration was held constant and the percentage of monocytes was increased, similar dose-dependent monocyte suppression of *F. nucleatum*-PBA was observed (Fig. 2).

F. nucleatum-PBA is influenced by the T/B cell ratio in the cultures (23). We considered the possibility that the T/B cell ratio was altered by the G-10 fractionation, thereby enhanc-

ing the responsiveness to *F. nucleatum* and decreasing the responsiveness to PWM. Thus, unfractionated and G-10-nonadherent lymphocytes were assayed for the total number of sheep erythrocyte rosette-positive cells (T cells) and surface immunoglobulin-positive cells (B cells). In both cell preparations, the ratio of T to B cells was approximately 3:1. Dose-dependent monocyte suppression of *F. nucleatum*-PBA could also be demonstrated in cultures containing isolated T and B cells at a ratio of 3:1 (Fig. 3), the optimal ratio for *F. nucleatum*-PBA (23).

Removal of monocytes with monoclonal antibody. OM-1 monoclonal antibody, which reacts with blood monocytes (5), was used to deplete monocytes from unfractionated cells and minimize the concomitant depletion of nonmonocytic cells which occurs on G-10 columns (13). Treatment of unfractionated cells with OM-1 plus complement resulted in a decrease of monocytes from 19 to 3% and a dramatic increase in *F. nucleatum*-PBA (Fig. 4). In contrast, OM-1 treatment completely abrogated the relatively weak PWM-PBA. The addition of isolated monocytes to the OM-1-treated cells reduced *F. nucleatum* stimulation. In this experiment, the number of monocytes added back to these cultures was insufficient to completely abrogate *F. nucleatum*-PBA or substantially enhance PWM-PBA. OM-1-treated, unfractionated cells produced approximately the same amount of *F. nucleatum*-PBA as G-10-fractionated lymphocytes.

Removal of monocytes with Leu-O-Me. Recently, Thiele et al. (32) reported that treatment of unfractionated mononuclear cells with Leu-O-Me resulted in the selective destruction of monocytes. Using this treatment, we reduced the number of monocytes in the unfractionated cells from 19 to 6%. As expected, the Leu-O-Me-treated, unfractionated cells showed increased *F. nucleatum*-PBA and decreased PWM-PBA (Table 1). The addition of fresh monocytes to the treated cells depressed *F. nucleatum*-PBA and enhanced PWM-PBA.

The amount of *F. nucleatum*-PBA in G-10-fractionated lymphocyte cultures was over 10 times the amount produced in Leu-O-Me-treated cell cultures. This may indicate that the residual 6% monocytes in the Leu-O-Me-treated cultures suppressed *F. nucleatum*-PBA. In addition, the Leu-O-Me

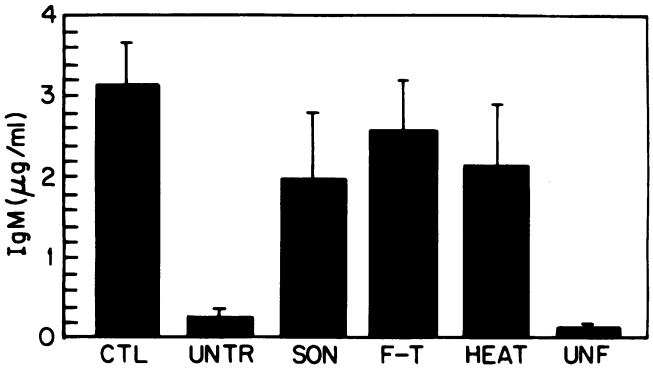

FIG. 6. Monocytes were held on ice (untreated control, UNTR), sonicated for 10 s (SON), freeze-thawed twice (F-T), or heated at 56°C for 15 min (HEAT). Lymphocytes (1.6×10^5) alone (CTL) or with treated monocytes (0.6×10^5), and unfractionated cells (2×10^5) (UNF) were stimulated with *F. nucleatum* (5 μg/ml). The means (± standard errors of the mean) of three replicate cultures are shown. The results are representative of three similar experiments with different donors.

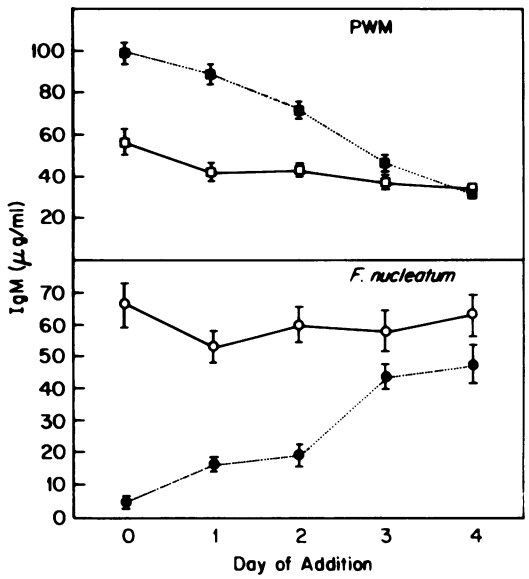

FIG. 7. Lymphocytes (1.5×10^5) were stimulated with eiher *F. nucleatum* (0.5 μg/ml) or PWM (1%) on day zero. Freshly isolated monocytes (0.4×10^5) (●) or an equal volume of medium (○) were added on the days indicated. IgM concentrations were determined on day 9. The means (± standard errors of the mean) of eight replicate cultures are shown. There was no significant difference ($P > 0.05$) between the data points of stimulated and unstimulated cultures on day 3 or 4. The results are representative of four similar experiments with different donors.

may have functionally damaged the lymphocytes and decreased the capacity of B cells to be activated. Monocyte-depleted lymphocytes treated with Leu-O-Me showed a 25% decrease in *F. nucleatum*-PBA (experiment 2 in Table 1), indicating that Leu-O-Me treatment diminishes the *F. nucleatum*-PBA response.

Effect of allogeneic monocytes. To test whether allogeneic monocytes can suppress *F. nucleatum*-PBA, monocytes were purified from two donors and added to G-10-fractionated lymphocytes from each donor. The addition of 0.4×10^5 autologous or allogeneic monocytes to the lymphocytes from either donor abrogated *F. nucleatum* stimulation (Fig. 5). However, autologous monocytes were more suppressive than allogeneic monocytes when lower numbers ($<0.4 \times 10^5$) of monocytes were added.

Intact, viable monocytes are required. Monocytes were treated by several physical procedures to determine whether suppression required intact, viable monocytes. Heated (56°C, 15 min), sonicated (>95% disruption), or freeze-thawed monocyte preparations contained <5% viable cells. These treatments resulted in the abrogation of suppression of *F. nucleatum*-PBA (Fig. 6), indicating that suppression is induced by intact, viable monocytes.

Kinetic studies. Fresh monocytes were added daily to lymphocyte cultures stimulated with *F. nucleatum* or PWM to determine the kinetics of monocyte effects on PBA (Fig. 7). The addition of monocytes to the cultures during the first 2 days resulted in suppression of *F. nucleatum*-PBA and enhancement of PWM-PBA. By day 3, the addition of fresh monocytes had no effect on either *F. nucleatum*-PBA or PWM-PBA, indicating that the early stages of B cell activation are affected by monocytes.

***F. nucleatum*-stimulated monocytes release a soluble suppressive factor.** The mechanism by which monocytes sup-

TABLE 2. *F. nucleatum*-stimulated monocytes release a soluble suppressive factor[a]

No.	Preculture: Monocytes	Preculture: *F. nucleatum*	IgM production [μg/ml (SEM)] in lymphocyte cultures[b]: Medium	IgM production [μg/ml (SEM)] in lymphocyte cultures[b]: *F. nucleatum*
1	−	−	0.45 (0.11)	27.58 (7.32) (n = 2)
2	−	+	31.92 (4.61)[c]	34.46 (1.28)[d]
3	+	−	2.38 (1.30)[d]	25.29 (1.58)[d]
4	+	+	9.16 (5.31)[d]	8.25 (2.22)[c]

[a] Isolated monocytes (6×10^4 in 0.2 ml of medium) were stimulated with medium alone or with *F. nucleatum* (5 μg/ml) (precultures 3 and 4, respectively). Control cultures lacked monocytes (precultures 1 and 2). After 48 h, the precultures were centrifuged, and 0.1 ml of the cell-free supernatants was added to 2×10^5 fresh lymphocytes suspended in 0.1 ml of fresh medium (final culture volume, 0.2 ml). The cultures were stimulated with either medium alone or *F. nucleatum* (1 μg/ml) and incubated for 9 days. Except where indicated $n = 3$. The results are representative of three similar experiments with different donors.

[b] Lymphocyte cultures suspended in fresh medium alone (i.e., receiving no preculture supernatants) produced 0.27 ± 0.08 μg of IgM per ml when stimulated with medium and 21.60 ± 4.12 μg of IgM per ml when stimulated with *F. nucleatum*.

[c] Significantly different from preculture 1 ($P \leq 0.05$).

[d] Not significantly different from preculture 1 ($P > 0.05$).

press *F. nucleatum*-PBA is unknown. We tested the possibility that monocytes release a soluble suppressive factor into the culture medium. Knowing that the monocytes suppress *F. nucleatum*-PBA during the first few days of stimulation, we speculated that a putative soluble factor would be produced within the first 48 h. To test for such a suppressive factor, purified monocytes were incubated in media with or without *F. nucleatum*. Control wells were set up without monocytes. After 48 h, the cell-free supernatant from these four precultures were added to fresh lymphocyte cultures, which were then stimulated with media or with *F. nucleatum* (Table 2). Supernatants from precultured media alone (preculture 1), media with *F. nucleatum* (preculture 2), or monocytes alone (preculture 3) had little effect on *F. nucleatum*-PBA (Table 2). In contrast, the supernatants from monocytes incubated 48 h with *F. nucleatum* (preculture 4) suppressed *F. nucleatum*-PBA.

DISCUSSION

Removal of esterase-positive, adherent cells (monocytes) from peripheral blood mononuclear cells enhanced *F. nucleatum*-induced nonspecific immunoglobulin production (i.e., PBA) (23). We initiated monocyte depletion as a routine procedure several years ago while studying PBA induced by *Actinomyces viscosus* (22) and other microorganisms associated with periodontal diseases (unpublished data). Unable to stimulate significant concentrations of immunoglobulin in unfractionated mononuclear cell cultures, we found that passage of the cells through a Sephadex G-10 column resulted in a population of cells that produced 10 to 100 times more immunoglobulin than in unfractionated cell cultures. The monocyte-depleted lymphocytes consistently yielded higher amounts of immunoglobulin and converted nonresponder donors into responder donors. Recently, Carpenter et al. (7) examined the role of adherent cells in polyclonal immunoglobulin production stimulated by insoluble extracts of *Streptococcus sanguis* and *Wolinella* sp. HVS, two bacteria associated with periodontal diseases. They found that total IgM and IgG production were consistently enhanced in monocyte-depleted cultures from all persons tested. Furthermore, they showed that purified monocytes could inhibit PBA stimulation by these bacteria. Other studies reported that monocyte depletion also enhanced human polyclonal B-cell activation induced by streptolysin (12), staphylococcal phage lysate (12), *Nocardia* sp. water-soluble mitogen (12), Epstein-Barr virus (12), *Staphylococcus aureus* (33), staphylococcal protein A (25, 33), concanavalin A (25), lipopolysaccharide (20), tetanus toxoid (11), and keyhole limpet hemocyanin (11). In contrast, PWM-PBA was optimal in cultures containing monocyte concentrations of approximately 10% and was diminished by thorough monocyte depletion (<4%) (7, 10–12, 18, 23, 25, 28, 29), a finding we also confirmed in the present study.

The role of the monocyte in suppression of *F. nucleatum*-PBA was confirmed by the capacity of highly purified monocytes (>85% esterase-positive cells) to inhibit in a dose-dependent manner IgM production in thorougly monocyte-depleted lymphocyte cultures (<0.25% esterase-positive cells). Furthermore, *F. nucleatum*-PBA was obtained in unfractionated cells, which are normally refractive to *F. nucleatum* stimulation, after these cells were treated with anti-monocyte specific OM-1 antibody plus complement (5) or with Leu-O-Me, a compound which selectively destroys lysosome-rich cells (32).

Allogeneic monocytes suppressed *F. nucleatum*-PBA when added in relatively large numbers (0.4×10^5) to lymphocyte cultures. However, in lower numbers, autologous monocytes were more suppressive than allogeneic monocytes. Bona et al. (4) also reported that allogeneic monocytes suppress *Nocardia* sp.-induced PBA and mentioned that autologous monocytes appeared to be slightly more suppressive than allogeneic cells. Monocyte suppression of PBA may therefore be composed of genetically restricted and unrestricted mechanisms. The genetically restricted suppression appears to be evident only at low concentrations of monocytes. However, the decreased suppression in cultures receiving allogeneic monocytes may represent a concomitant allogeneic stimulation of the lymphocytes.

Kinetic studies showed that it was necessary for monocytes to be present in the culture within the first 2 days to suppress *F. nucleatum*-PBA or enhance PWM-PBA. Gmelig-Meyling and Waldmann (12) similarly showed that monocytes must be added to unfractionated cells near the beginning of the culture to suppress PWM-PBA. These results indicate that monocytes suppress the early stages of B-cell activation.

The mechanism(s) by which monocytes suppress *F. nucleatum*-PBA is not known. The suppression was not due to a cytotoxic effect of the isolated monocytes on the lymphocytes, since the monocytes were able to concomitantly restore PWM-PBA in a dose-dependent fashion, as previously reported by other investigators (7, 12, 18, 28). In addition, the monocyte suppression did not reflect an inhibitory increase in cell density in the cultures for the following reasons: (i) lymphocytes were added to the cultures in suboptimal concentrations so that the addition of monocytes would yield a more optimal cell density for *F. nucleatum*-PBA (23); (ii) control cultures containing equivalent numbers of monocyte-depleted lymphocytes were not suppressed; (iii) cultures in which the total cell concentration was held constant while the percentage of monocytes varied showed a similar dose-dependent suppression by monocytes; and (iv) the addition of intact, heat-inactivated monocytes to the cultures did not suppress the PBA response. Finally, the monocyte suppression of *F. nucleatum*-PBA was not a result of monocyte degradation or removal of in vitro-synthesized IgM, since (i) monocytes added to cultures after 4 days failed

to decrease IgM concentrations, (ii) monocytes enhanced IgM production in PWM cultures, and (iii) monocytes also suppressed the total number of hemolytic plaque-forming (immunoglobulin-secreting) cells in lymphocyte cultures stimulated with *F. nucleatum* (data not presented).

The enhanced IgM production in G-10 monocyte-depleted cultures stimulated with *F. nucleatum* did not appear to result from a change in the T/B cell ratio, a parameter which can influence *F. nucleatum*-PBA responses (23). The ratio of sheep erythrocyte rosette-positive cells (T cells) to sheep erythrocyte rosette-negative, surface immunoglobulin-positive cells (B cells) in both unfractionated and G-10-depleted cells was roughly 3:1, a finding reported by others (14). Furthermore, we showed that cultures containing isolated T and B cells combined in a ratio of 3:1, an optimal ratio for *F. nucleatum*-induced immunoglobulin production (23), were suppressed by monocytes in a similar dose-dependent manner.

Supernatants from monocyte cultures stimulated with *F. nucleatum* for 2 days suppressed *F. nucleatum*-PBA, whereas the supernatants from unstimulated monocyte cultures were not suppressive. Since control supernatants containing *F. nucleatum* (preculture 2, Table 2) did not inhibit *F. nucleatum*-PBA in these experiments, the maximum concentration of *F. nucleatum* in the lymphocyte cultures (approximately 6 μg/ml) was not inhibitory. Therefore it appears that a soluble suppressive factor was produced during the 48-h monocyte-*F. nucleatum* interaction. Monocytes release a number of factors that can influence lymphocyte activation (1, 26), including interleukin 1 and prostaglandins. Preliminary studies (unpublished data) indicated that purified interleukin 1 can partially suppress *F. nucleatum*-PBA. Prostaglandins, however, do not seem to mediate the suppression since treatment of unfractionated cells with indomethacin, an inhibitor of prostaglandin synthesis, did not enhance immunoglobulin production stimulated by *F. nucleatum*. It is also possible that the suppressive factor in the monocyte-*F. nucleatum* supernatant is a breakdown product of the *F. nucleatum* stimulant. We have found that the soluble fraction of sonicated *F. nucleatum* can inhibit *F. nucleatum*-PBA and PWM-PBA (unpublished data). A detailed study on the production and characterization of the suppressive factors in the *F. nucleatum*-monocyte supernatants is currently underway.

The results of this study do not eliminate the possibility that the suppression of *F. nucleatum*-PBA in unfractionated cell cultures may be due to cells other than monocytes. The role of suppressor T cells, NK cells, and other immunosuppressive lymphocytes on *F. nucleatum*-PBA has not been determined. However, since treatment of unfractionated cell populations by three methods that remove primarily monocytes enhanced the PBA response to *F. nucleatum*, and since highly purified monocytes were capable of inhibiting this stimulation, the monocyte appears to play a significant role in the regulation of *F. nucleatum*-PBA.

The role of monocyte-mediated suppression of PBA in vivo has yet to be determined. The induction of suppressor monocytes (or monocyte-mediated suppressive factors) might be advantageous to the host by preventing an exaggerated immunoglobulin response caused by microbial PBA factors. On the other hand, suppressor monocytes have been associated with diseases such as systemic lupus erythematosus (19), sarcoidosis (16), multiple myeloma (6), tuberculosis (9, 17), and fungal diseases (31). Large numbers of suppressor monocytes in vivo might shut down the immune responses required for the successful removal of cancerous cells or pathogenic bacteria from the host. Microbe-induced suppressor monocytes (or monocyte-mediated suppressive factors) may be a significant pathogenic mechanism in disease.

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LITERATURE CITED

1. **Allison, A. C.** 1978. Mechanisms by which activated macrophages inhibit lymphocyte responses. Immunol. Rev. **4**:3–27.
2. **Alonso, C. M., R. R. Bernabe, E. Moreno, and F. D. DeEspada.** 1978. Depletion of monocytes from human peripheral blood leucocytes by passage through Sephadex G-10 columns. J. Immunol. Methods **22**:361–368.
3. **Bick, P. H., A. B. Carpenter, L. V. Holdeman, G. A. Miller, R. R. Ranney, K. G. Palcanis, and J. G. Tew.** 1981. Polyclonal B cell activation induced by extracts of gram-negative bacteria isolated from periodontally diseased sites. Infect. Immun. **34**:43–49.
4. **Bona, C., S. Broder, A. Dimitriu, and T. A. Waldmann.** 1979. Polyclonal activation of human B lymphocytes by nocardia water soluble mitogen (NWSM). Immunol. Rev. **45**:69–92.
5. **Breard, J., E. L. Reinherz, P. C. Kung, G. Goldstein, and S. F. Schlossman.** 1980. A monoclonal antibody reactive with human peripheral blood monocytes. J. Immunol. **124**:1943–1948.
6. **Broder, S., R. Humphrey, M. Durm, M. Blackman, B. Meade, C. Goldman, W. Strober, and T. A. Waldmann.** 1975. Impaired synthesis of polyclonal (non-paraprotein) immunoglobulins by circulating lymphocytes from patients with multiple myeloma. N. Engl. J. Med. **293**:887–892.
7. **Carpenter, A. B., E. C. Sully, K. G. Palcanis, and P. H. Bick.** 1983. Role of monocytes in polyclonal immunoglobulin production stimulated by sonicates of periodontally associated bacteria. Infect. Immun. **42**:853–862.
8. **Delfraissy, J. F., P. Galanaud, J. Dormont, and C. Walton.** 1978. Primary *in vitro* antibody response of human peripheral blood lymphocytes: role of phagocytic mononuclear cells. J. Immunol. **120**:1283–1288.
9. **Ellner, J. J.** 1978. Suppressor adherent cells in human tuberculosis. J. Immunol. **121**:2573–2579.
10. **Fauci, A. S., H. C. Stevenson, G. Whalen, and P. Andrysiak.** 1982. Monocyte-mediated immunoregulation of human B-cell function, p. 275. *In* A. S. Fauci and R. E. Ballieux (ed.), Human B lymphocyte function: activation and immunoregulation. Raven Press, New York.
11. **Gerrard, T. L., and A. S. Fauci.** 1982. Activation and immunoregulation of antigen-specific human B lymphocyte responses: multifaceted role of the monocyte. J. Immunol. **128**:2367–2372.
12. **Gmelig-Meyling, F., and T. A. Waldmann.** 1981. Human B cell activation *in vitro*: augmentation and suppression by monocytes of the immunoglobulin production induced by various B cell stimulants. J. Immunol. **126**:529–537.
13. **Hoffmann, M. K., S. Pollack, S. E. Krown, and R. S. Mittler.** 1982. Deletion of active human suppressor T lymphocytes from peripheral blood by Sephadex G-10 filtration. J. Immunol. Methods **55**:327–336.
14. **Jerrells, T. R., J. H. Dean, G. L. Richardson, and R. B. Herberman.** 1980. Depletion of monocytes from human peripheral blood mononuclear leukocytes: comparison of the Sephadex G-10 column method with other commonly used techniques. J. Immunol. Methods **32**:11–29.
15. **Kaplan, M. E., and C. Clark.** 1974. An improved rosetting assay for detection of human T lymphocytes. J. Immunol. Methods

5:131–135.
16. **Katz, P., and A. S. Fauci.** 1978. Inhibition of polyclonal B-cell activation by suppressor monocytes in patients with sarcoidosis. Clin. Exp. Immunol. **32**:554–562.
17. **Katz, P., R. A. Goldstein, and A. S. Fauci.** 1979. Immunoregulation in infection caused by *Mycobacterium tuberculosis*: the presence of suppressor monocytes and the alteration of subpopulations of T lymphocytes. J. Infect. Dis. **140**:12–21.
18. **Knapp, W., and G. Baumgartner.** 1978. Monocyte-mediated suppression of human B lymphocyte differentiation *in vitro*. J. Immunol. **121**:1177–1183.
19. **Laughter, A. H., M. D. Lidsky, and J. J. Twomey.** 1979. Suppression of immunoglobulin synthesis by monocytes in health and in patients with systemic lupus erythematosus. Clin. Immunol. Immunopathol. **14**:435–440.
20. **Levitt, D., D. Duber-Stull, and A. R. Lawton.** 1981. T-cell-dependent and independent plasma cell differentiation induced by *Escherichia coli* lipopolysaccharide in human peripheral blood lymphocytes. Clin. Immunol. Immunopathol. **18**:309–321.
21. **Mackler, B. F., K. B. Frostad, P. B. Robertson, and B. M. Levy.** 1977. Immunoglobulin bearing lymphocytes and plasma cells in human periodontal disease. J. Periodont. Res. **12**:37–45.
22. **Mangan, D. F., and D. E. Lopatin.** 1981. *In vitro* stimulation of immunoglobulin production from human peripheral blood lymphocytes by a soluble preparation of *Actinomyces viscosus*. Infect. Immun. **31**:236–244.
23. **Mangan, D. F., and D. E. Lopatin.** 1983. Polyclonal activation of human peripheral blood B lymphocytes by *Fusobacterium nucleatum*. Infect. Immun. **40**:1104–1111.
24. **Mangan, D. F., T. Won, and D. E. Lopatin.** 1983. Nonspecific induction of immunoglobulin M antibodies to periodontal disease-associated microorganisms after polyclonal human B-lymphocyte activation by *Fusobacterium nucleatum*. Infect. Immun. **41**:1038–1045.
25. **Montazeri, G., N. Chiorazzi, S. M. Fu, and H. G. Kunkel.** 1980. Regulatory role of circulating monocytes in the differentiative and proliferative response of human B lymphocytes. Clin. Immunol. Immunopathol. **16**:1–10.
26. **Page, R. C., P. Davies, and A. C. Allison.** 1974. Participation of mononuclear phagocytes in chronic inflammatory diseases. RES J. Reticuloendothel. Soc. **15**:413–438.
27. **Rinehart, J. J., B. J. Gormus, P. Lange, and M. E. Kaplan.** 1978. A new method for isolation of human monocytes. J. Immunol. Methods **23**:207–212.
28. **Rosenberg, S. A., and P. E. Lipsky.** 1979. Monocyte dependence of pokeweed mitogen-induced differentiation of immunoglobulin secreting cells from human peripheral blood mononuclear cells. J. Immunol. **122**:926–931.
29. **Rosenberg, S. A., and P. E. Lipsky.** 1982. The role of monocytes in human B-cell activation, p. 263. *In* A. S. Fauci and R. E. Ballieux (ed.), Human B-lymphocyte function: activation and immunoregulation. Raven Press, New York.
30. **Seymour, G. J., and J. S. Greenspan.** 1979. The phenotypic characterization of lymphocyte populations in established human periodontal disease. J. Periodont. Res. **14**:39–46.
31. **Stobo, J. D.** 1977. Immunosuppression in man: suppression by macrophages can be mediated by interactions with regulatory T cells. J. Immunol. **119**:918–924.
32. **Thiele, D. L., M. Kurosaka, and P. E. Lipsky.** 1983. Phenotype of the accessory cell necessary for mitogen-stimulated T and B cell responses in human peripheral blood: delineation by its sensitivity to the lysosomotropic agent, L-leucine methyl ester. J. Immunol. **131**:2282–2290.
33. **Tsokos, G. C., C. B. Christian, and J. E. Balow.** 1983. Induction of monocytic suppression after stimulation of peripheral human mononuclear cells with staphylococcal protein A and *Staphylococcus aureus*. Cell. Immunol. **78**:144–151.
34. **Tucker, S. B., R. V. Pierre, and R. E. Jordon.** 1977. Rapid identification of monocytes in a mixed mononuclear cell preparation. J. Immunol. Methods **14**:267–269.