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In Vitro Stimulation of Immunoglobulin Production from Human Peripheral Blood Lymphocytes by a Soluble Preparation of *Actinomyces viscosus*

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In this paper, we report the ability of a soluble, ultrasonicate preparation of *Actinomyces viscosus* to stimulate in vitro immunoglobulin production by human peripheral blood lymphocytes. Immunoglobulin A (IgA), IgG, and IgM concentrations were determined with a highly sensitive and specific micro-enzyme-linked immunosorbent assay (micro-ELISA). *A. viscosus*-induced immunoglobulin production was T lymphocyte dependent and was inhibited by monocytes. The immunoglobulin response was optimal when *A. viscosus* (1,000 μg/ml) was removed from the cultures on day 2 of incubation. Antibodies specific for *A. viscosus* were not detected, indicating that *A. viscosus* was activating a polycolonal B-lymphocyte response. Since periodontal lesions in humans are characterized by the accumulation of a large number of B lymphocytes, the polyclonal activation of these cells by *A. viscosus* may affect the severity and resolution of the disease.

Actinomyces viscosus is a filamentous, gram-positive bacterium associated with inflammatory periodontal lesions in humans (20, 39, 40, 43) and experimental rodents (10, 17). This organism comprises a large percentage of the supragingival dental plaque which develops during experimental gingivitis in humans, and its prevalence increases as the plaque ages (40). *A. viscosus* is also a major component of subgingival plaque associated with periodontitis (43) and is the major cultivatable organism in mature and calcifying plaque (15). *A. viscosus* induces neutrophil chemotaxis (12) and lysosomal enzyme release from neutrophils (41) and macrophages (31)—events which are associated with exacerbation of the inflammatory response and subsequent host tissue damage. The association of *A. viscosus* with periodontal disease, therefore, seems well-founded.

Actinomycotic (5) and periodontal (26, 37) lesions are histologically characterized by an accumulation of neutrophils, macrophages, and lymphocytes. The reports of Mackler et al. (26) and Seymour and Greenspan (37) show that the majority of the lymphocytes within chronic inflammatory periodontal lesions in humans are B cells, though some T cells are also present. In the murine system, *A. viscosus* is mitogenic for normal spleen cells (6, 7, 8, 11), augments an antibody response to sheep erythrocytes (7), and stimulates a polyclonal antibody response (6, 8). In the human system, however, *A. viscosus* is reported to stimulate sensitized lymphocytes from patients with gingivitis (32) and periodontal disease (2, 32, 33). Cord blood leukocytes (33) and lymphocytes from healthy individuals (2, 32, 33) respond poorly to *A. viscosus*, suggesting the stimulation is antigenic (specific) rather than mitogenic (nonspecific). However, in a recent study from our laboratory, *A. viscosus* was shown to be a potent stimulant of deoxyribonucleic acid (DNA) synthesis in peripheral blood T and B lymphocytes from healthy donors, provided a pokeweed mitogen (PWM)-induced helper cell was available in the culture (D. E. Lopatin et al., submitted for publication). Smith et al. (38) have recently reported that a nonsoluble, cell wall preparation from *A. viscosus* stimulates an in vitro polyclonal antibody response from normal human lymphocytes. Their report, however, did not examine the cellular requirements or measure the immunoglobulin subclasses produced.

In this paper, we report the ability of a soluble extract of *A. viscosus* to induce in vitro immunoglobulin production by lymphocytes from healthy (non-periodontally diseased) adults. Immunoglobulins A, G, and M (IgA, IgG, and IgM) were measured with the highly sensitive and specific micro-enzyme-linked immunosorbent assay (micro-ELISA). *A. viscosus*-induced immunoglobulin production did not require mitogen-induced helper cell activity, did require T-cell participation, and was suppressed by monocytes. Since periodontal lesions are populated by large numbers of immunoglobulin-secreting B cells (26, 37), nonspecific activation of these lymphocytes by *A. viscosus* may ultimately af-

fect the severity and resolution of the disease.

MATERIALS AND METHODS

***A. viscosus* ultrasonicate preparation.** The preparation and characterization of the *A. viscosus* ultrasonicate preparation has already been reported (23). Briefly, an isolate of *A. viscosus* (GA), obtained from a naturally occuring gingivitis site, was cultured under anaerobic conditions. The cells were harvested from batch cultures by centrifugation at 12,000 × *g* for 30 min. The cell pellets were washed in sterile phosphate-buffered saline (PBS; 0.15 M NaCl–0.05 M PO_4 [pH 7.4]) and, finally, suspended in sterile distilled water. The washed cells were subjected to a total of 40 min of ultrasonic disruption while in an ice bath, delivered in 5-min intervals with alternating periods of cooling. The cellular debris was removed by centrifugation at 12,000 × *g* for 30 min at 4°C. The cell-free supernate was dialyzed against distilled water at 4°C and lyophilized.

Mononuclear cell isolation. Mononuclear leukocytes from heparinized venous blood drawn from healthy (non-periodontally diseased) volunteers between the ages of 20 and 35 were isolated by previously described procedures (22). The buffy coat layer, obtained from whole blood by centrifugation at 350 × *g* for 30 min at 23°C, was aspirated, diluted in sterile PBS, layered onto Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, N.J.), and centrifuged at 500 × *g* for 30 min at 23°C. The mononuclear leukocytes which banded at the interface were aspirated, washed three times in PBS, and resuspended in RPMI 1640 (RPMI) culture medium (GIBCO, Grand Island, N.Y.), supplemented with 30 mM *N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer, 2 mM glutamine, and gentamicin (50 μg/ml; Schering Corp., Kenilworth, N.J.).

Monocyte depletion and isolation. Mononuclear cell suspensions were depleted of monocytes by the Sephadex G-10 column method previously described (21). Passage through such columns resulted in lymphocyte suspensions having less than 4% monocyte contamination as determined by latex particle uptake and nonspecific esterase staining (21).

Monocytes were recovered from G-10 columns by first washing with 20 ml of RPMI and then incubating with 15 ml of a 0.5% lidocaine solution in RPMI. After incubation at 22 to 24°C for 15 min, the columns were eluted with an additional 15 ml of lidocaine solution. A second elution was performed, the eluates were pooled, and the cells were washed twice in Hanks balanced salt solution and resuspended in RPMI.

T- and B-lymphocyte isolation. T cells were prepared from monocyte-depleted leukocyte suspensions by rosetting with 2-aminoethylisothiouronium bromide-treated sheep erythrocytes (21). Such T-cell preparations were typically free of B-cell and monocyte contamination (>99% T cells). The fraction of nonrosetting cells were typically >90% B cells (T-cell contamination was 1 to 3%). B lymphocytes were identified by rosetting with anti-human immunoglobulin-coupled polyacrylamide beads (Immunobeads, Bio-Rad Laboratories, Richmond, Calif.), and by lack of nonspecific esterase staining (21). The B- and T-cell preparations were washed twice in Hanks balanced salt solution and resuspended in RPMI.

In vitro immunoglobulin synthesis. Lymphocytes were cultured in RPMI media supplemented with 10% fetal bovine serum (FBS; Hyclone lot 100185, Sterile Systems, Inc., Logan, Utah). Since unstimulated control cultures occasionally yielded substantial immunoglobulin synthesis, the FBS was heated to 80°C for 10 min to inactivate nonspecific mitogens in the serum (9). The lymphocytes (10^6 cells/ml) were incubated with *A. viscosus*, PWM (GIBCO, Grand Island, N.Y.), or media (control) in 1.0-ml volumes in polypropylene tubes (12 by 75 mm; Falcon, Oxnard, Calif.). Preliminary experiments indicated the continued presence of *A. viscosus* in the cultures suppressed immunoglobulin production. Therefore, in most experiments, the cultures were centrifuged (250 × *g*, 22°C, 10 min) after 2 days, the supernate was discarded, and the cells were resuspended with 1.0 ml of fresh FBS-RPMI without stimulants. The cultures were incubated for a total of 9 days at 37°C in 5% CO_2 and 100% humidity unless otherwise noted.

Micro-ELISA. Micro-ELISA was used to measure immunoglobulin (IgA, IgG, IgM) production (42). The wells of polystyrene substrate plates (Immulon, no. 1-223-29, Dynatech Laboratories, Inc., Alexandria, Va.) were coated (sensitized) with 0.2 ml of heavy-chain specific rabbit anti-human immunoglobulin (Bio-Rad Laboratories) diluted to 2 μg/ml in 0.05 M sodium carbonate (pH 9.6), containing 0.02% NaN_3. The plates were sealed with cellophane tape and incubated at room temperature (22 to 24°C) for 16 to 20 h in a closed chamber with high humidity. The plates were then washed with PBS containing polyoxyethylene (20) sorbitan monolaurate (Tween 20; Matheson, Coleman, and Bell, Norwood, Ohio) (PBS-T), five times at 4-min intervals. Lymphocyte culture supernates and IgA, IgG, or IgM standards were diluted in PBS-T, and 0.2 ml of each dilution was added to duplicate sensitized wells. After a 3-h incubation at room temperature, the plates were washed as described above. Alkaline phosphatase (calf intestine, type VII; Sigma Chemical Co., St. Louis, Mo.) was conjugated to heavy-chain specific rabbit anti-human immunoglobulin by glutaraldehyde treatment (42). The conjugates were diluted in PBS-T, and 0.2 ml was added to the wells. After incubation at room temperature for 16 to 20 h, the plates were again washed with PBS-T, and 0.2 ml of alkaline phosphatase substrate (Sigma 104, Sigma Chemical Co.) at 1 mg/ml in 0.05 M sodium carbonate buffer (pH 9.8), containing 1 mM $MgCl_2$, was added to all the wells. After 15 min at room temperature, the hydrolysis reaction was stopped by adding 0.05 ml of 3 N NaOH, and the absorbance at 405 nm was measured with a spectrophotometer (Multiskan, Flow Laboratories, Arlington, Va.). The concentration of immunoglobulin in the cultures was determined by comparing the absorbancy of the samples with absorbancy curves obtained with the immunoglobulin standards.

Antibodies specific for *A. viscosus* were determined by a modification of the above procedure. *A. viscosus*, diluted to 5 μg/ml in the carbonate coating buffer, was used to sensitize the plates. The remainder of the micro-ELISA procedures were identical to those described above, with the exception that the substrate hydrolysis was extended to 60 min. The assay can detect less than 1 ng of antibody specific for *A. visco-*

sus per ml of culture (S. Doty, D. E. Lopatin, and F. Smith, manuscript in preparation).

Statistical analysis. The test results were statistically compared to the controls using a one-sided Student *t* test.

RESULTS

Removal of *A. viscosus* is required for optimal immunoglobulin production. To investigate the ability of a soluble, ultrasonicated preparation of *A. viscosus* to induce in vitro immunoglobulin production from human peripheral blood lymphocytes, we stimulated cells from healthy adults with *A. viscosus* for 9 days. For comparison, PWM, a frequently employed nonspecific immunoglobulin stimulant (13), was also included in many of the experiments. IgA, IgG, and IgM concentrations were determined using a micro-ELISA assay specific for human immunoglobulin heavy chains.

Preliminary experiments, in which *A. viscosus* (1 to 1,000 μg/ml) was continuously present in lymphocyte cultures for the 9-day period, were frequently characterized by the secretion of little or no additional immunoglobulins into the culture supernates compared to nonstimulated control cultures. However, if the culture medium containing *A. viscosus* was replaced with fresh media lacking *A. viscosus* and then incubated as usual for a total of 9 days, immunoglobulin responses to *A. viscosus* were consistently obtained.

The inhibition of *A. viscosus*-induced immunoglobulin synthesis by the continual presence of *A. viscosus* in the 9-day cultures was examined by (i) maintaining *A. viscosus* continuously in cultures and (ii) re-adding *A. viscosus* to cultures previously exposed to *A. viscosus* for 2-days. The continuous presence of *A. viscosus* in the cultures resulted in lower immunoglobulin production compared to cultures washed free of *A. viscosus* after 2 days (Table 1).

Optimal time of stimulation with *A. viscosus*. Since the continuous presence of *A. viscosus* inhibited the immunoglobulin response, the optimal time during which *A. viscosus* was required to stimulate immunoglobulin synthesis was determined. Lymphocytes were stimulated with *A. viscosus* for 1 to 4 days, washed, and incubated for 9 days. *A. viscosus*-induced immunoglobulin synthesis was maximal after a 2-day stimulation with *A. viscosus* (Fig. 1). Similar results were obtained in subsequent experiments (data not shown) in which lymphocytes were stimulated with *A. viscosus* for 1 to 4 days, washed, and then incubated for 7 additional days after removing *A. viscosus*. This indicates that cultures stimulated with *A. viscosus* for 2 days did not have a higher immunoglobulin concentration than the 3- or 4-day *A. viscosus*-stimulated cultures simply because the interval after the wash was longer, allowing more time to accumulate the immunoglobulins.

TABLE 1. *Inhibition of A. viscosus-induced immunoglobulin synthesis by the continous presence of A. viscosus in the cultures*[a]

Stimulant	Cultures washed	*A. viscosus* readded	ng/ml		
			IgA	IgG	IgM
None	−	−	95	329	1,400
None	+	−	42	94	912
A. viscosus	−	−	161	746	3,121
A. viscosus	+	+	257	763	4,659
A. viscosus	+	−	344	1,180	6,683

[a] Monocyte-depleted lymphocytes (10^6) were cultured with *A. viscosus* (1,000 μg/ml) or media (control) for 2 days. As indicated, cells were then washed and resuspended in fresh media with or without *A. viscosus* (1,000 μg/ml). Incubation was continued for an additional 7 days. Each value represents the average of duplicate tests. The data are representative of three such experiments.

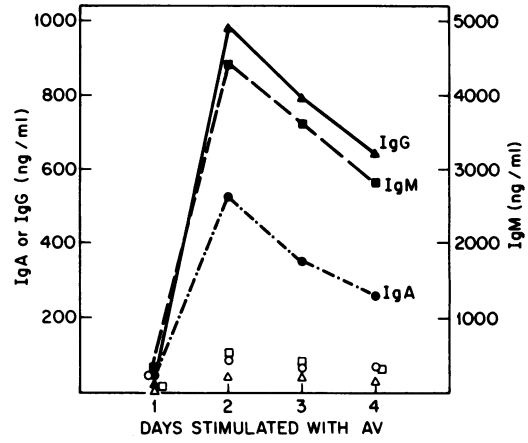

FIG. 1. *Optimal time of stimulation with A. viscosus (AV). Monocyte-depleted lymphocytes (10^6) were cultured with AV (1,000 μg/ml; closed symbols) or media (control; open symbols). After the number of days indicated, the cells were washed and resuspended in fresh media without AV. All cultures were terminated on day 9. Each value represents the average of duplicate tests. The data represent three such experiments.*

Optimal concentration of *A. viscosus*. The optimal concentration of *A. viscosus* required to induce an immunoglobulin response was examined. A concentration of 1,000 μg of *A. viscosus* per ml of culture (wt/vol) was shown to be optimal for stimulating immunoglobulin synthesis (Fig. 2). This concentration of *A. viscosus* contains approximately 100 μg of protein per ml as determined by the method of Lowry et al.

(24), using bovine serum albumin as a standard.

Kinetics of immunoglobulin synthesis. The production of immunoglobulins in cultures stimulated with *A. viscosus* (1,000 μg/ml) for 2 days was examined over a 15-day period (Fig. 3). Immunoglobulin production was first detected on day 5 (IgM) or day 7 (IgA and IgG) and increased to a maximum accumulation on day 15. When the rate of *A. viscosus*-induced immunoglobulin production was calculated (Fig. 4), the maximum IgM synthesis occurred on day 7. There were two periods of increased synthesis of IgA and IgG: one peak occurring between days 7 and 9 and the other peak on day 15. In comparison, PWM-stimulated lymphocytes yielded only one peak period of synthesis between days 7 and 9 for all immunoglobulin subclasses.

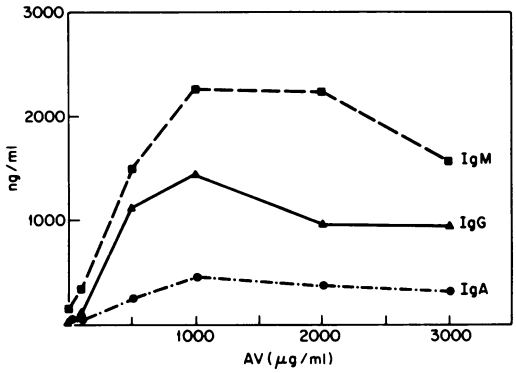

FIG. 2. *Immunoglobulin response to various concentrations of A. viscosus (AV). Monocyte-depleted lymphocytes (10^6) were cultured with various concentrations of AV or media alone (control) for 2 days. The cells were then washed, resuspended in fresh media without AV, and incubated for an additional 7 days. Each value represents the average of triplicate tests minus the control value. The data represent four such experiments.*

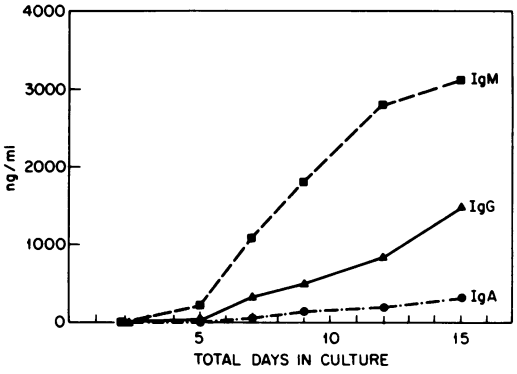

FIG. 3. *Kinetics of the immunoglobulin response to A. viscosus. Monocyte-depleted lymphocytes (10^6) were cultured with A. viscosus (1,000 μg/ml) or media alone (control) for 2 days. The cells were washed and resuspended in fresh media without A. viscosus. Periodically, 0.02 ml was removed from quadruplicate test cultures, pooled, and assayed for immunoglobulins. Control values at each time point were subtracted from the test value. The data represent four such experiments.*

Effect of monocytes on *A. viscosus*-induced immunoglobulin synthesis. The requirement for monocytes in *A. viscosus*-induced immunoglobulin synthesis was determined. Monocytes were added in increasing numbers to a constant number of monocyte-depleted lymphocytes. Increasing the number of monocytes in the cultures decreased the immunoglobulin response to *A. viscosus* (Table 2). The immunoglobulin response to PWM was less sensitive to increased numbers of monocytes, but was depressed at relatively high ratios of monocytes to lymphocytes (1:2). Normally, in unseparated lymphocytes preparations, the ratio of monocytes to lymphocytes was ca. 1:5. At this ratio the immunoglobulin response to PWM was only slightly affected while the response to *A. visco-*

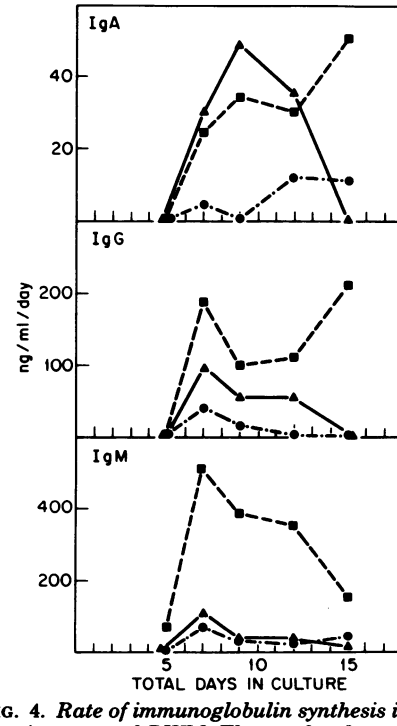

FIG. 4. *Rate of immunoglobulin synthesis induced by A. viscosus and PWM. The results shown in Fig. 3 were used to obtain these data. The values represent the difference in immunoglobulin concentrations between consecutive assay days divided by the number of days between the assays. Symbols: ■, A. viscosus; ▲, PWM; ●, media control.*

TABLE 2. *Monocyte effect on A. viscosus-induced immunoglobulin synthesis*[a]

Stimulant	Monocytes/100 lymphocytes	ng/ml		
		IgA	IgG	IgM
None	4	223	172	426
	25	42	0	0
	50	23	0	0
A. viscosus (1,000 μg/ml)	4	89	783	837
	25	39	11	0
	50	10	0	0
PWM (1:100)	4	1,039	766	1,857
	25	1,030	1,040	1,377
	50	443	488	430

[a] Monocytes were added in increasing numbers to 10^6 Sephadex G-10 monocyte-depleted lymphocytes. Incubation was continuous for 9 days. Each value represents the average of duplicate tests. The data are representative of two such experiments.

sus was highly suppressed. Therefore, cultures were routinely depleted of monocytes for *A. viscosus* stimulation studies.

T-lymphocyte dependency. The requirement for T lymphocytes during *A. viscosus*-induced immunoglobulin stimulation was determined by adding increasing numbers of sheep erythrocyte-rosetted T lymphocytes to a constant number of B lymphocytes. The results (Table 3) indicate that the immunoglobulin response to both *A. viscosus* and PWM increased in proportion to the number of T lymphocytes added. *A. viscosus*-induced immunoglobulin production was more T-lymphocyte dependent than was PWM stimulation, since the PWM-induced response was detected in cultures with lower numbers of T lymphocytes.

***A. viscosus*-induced immunoglobulin production from healthy adults.** The general nature of *A. viscosus*-induced immunoglobulin stimulation was assessed using lymphocytes from 15 adult donors free of periodontal disease. The immunoglobulin response to PWM was also assessed under the same conditions. Both *A. viscosus* and PWM induced significant stimulation of IgA, IgG, and IgM compared to unstimulated control cultures (Table 4). PWM induced higher levels of IgA and IgG in these experiments, whereas *A. viscosus* generally stimulated higher levels of IgM. Responses to *A. viscosus* and PWM varied from individual to individual. For example, donors 238, 282, and 283 responded better to *A. viscosus* than to PWM, donor 274 responded better to PWM than to *A. viscosus*, and donors 245-A, 245-B, and 273 responded equally well to both *A. viscosus* and PWM.

Ability of *A. viscosus* T14-V to induce immunoglobulin synthesis. The ability of a strain of *A. viscosus* other than our strain GA to induce immunoglobulin production was tested. *A. viscosus* strain T14-V, kindly provided by Benjamin F. Hammond, University of Pennsylvania, was grown and a soluble ultrasonicated preparation was made exactly as described for strain GA in Materials and Methods. Both *A. viscosus* (GA) and *A. viscosus* (T14-V) were able to stimulate immunoglobulin synthesis, though in this experiment, strain GA induced a somewhat better immunoglobulin response than did strain T14-V (Table 5).

Specificity of immunoglobulins. *A. viscosus*-induced immunoglobulins were assayed for specific antibodies to *A. viscosus* (GA). The micro-ELISA system used had a sensitivity of at least 1 ng of antibody to *A. viscosus* per ml of culture supernate (S. Doty, D. E. Lopatin, and F. N. Smith, submitted for publication). Nevertheless, we repeatedly failed to detect IgA, IgG, or IgM specific for *A. viscosus* in any of the *A. viscosus*-stimulated cultures, though these same cultures had as much as 7,000 ng of immunoglobulin per ml. These findings suggest that *A. viscosus* is a polyclonal B-lymphocyte activator.

TABLE 3. *T-lymphocyte dependency of A. viscosus-induced immunoglobulin production*[a]

Lymphocytes	Ratio (B/T)	ng/ml		
		IgA	IgG	IgM
Unsep	1:5	<488	<125	264
Unsep + *A. viscosus*	1:5	2,894	1,195	8,914
Unsep + PWM	1:5	6,363	1,950	12,670
B	33:1	<488	<125	<243
B + *A. viscosus*	33:1	<488	<125	248
B + PWM	33:1	<488	298	5,243
B + T_1	10:1	<488	<125	243
B + T_1 + *A. viscosus*	10:1	<488	<125	592
B + T_1 + PWM	10:1	5,528	>2,000	>54,973
B + T_2	5:1	520	<125	561
B + T_2 + *A. viscosus*	5:1	<488	<125	1,389
B + T_2 + PWM	5:1	6,068	>2,000	41,086
B + T_3	1:1	<488	<125	1,266
B + T_3 + *A. viscosus*	1:1	877	1711	16,428
B + T_3 + PWM	1:1	15,503	>2,000	53,122
T_3	1:99	<488	<125	<243
T_3 + *A. viscosus*	1:99	<488	<125	<243
T_3 + PWM	1:99	<488	<125	<243

[a] Monocyte-depleted, unseparated (Unsep), and sheep erythrocyte rosette-separated B and T lymphocytes were cultured with *A. viscosus* (1,000 μg/ml), PWM (1/100), or media alone (control) for 4 days. The cells were then washed, resuspended in fresh media without stimulants, and incubated for an additional 5 days. Cell concentrations: Unsep = 1×10^6/ml; B = 1×10^6/ml; $T_1 = 0.1 \times 10^6$/ml; $T_2 = 0.2 \times 10^6$/ml; $T_3 = 1 \times 10^6$/ml. Results are the average of duplicate tests. The data represent two such experiments.

TABLE 4. *A. viscosus-induced immunoglobulin synthesis by lymphocytes from normal healthy individuals*[a]

Donor no.	Control			*A. viscosus* (1,000 μg/ml)			PWM (1:100)		
	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM
238	96[b]	883	985	803	2,471	6,061	165	864	1,156
245-A	25	251	415	450	1,216	4,665	510	1,214	3,379
245-B	205	784	1,212	275	1,081	2,544	654	1,188	2,300
249	171	547	510	289	1,092	1,248	1,241	2,320	2,275
252-B	0	0	216	0	323	932	15	450	2,764
259	0	585	2,723	36	1,339	7,316	321	1,141	3,608
273	0	14	0	19	685	1,082	155	764	777
274	51	137	284	80	619	721	650	7,998	6,963
275-A	ND[c]	19	ND	ND	2,081	ND	ND	4,439	ND
275-B	ND	110	ND	ND	722	ND	ND	2,861	ND
278-A	ND	174	ND	ND	550	ND	ND	562	ND
278-B	ND	108	ND	ND	2,128	ND	ND	1,937	ND
280	223	172	426	89	783	837	1,039	766	1,857
282	84	38	576	523	981	4,416	134	47	92
283	86	58	1,094	671	1,476	6,140	366	178	255
Mean	79	259	767	294	1,123	3,269	477	1,782	2,311
SEM[d] ±	26	76	226	85	177	756	118	535	586
P (*t*-test)[e]	—	—	—	0.01	0.005	0.005	0.01	0.01	0.05

[a] Monocyte-depleted lymphocytes (10^6) were incubated with *A. viscosus* (1,000 μg/ml), PWM (1/100), or media (control) for 2 days. The cells were washed, resuspended in fresh media lacking stimulants, and incubated for an additional 7 days.

[b] Nanograms per milliliter.

[c] ND, Not determined.

[d] SEM, Standard error of the mean.

[e] Statistical difference from controls ($P<$).

TABLE 5. *Immunoglobulin response to two A. viscosus strains*[a]

Stimulant	μg/ml	ng/ml		
		IgA	IgG	IgM
None		86	58	1,094
A. viscosus	1,000	671	1,476	6,140
(GA)	100	190	437	1,428
	10	163	126	1,169
A. viscosus	1,000	310	687	3,686
(T14-V)	100	133	304	971
	10	163	123	1,046

[a] Monocyte-depleted lymphocytes (10^6) were cultured with various concentrations of the *A. viscosus* preparations or with media alone (control) for 2 days. The cells were washed, resuspended in fresh media without *A. viscosus*, and incubated for an additional 7 days. The results are the average of duplicate tests. The data are representative of a single experiment.

DISCUSSION

A. viscosus is a nonspecific activator of in vitro DNA synthesis (11) and antibody formation in murine B lymphocytes (6, 8). However, in the human system, *A. viscosus* is a weak stimulant of peripheral blood lymphocytes from healthy individuals, though a more effective stimulant of cells from donors with periodontal disease (2, 32, 33). Previous studies from our laboratory show that *A. viscosus* stimulates low levels of DNA synthesis in lymphocytes from healthy individuals (23) which can be enhanced by adding PWM-induced helper lymphocytes to the cell culture (D. E. Lopatin et al., submitted for publication). Our current study reports that, even without PWM-induced helper activity, *A. viscosus* is a potent stimulator of in vitro immunoglobulin synthesis by peripheral blood lymphocytes from non-periodontally diseased adults. The immunoglobulin stimulation appears to be of the nonspecific, polyclonal type because: (i) we were unable to detect (<1 ng/ml) antibodies specific for *A. viscosus*, and (ii) the magnitude of the immunoglobulin secretion induced by *A. viscosus* was frequently equal to or greater than that induced by PWM, a known polyclonal B-cell activator (13).

Though *A. viscosus* nonspecifically stimulates polyclonal B-cell immunoglobulin production, the kinetics of *A. viscosus*-induced DNA synthesis by peripheral blood lymphocytes from healthy donors suggest that *A. viscosus* acts as an antigenic stimulus and not as a nonspecific mitogen (23). Together, these results indicate that *A. viscosus* can nonspecifically stimulate B-cell immunoglobulin production without inducing a corresponding nonspecific (mitogenic) increase in DNA synthesis. This paradox in B-cell biology is not unusual. Many investigators have reported that B cells have distinct pathways for

activation of DNA synthesis and immunoglobulin synthesis (8, 14, 18, 35). For example, Ringdén et al. (35) reported that *Staphylococcus aureus* Wood 46 and lipopolysaccharide induce immunoglobulin secretion in the absence of B-cell DNA synthesis. Whether DNA synthesis and immunoglobulin production represent activation of different subpopulations of B cells or a differential activation of the same B cell remains to be determined. Nevertheless, these results emphasize the importance of examining several forms of B-cell stimulation in identifying a polyclonal B-cell activator.

It is notable that *A. viscosus* and *Nocardia* are taxonomically related organisms and that both organisms possess the ability to stimulate polyclonal human B-cell activation (4, 19). This may indicate that these organisms share a common cell component(s) involved in immunoglobulin stimulation. However, since many bacteria are reported to non-specifically activate B cells (3), the biologically active factor is probably not unique to *Actinomyces* and *Nocardia* species. An immunoglobulin stimulating factor appears to be common to at least two *A. viscosus* strains (Table 5). Current studies in our laboratory are investigating the biochemical nature of the *A. viscosus* active component.

Unlike *Nocardia* (4, 19), immunoglobulin stimulation by *A. viscosus* is T cell dependent. This finding parallels other T-cell-dependent activators of murine B cells, such as purified protein derivative (30) and lipopolysaccharide (18), which require T-cell participation to activate human B cells.

Increased immunoglobulin production was obtained when, after 2 days of incubation, *A. viscosus* was removed from the cultures. An explanation for this phenomenon is as yet unavailable. We have found in previous studies that lymphocytes cultured with *A. viscosus* (1,000 μg/ml) for up to 3 weeks do not differ from unstimulated control cultures in viability as determined by trypan blue exclusion (unpublished data). Therefore, *A. viscosus* toxicity does not seem to be a likely explanation for decreased immunoglobulin production. It can be speculated that since *A. viscosus*-induced immunoglobulin synthesis is T cell dependent, the B-cell activation may be influenced by immunostimulatory (helper) T cells early (during the first 2 days) in culture and by immunosuppressive (suppressor) T cells later (after 2 days) in culture. Suppressor T cells comprise a small percentage of circulating T cells, whereas helper T cells are present in higher numbers (36). *A. viscosus* may stimulate suppressor T-cell proliferation which, only after several days in culture, results in the generation of enough suppressor cells in the culture to inhibit immunoglobulin production. This supposition, however, assumes that (i) *A. viscosus* either does not activate helper T-cell proliferation or activates a much faster rate of suppressor T-cell proliferation, and (ii) *A. viscosus* must be continuously present in the cultures to stimulate suppressor T cells. Preliminary experiments in our laboratory indicate that *A. viscosus* can generate such suppressor cells. Alternatively, constant stimulation of lymphocytes by *A. viscosus* for 9 days may cause a substantial depletion of necessary nutrients in the culture media, resulting in lower immunoglobulin secretion. An explanation for the suppressed immunoglobulin response is currently under investigation in our laboratory.

The removal of monocytes from the lymphocyte preparations substantially enhanced the immunoglobulin response to *A. viscosus*. Antibody secretion stimulated by lipopolysaccharide (18), PWM, staphylococcal protein A, concanavalin A, and phytohemagglutinin (27) is also enhanced by monocyte depletion. Monocytes secrete soluble mediators, including prostaglandins, cyclic adenosine monophosphate, and interferon, which suppress lymphocyte activity (1). The activation of monocytes by *A. viscosus* (31) may induce release of such factors which suppress B-cell immunoglobulin production.

The mechanisms of B-cell activation by *A. viscosus* and PWM appear to be somewhat different when examined under identical culture conditions. Compared to activation by PWM, B-cell activation by *A. viscosus* is more T cell dependent and more susceptible to monocyte inhibition. In addition, *A. viscosus*-induced immunoglobulin production shows two peak periods of IgA and IgG synthesis and one peak for IgM synthesis (Fig. 4). Our results, as well as the results of others (34), indicate that PWM induces only one peak period of synthesis for all three immunoglobulin subclasses. Furthermore, the presence of *A. viscosus* in the cultures for longer than 2 days tends to lower immunoglobulin production, whereas immunoglobulin production induced by PWM appears to be enhanced when PWM remains in the cultures for the entire 9 days (data not shown).

Actinomycotic (5) and periodontal (26, 37) lesions are characterized by the accumulation of a large number of B lymphocytes. Induction of B-cell immunoglobulin production may exacerbate the inflammatory response leading to tissue damage and bone resorption. Immunoglobulins produced during periodontal disease have blocking and unblocking effects on the immune response to antigens (16), form immune complexes (29), activate complement (29), stimulate B-cell lymphokine release (25), and participate in an-

aphylactic-type allergic reactions (28). Thus, the investigation of polyclonal B-cell activation by *A. viscosus* may provide a better understanding of the complex mechanisms involved in the etiology of periodontal disease.

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