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Chlamydia trachomatis Stimulates Human Peripheral Blood B Lymphocytes to Proliferate and Secrete Polyclonal Immunoglobulins In Vitro

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Infectious *Chlamydia trachomatis* (LGV strain), obligate intracellular bacteria, stimulated human peripheral blood lymphocytes to proliferate and secrete immunoglobulins in vitro. In contrast, mock-infected preparations were unable to induce similar responses in peripheral blood lymphocytes. Although levels of immunoglobulin secreted into the media of LGV-stimulated cultures were greater than 10 μg/ml, we estimated that less than 1% of these molecules were directed against the bacteria itself, suggesting polyclonal antibody production. Since stimulation with Formalin-killed bacteria resulted in comparable numbers of plaque-forming cells (PFC) as infectious particles, we concluded that the polyclonal immunoglobulin response was not dependent on the in vitro chlamydial infectious process. The polyclonal PFC response induced by LGV was highly sensitive to monocyte inhibition. Although LGV induced proliferation of predominantly B cells, the numbers of generated PFC was increased by the addition of autologous T cells. Neither lymphocyte proliferation nor PFC responses of normal human volunteers correlated significantly with the presence or titer of antichlamydial antibodies in their sera.

Chlamydia trachomatis are obligate intracellular bacteria whose various serotypes are associated with a variety of human diseases (e.g., trachoma, inclusion conjunctivitis, nongonococcal urethritis, lymphogranuloma venereum [LGV], and infant pneumonia) (37). Chlamydiae have a complex life cycle involving an infectious nonmetabolically active form (elementary body) and a noninfectious metabolically active stage (reticulate body). This growth pattern may be responsible for latency or inapparent infections as well as chronic, persistent infections that are commonly observed in human chlamydia-associated diseases.

The role of the immune system in protecting individuals against reinfection or in resolving ongoing infections by chlamydiae is unclear. Both humoral and cell-mediated immunity induced by chlamydiae have been proposed as major factors involved in limiting chlamydial infections in animals (24, 25, 43, 47). However, caution should be exercised when extrapolating from these systems to humans; many of the strains of chlamydiae as well as the natural history of illness in animal models differ in human infections (23, 31, 37).

Chlamydiae can induce human cell-mediated immune responses in vivo (Frei skin test for delayed-type hypersensitivity) and in vitro (blast transformation and chlamydiacidal effect by polymorphonuclear leukocytes) (8, 15, 49). Antibodies to *C. trachomatis* have been detected in serum, tears, and genital secretions in humans. Individuals with the disease LGV often show profound hypergammaglobulinemia, especially of the immunoglobulin A (IgA) isotype (26). Elevated serum immunoglobulin (total immunoglobulin) is also characteristic of infant pneumonitis associated with *C. trachomatis* infections (4, 44). Such infants possess dramatic elevations of peripheral blood B lymphocytes and plasma cells and secrete large quantities of immunoglobulins spontaneously in vitro (D. Levitt, R. W. Newcomb, and M. O. Beem, Clin. Immunol. Immunopathol., in press).

To further define the mechanisms by which chlamydiae perturb the human immune system, we have undertaken an investigation of both the antibody and proliferative responses of normal human peripheral blood lymphocytes (PBL) to *C. trachomatis* in vitro. Our results demonstrate that *C. trachomatis* is a potent polyclonal B-cell activator of human PBL for both immunoglobulin secretion and DNA synthesis. We define several of the mechanisms of this activation and compare responses induced by *C. trachomatis* with those stimulated by two other polyclonal activators, pokeweed mitogen (PWM) and *Staphylococcus aureus* Cowan I.

MATERIALS AND METHODS

Isolation and purification of chlamydiae. The LGV strain (L_2/434/Bu) of *C. trachomatis* was obtained from J. Schachter, University of California, San Francisco. McCoy cells, a mouse fibroblast cell line, were infected with 10 50% infectious doses of *C. trachomatis* such that more than 95% of the host cells were infected, according to Hatch's method for titrating chlamydial infectivity (16). Monolayers were incubated for 1.5 h at 37°C on a shaker to allow attachment of bacteria. Fifteen milliliters of growth medium (modified Eagle medium containing 10% fetal calf serum [FCS] and 250 μg of streptomycin sulfate per ml) plus 0.5 μg of cycloheximide per ml (35) was added to washed monolayers. Chlamydiae were harvested after 48 to 60 h, at which time cell lysis or inclusion bodies within all attached cells were observed by phase-contrast microscopy. Mock-infected preparations (MI) used as controls in this study were initiated as described above, except that medium without bacteria was added to monolayers. All subsequent steps were performed at 4°C.

The method for isolation and purification of chlamydiae was modified, as follows, from Lee (27). (i) Human serum albumin (0.5%) was used instead of 2% FCS. (ii) A 60:32:20% Renografin-step gradient (Renografin [76%],

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methylglucamine diatrizoate; E. R. Squibb & Sons, Princeton, N.J.) centrifuged at 90,000 × g for 2 h was used to purify LGV or MI instead of a 5 to 60% continuous gradient centrifuged for 4 h at 100,000 × g.

The interface between 60 and 32% containing LGV elementary bodies was collected, pooled, washed twice, and suspended in sucrose-phosphate buffer (0.2 M sucrose in 0.2 M potassium phosphate buffer [pH 7.2]) with 0.5% human serum albumin. The samples were then divided into portions and stored at −70°C.

Preparation of PBMC. Peripheral blood from normal adult donors was sedimented on Ficoll-Hypaque gradients to separate mononuclear leukocytes (PBMC) (5). Samples were obtained from healthy adult blood bank donors (age and sex unknown) and laboratory personnel (male to female ratio, 60:40; ages, 22 to 60 years). Cells from a single known donor used in different experiments were usually obtained on different days. Cells were washed extensively with Hanks balanced salt solution plus 10% FCS and suspended in complete medium (RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 250 μg of streptomycin sulfate per ml).

In most experiments, monocytes were removed from PBMC by either adherence to plastic for 1 h at 37°C or passage through Sephadex G-10 (18). Nonadherent cells contained fewer than 5% monocytes, as determined by fluorescent staining with anti-human monocyte antibody OKM1.

Fluorescent staining of cells. Characterization of PBMC subpopulations was performed by reacting PBMCs with fluorochrome-conjugated antibodies (13). B cells were detected by using fluorescein-conjugated goat antihuman immunoglobulin (polyvalent; Meloy Lab; Springfield, Va.) or antihuman μ (μ-chain specific; affinity purified); percentages of total T cells and monocytes/null cells were determined by using monoclonal antibodies OKT3 and OKM1, respectively (both from Ortho Diagnostics, Raritan, N.J.), followed by fluorescein-conjugated goat antimouse IgG (Bionetics, Kensington, Md.) absorbed twice with human gamma globulins.

Separation of T- and B-cell fractions. T- and B-cell fractions were obtained by rosetting monocyte-depleted PBMC with aminoethylisothiouronium bromide-treated sheep erythrocytes, followed by separation on Ficoll-Hypaque gradients (33). The nonrosetted fraction contained 40 to 80% immunoglobulin-positive B cells, 20 to 40% $OKM1^+$ cells, and less than 2% T Cells ($OKT3^+$ cells). The rosetted cells were contaminated with fewer than 2% surface immunoglobulin-positive cells and less than 1% $OKM1^+$ cells. B-cell fractions were further separated into μ^+ and μ^- populations by using a fluorescence-activated cell sorter (FACS-II; Becton-Dickinson, Mountain View, Calif.). A total of 50×10^6 to 100×10^6 cells from the B-cell fraction were incubated with sterile, affinity-purified fluorescein-conjugated goat antihuman μ in Hanks balanced salt solution plus 5% FCS for 30 min at 0°C. After washing, cells were separated into μ^+ and μ^- populations sterilely with the fluorescence-activated cell sorter (30). Reanalysis of sorted cells demonstrated 95% purity of the μ^+ population and less than 5% contamination of μ^- cells with μ^+ B lymphocytes.

Culture conditions. PBMC ± monocytes were cultured in either 96-well microtiter plates or 24-well tissue culture plates (Costar, Cambridge, Mass.) at a density of 10^6 cells per ml (0.2 or 1.0 ml, respectively, final volume per well) for indicated culture periods in the presence or absence of stimuli.

Determination of DNA synthesis. To detect proliferative responses induced by LGV, cells were cultured for the indicated period in triplicate wells of a 96-well microtiter plate at a density of 10^6 or 0.5×10^6 cells per ml (0.2 ml final volume per well). [^{3}H]thymidine (1 μCi; 6.7 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) was added to each well, and cells were harvested 18 h later with a Titertek cell harvester (Flow Laboratories, McClean, Va.). Incorporation of label into DNA was measured with a Beckman liquid scintillation spectrophotometer.

Assays for measuring immunoglobulin. Two methods were used to analyze immunoglobulin secreted by cultured human PBLs. (i) total amounts of each immunoglobulin isotype (IgM, IgG, and IgA) were quantitated in culture medium after 7 days in vitro by using a solid-phase enzyme-linked immunosorbent assay (ELISA) as previously described in detail (28). Standard curves plotted on semilog graph paper were linear between 7.5 and 500 ng/ml for each immunoglobulin isotype. (ii) Individual immunoglobulin-secreting cells were determined by using the reverse hemolytic plaque-forming cell (PFC) assay (12, 14). Isotype-specific PFC responses were determined by substituting class-specific rabbit antihuman heavy-chain antisera (Dako, San Diego, Calif.) for the polyvalent antiserum.

For all antisera, specificity controls consisted of (i) reactivity against purified human myeloma proteins by Ouchterlony gel diffusion and (ii) ability to develop PFC from human lymphoblastoid cell lines secreting only single immunoglobulin classes (IgG, IgM, or IgA). In all cases, optimal titers and precise specificities of antisera were determined initially on the lymphoblastoid cell lines.

LGV and other activators. Purified *C. trachomatis* LGV was freshly thawed and diluted with complete medium for each experiment. The quantity of bacteria added to culture was determined by titration of infectivity in McCoy cells (see below). In addition, protein concentration of bacteria was determined by the method of Bradford (6) (Bio-Rad Laboratories, Richmond, Calif.). Thus, a dilution of 1:200 of purified *C. trachomatis* yielded 36% inclusion-containing McCoy cells and resulted in 0.8 μg of protein added to the 24-well plates and 0.16 μg protein added to the 96-well microtiter plates. A 1:200 dilution of the MI corresponded to 67.5 ng of protein added to the 24-well plates and 13.5 ng of protein in the 96-well microtiter plates. The addition of MI to McCoy cells yielded no inclusion-containing cells.

S. aureus Cowan I (Pansorbin; Calbiochem, La Jolla, Calif.) was fixed in 2% Formalin, washed three times, and suspended to 10% (vol/vol) in phosphate-buffered saline. Optimal responses were assessed by using PBL from different donors. PWM (GIBCO Laboratories, Grand Island, N.Y.) was diluted according to the manufacturer's instructions, and optimum dilutions were determined.

Determination of chlamydial infectious units. The amount of bacteria added to the culture was assayed by determining the infectivity of the chlamydiae preparations in McCoy cells by the method of Hatch (16). At least 300 cells over six fields were scored for the absence or presence of chlamydial inclusions. Results are expressed as percentage of McCoy cells containing inclusion bodies.

Determination of anti-LGV antibodies. To assess the presence of antibodies directed toward *C. trachomatis* in donor serum or culture medium, the indirect immunofluorescence method of Beem and Saxon (4) was employed. Ten-microliter portions of either fourfold serial dilutions of sera from donors or twofold serial dilutions of culture supernatant were used for each determination. Slides counterstained

with Evans blue dye were examined for fluorescence localized to cytoplasmic inclusion bodies with a Leitz Orthoplan fluorescent microscope. Fluorescence was graded 0 to ++++, where 0 = nil, + = dull, ++ = moderately bright, +++ = bright, and ++++ = very bright. Endpoint equivalence was defined as the highest dilution of serum that still exhibited +++ or greater intensity.

RESULTS

Quantitative polyclonal antibody secretion by human PBL stimulated with *C. trachomatis* determined by ELISA. The production and secretion of immunoglobulins in response to *C. trachomatis* was compared to immunoglobulin secretion induced by the extracellular bacteria *S. aureus* Cowan I (19, 34, 41) (Table 1). When purified infectious *C. trachomatis* was added to cultured human PBL, there were significantly elevated levels of IgG, IgM, and IgA secreted into the medium, compared to unstimulated cultures, as measured by ELISA. *S. aureus* induced significant increases in immunoglobulin secretion of all three isotypes, with quantities of immunoglobulin that were generally equivalent to or slightly greater than immunoglobulin secreted after chlamydial stimulation. The amount of immunoglobulin secreted varied among donors; six of nine volunteers responded to *C. trachomatis* by significant increases in immunoglobulin secretion (four representative examples are shown in Table 1). The range of stimulation by *C. trachomatis* was: IgG, 5- to >40-fold; IgM, 10- to >80-fold; and IgA, 2- to 40-fold. IgM was secreted in the greatest quantities relative to other immunoglobulin classes after *C. trachomatis* activation. MI did not induce significant immunoglobulin production above controls. Therefore, these data suggest that chlamydiae, similar to *S. aureus*, stimulate significant increases in immunoglobulin secretion of all three isotypes by human PBL in vitro.

Evaluation of the number of immunoglobulin-secreting cells stimulated by *C. trachomatis* in human peripheral blood. The ELISA measures total immunoglobulin secreted during culture periods. To analyze the total number of immunoglobulin-secreting cells induced by chlamydiae in vitro, we used a reverse hemolytic PFC assay. The PFC response peaked on day 5, followed by either a dramatic (1 day) or gradual (2 to 4 days) decrease to background levels. The peak response for PFC induced by another bacterium, *S. aureus*, also occurred on day 5 and for PWM between days 5 and 7, followed by a gradual decrease in numbers of PFC (data not shown).

TABLE 2. Isotype distribution of PFC response to *C. trachomatis*

Donor	Isotype[a]	PFC/culture[b]			
		None	*C. trachomatis*	PWM	*S. aureus*
LC_1	Total	170	4,920	3,240	7,830
	IgM	60 (43)	4,080 (67)	90 (3)	2,340 (29)
	IgG	50 (36)	1,320 (22)	1,620 (56)	5,160 (64)
	IgA	30 (21)	660 (11)	1,160 (41)	520 (7)
DR_1	Total	40	1,590	ND[c]	1,620
	IgM	10 (20)	990 (61)		630 (32)
	IgG	10 (20)	210 (13)		660 (33)
	IgA	30 (60)	420 (26)		690 (35)
JB_1	Total	130	6,020	ND	11,280
	IgM	20 (40)	3,360 (66)		3,640 (41)
	IgG	20 (40)	1,160 (23)		4,020 (45)
	IgA	10 (20)	600 (11)		1,180 (14)
LW_1	Total	100	5,420	ND	4,530
	IgM	90 (64)	4,020 (80)		2,130 (51)
	IgG	10 (7)	320 (6)		1,920 (46)
	IgA	40 (29)		700 (14)	120 (3)
SN	Total	70	1,380	740	7,410
	IgM	10 (18)	1,120 (67)	160 (25)	1,170 (17)
	IgG	15 (27)	120 (7)	230 (35)	4,230 (63)
	IgA	30 (55)	420 (26)	260 (40)	1,290 (20)

[a] Total immunoglobulin PFC were detected by using polyvalent developing antisera. PFC responses of each isotype were assayed by using antisera specific for each heavy-chain isotype.

[b] Monocyte-depleted PBL were cultured for 5 days in 24-well plates in the presence of stimulus. At the end of the culture period, cells were harvested and diluted 10-, 20-, or 30-fold, and PFC were determined by the reverse hemolytic plaque assay as described in the text. *C. trachomatis*, *S. aureus*, and PWM were used at final dilutions (vol/vol) of 1:2,000, 1:100,000, and 1:400, respectively. Values in parentheses represent percentage of total immunoglobulin PFC response.

[c] ND, Not done.

Similar to results from ELISAs, IgM was the predominant isotype secreted by PFC stimulated with *C. trachomatis* (Table 2). In all individuals, IgM-PFC accounted for more than 60% of the total PFC. In contrast, IgM-PFC were usually less than 50% of the total PFC induced by either *S. aureus* or PWM (Table 2).

TABLE 1. Induction of immunoglobulin (IgG, IgM, and IgA) secretion in human PBL by *C. trachomatis* in vitro

Stimulant	Immunoglobulin secretion[a] (ng/ml)											
	Donor RW_1			Donor MB_1			Donor BB_1			Donor DL_1		
	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA
None	680	1,220	100	40	190	100	110	520	610	140	<60	100
LGV[b]	7,200	12,800	3,700	460	>16,000	2,840	>4,000	>16,000	1,300	740	1,930	280
MI[c]	750	2,010	150	30	145	110	200	380	380	ND[d]	ND	ND
S. aureus[e]	1,610	>16,000	3,040	>4,000	>16,000	3,160	>4,000	>16,000	>8,000	3,360	10,000	1,070

[a] The media from monocyte-depleted human PBL cultured for 7 days in 24-well plates were assayed for immunoglobulin secretion by ELISA. Data are given in as nanograms of immunoglobulin per milliliter of culture supernatant. Cells from a single known donor used in different experiments were usually obtained on different days, as indicated by a subscript next to the donor's initials (e.g., cells from DL_1 were obtained on a different day than DL_2 cells).

[b] *C. trachomatis* LGV was used at a final dilution (vol/vol) of 1:240 for donors 1 to 3 and 1:200 for donor 4.

[c] Same final dilution as for *C. trachomatis*.

[d] ND, Not done.

[e] *S. aureus* was used at a 1:100,000 (vol/vol) final dilution.

The PFC response to chlamydiae varied greatly among individuals (Table 3). However, no person was completely unresponsive to the stimulatory effect of *C. trachomatis*. The relative numbers of PFC generated by *C. trachomatis* were similar to those induced by another bacteria, *S. aureus*. Individuals producing large numbers of PFC after *C. trachomatis* stimulation in vitro also produced elevated numbers of PFC after *S. aureus* stimulation. In contrast, PWM, a nonbacterial plant lectin, did not reveal a pattern similar to either bacteria. Certain individuals produced no increases in PFC after PWM stimulation (Table 3, donors DR_1 and BB5). Therefore, a similar mechanism for PFC induction may exist for both bacteria.

Infectious chlamydiae are not necessary for generation of polyclonal PFC responses. To analyze whether viable or infectious bacteria were needed to induce immunoglobulin secretion by PBL, a sample of *C. trachomatis* was treated with 2% Formalin in phosphate-buffered saline for 1 h. Bacteria were washed, resuspended to their original volume, and added to cultures. Mock fixations of *C. trachomatis* were performed with phosphate-buffered saline instead of Formalin simultaneously. As shown in Table 4, Formalin fixation failed to prevent PFC induction by *C. trachomatis* compared to mock-fixed bacteria. The diminution of PFC compared to untreated samples appeared to be caused by loss of bacteria during sample handling, as indicated by a reduction in infectivity of mock-fixed preparations (Table 4). The Formalin-fixed bacteria did not produce any inclusion-containing McCoy cells, demonstrating their noninfectious nature. Thus, infectious particles are not necessary for the generation of PFC after *C. trachomatis* stimulation.

Supernatants from *C. trachomatis*-stimulated cultures do not contain detectable antichlamydial antibodies. To analyze the contribution that antichlamydial antibodies make to the total immunoglobulin secreted by cultured cells, indirect immunofluorescent assays were performed on culture supernatants. We have calculated that this assay can detect positive fluorescence when as little as 1.5 ng of total serum IgG is analyzed. Despite evaluating cultures in which as much as 200 ng of immunoglobulin was assayed, no positive fluorescence could be detected. Positive standard serum samples (generously provided by M. Beem, University of Chicago) revealed intense fluorescence, even at a dilution of 1:65,000. Therefore, less than 1% of the total culture immunoglobulin contained antichlamydial antibodies.

TABLE 3. PFC repsonses of human PBL stimulated in vitro

Donor	ΔPFC/culture[a]			
	MI	*C. trachomatis*	*S. aureus*	PWM
BB2	ND[b]	340	460	1,620
BB3	ND	670	600	130
DR_1	ND	1,550	1,580	0
PG	ND	1,920	910	600
BB4	0[c]	2,895	8,775	6,315
DL_2	100	3,620	7,690	1,680
BB_5	0	4,320	6,860	0
LC_1	ND	4,750	7,660	3,070
LW_1	ND	5,320	4,430	ND
BB6	270	5,870	11,430	2,870
JB_1	ND	5,890	11,150	ND

[a] Monocyte-depleted PBL were cultured for 5 days in 24-well plates in the presence of stimulus. At the end of the culture period, cells were harvested and assayed for PFC by using polyvalent developing antisera as described in the text. ΔPFC/culture = PFC per culture in the presence of stimulant − PFC per culture with medium alone (unstimulated). Numbers of PFC in unstimulated cultures ranged from 20 to 630 PFC per culture. Final dilutions of stimulants are given in footnote *b* of Table 2. MI were used at the same final dilution (vol/vol) as *C. trachomatis* samples.

[b] ND, Not done.

[c] If PFC per culture was <0, it was considered 0.

TABLE 4. PFC responses induced by Formalin-fixed *C. trachomatis*

Stimulation	Dilution	Fixation[a]	PFC/culture[b]				% Infectivity[c]
			JB_1	DL_2	DR_1	BL	
None			130	20	40	180	
LGV	1:200	−	6,020	3,640	1,590	2,940	36
LGV	1:500	−	4,920	3,380	1,040	1,600	18
LGV	1:200	+	4,420	3,040	920	1,360	0
LGV	1:200	*	4,140	2,740	840	1,400	11
MI	1:200	*	40	120	ND[d]	ND	0
MI	1:200	+	80	40	ND	ND	0

[a] +, Formalin fixed; −, untreated preparation; *, Mock Formalin fixed as described in the text.

[b] Culture conditions were the same as those described for Table 2.

[c] Values indicate percentage of McCoy cells (infected with the indicated dilution of fixed or untreated chlamydiae) containing inclusion bodies when examined after Giemsa stain as described in the text.

[d] ND, Not done.

Monocytes inhibit the PFC responses of human PBL to *C. trachomatis*. Monocytes have been shown to play critical roles during an immune response (45). Thus, we investigated the importance of monocytes during *C. trachomatis*-stimulated PFC responses. Monocytes were removed from the PBMC fraction by either passage through Sephadex G-10 or adherence to plastic dishes; the eluate or nonadhered cells represented the monocyte-depleted fraction. Greater than 90% of plastic-adhered cells were nonspecific esterase positive. As shown in Fig. 1, generation of PFC after C. trachomatis stimulation was significantly greater in monocyte-depleted cultures than undepleted samples ($P < 0.05$). In both cases, there was a significant increase in PFC after *C. trachomatis* stimulation compared with unstimulated MI cultures ($P < 0.05$ for both).

A similar effect of monocytes on *S. aureus*-driven PFC formation was also observed (Fig. 1). Significantly fewer PFC occurred before depletion of adherent cells than after passage through G-10 columns ($P < 0.01$). In contrast, removal of monocytes did not affect PFC generation by PWM-stimulated PBL (Fig. 1; $P > 0.1$). Thus, the effect of monocytes on PFC development differs substantially depending on the type of activation employed in vitro.

Polyclonal PFC response induced by *C. trachomatis* is enhanced by T cells. We analyzed whether T cells were necessary for induction of PFC in *C. trachomatis*-stimulated PBL cultures. As mentioned previously, T-cell fractions contained greater than 95% $OKT3^+$ cells and fewer than 2% immunoglobulin-positive cells, whereas B-cell fractions possessed 40 to 80% immunoglobulin-positive cells, 20 to 40% $OKM1^+$ cells (after passage of PBMC through G-10), and fewer than 2% $OKT3^+$ cells.

When the B-cell fraction was stimulated with *C. trachomatis*, increases in PFC above background occurred (Table 5). However, the addition of autologous T cells to B cells increased this response substantially. Maximal PFC generation was found at T to B cell ratios of 7:3 (similar to the T to B cell ratio in adult PBL). A small number of PFC developed in the B-cell fraction after PWM and *S. aureus* stimulation.

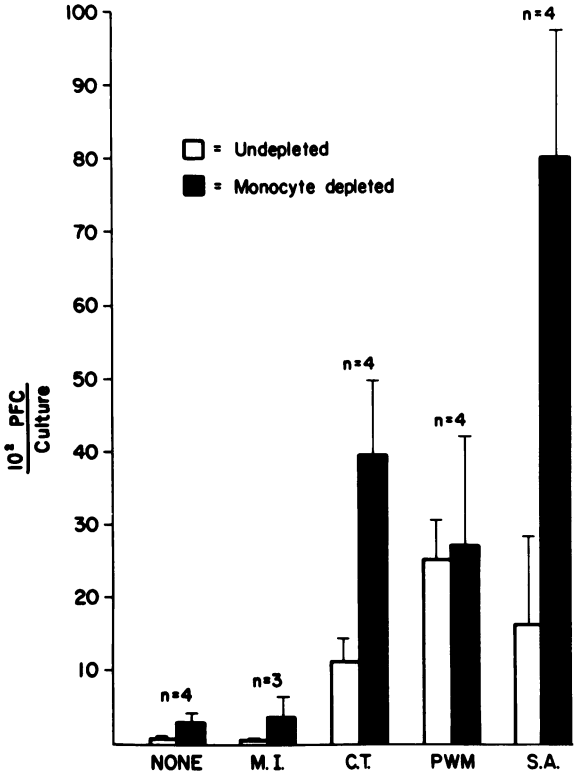

FIG. 1. Effect of monocytes on the PFC response of PBL to *C. trachomatis*. Human PBL were either depleted or not depleted of monocytes and cultured in the presence of the indicated stimuli. NONE, unstimulated; MI, 1:200 (vol/vol); C. T., *C. trachomatis*, 1:200 (vol/vol); PWM, 1:400 (vol/vol); and S. A., *S. aureus*, 1:200,000 (vol/vol). On day 5 of culture, cells were assayed for PFC. Bars represent mean PFC per culture ± standard error. The number of volunteers for each experiment is given above the bars.

The number of PFC increased, however, after T cells were added to cultures and was optimal at T to B cell ratios of 7:3 for *S. aureus* and 3:7 or lower for PWM. Thus, T lymphocytes markedly enhanced PFC development after *C. trachomatis* stimulation in a manner similar to the better characterized bacterial activator, *S. aureus* (19).

***C. trachomatis* stimulates proliferation predominantly in the B-cell fraction.** Other workers have demonstrated that *C. trachomatis* can stimulate DNA synthesis by human PBL (8, 15); however, it is not known which PBMC subpopulations can proliferate in response to *C. trachomatis*. We analyzed proliferation of the B-cell-enriched fractions and T-cell-enriched fractions after stimulation by *C. trachomatis*; these responses were compared with proliferation induced by PWM, a T-cell mitogen (46), and *S. aureus*, a B-cell mitogen (41, 46).

Unseparated PBL exhibited peak stimulation indices (S.I. = counts per minute of stimulated cultures/counts per minute of unstimulated cultures) by day 3 in vitro (data not shown). Thymidine incorporation plateaued at day 3, whereas unstimulated (background) DNA syntheses increased due to culture conditions. Thus, the peak stimulation of *C. trachomatis*-treated cultures occurred 48 h before maximal PFC development.

PWM induced the greatest incorporation of [^{3}H]thymidine (absolute increases in counts per minute or S.I.) in comparison to *S. aureus* and *C. trachomatis* in either unseparated PBL or T-cell fractions (Table 6). In contrast, *S. aureus* and *C. trachomatis* stimulated minimal proliferation in this fraction. Thus, *C. trachomatis* induced significantly greater increases in [^{3}H]thymidine incorporation in B-cell fractions than in T-cell fractions when either absolute counts per minute or S.I. were compared ($P < 0.01$ for both analyses).

It was unclear which cells in the B-cell fraction were proliferating in response to *C. trachomatis*. In addition, the absence of T-cell proliferation might be due to lack of help from cells within the B-cell fraction. We addressed these questions by using cell-sorting techniques to separate the B-cell-enriched fraction into μ^+ cells (surface IgM$^+$ B cells) and μ^- cells (other cells, including surface IgG- and IgA-positive B cells). Virtually the entire proliferative response to *C. trachomatis* resides in the surface IgM$^+$ B-cell fraction (Table 7). The IgM$^-$ cells were stimulated only 2- to 3-fold (possibly due to IgG$^+$ and IgA$^+$ B lymphocytes), whereas surface μ^+ cells demonstrated a greater than 20-fold increase in [^{3}H]thymidine incorporation. Again, T cells failed to proliferate when stimulated with *C. trachomatis*. This lack of DNA synthesis by T cells was not due to the absence of accessory cells that were present in the B-cell fraction. When μ^- cells were added to T cells in various ratios, significant enhancement of DNA synthesis was not observed (Table 7). Therefore, *C. trachomatis* stimulates proliferation predominantly if not exclusively by B cells, and the inability to stimulate T cells is not due to the absence of accessory cells which reside in the B-cell fraction.

We evaluated whether a correlation existed between the number of PFC and the amount of proliferation for each PBL sample stimulated with *C. trachomatis* in vitro. When data from 12 different volunteers were analyzed (Fig. 2), there was a significant correlation ($r = 0.864$) between the numbers of PFC and the amount of proliferation ([^{3}H]thymidine incorporation) in the responding population. Although this correlation is highly significant ($P < 0.01$), we do not imply a direct linear relationship between PFC response and proliferative response. In fact, the dispersion of data points about the straight line may suggest a more complex relationship, which could involve interactions of monocyte and T-cell populations with B cells.

Lack of correlation between anti-LGV antibody levels in serum and PFC or proliferative responses to *C. trachomatis*.

TABLE 5. Effect of T cells on PFC response to *C. trachomatis*

Donor	Activator[a]	PFC/culture[b]						
		T cells	T to B cell ratio:				B cells	PBL
			9:1	7:3	3:7	1:9		
MB_2	None	80	40	90	100	40	60	40
	LGV	110	770	2,510	2,160	1,140	900	1,880
DL_3	None	0	130	270	50	80	220	310
	LGV	60	1,220	3,810	2,620	1,910	1,040	3,180
	PWM	0	30	1,760	3,480	4,620	680	1,160
RW_2	None	0	40	90	160	180	190	160
	LGV	10	920	5,740	2,520	2,340	1,980	4,920
	PWM	0	170	1,620	3,900	3,620	460	1,710
	S. aureus	40	1,180	3,930	1,050	1,140	720	3,180

[a] Final dilutions of each stimulus were the same as those in Table 2.

[b] Monocyte-depleted PBL were separated into T-cell-enriched and B-cell-enriched fractions. Each fraction and combinations of the two fractions at various ratios were cultured at 10^6 cells per ml for 5 days. At the end of the culture period, cells were assayed for PFC as described in the text. PBL indicates unseparated cells.

TABLE 6. Proliferative responses induced by *C. trachomatis*

Stimulant[a]	[3H]thymidine incorporation (ΔKcpm)[b]					
	No. of donors	Unseparated PBL	No. of donors	B-cell-enriched fraction	No. of donors	T-cell-enriched fraction
LGV	12	15.1 ± 3.2 (8.9 ± 1.0)	5	13.4 ± 3.0 (8.2 ± 0.6)	5	0.6 ± 0.3 (2.9 ± 0.7)
PWM	8	39.9 ± 6.5 (27.6 ± 5.6)	4	3.6 ±1.6 (2.7 ± 0.6)	4	16.4 ± 11.1 (41.8 ± 13.0)
S. aureus	11	27.5 ± 4.7 (15.9 ± 2.0)	3	25.0 ± 4.2 (11.8 ± 2.7)	3	4.7 ± 0.7 (15.1 ± 6.3)

[a] Final dilutions are indicated in Table 2.

[b] Unseparated PBL or B- and T cell-enriched fractions were cultured at 10^6 cells per ml in triplicate wells of a 96-well microtiter plate (0.2 ml per well, final volume). Cells were labeled for 18 h with [3H]thymidine on day 2. Data are represented as mean Δcpm from indicated number of donors ± standard error of the mean. Δcpm = mean cpm per well of stimulated cultures − mean cpm per well of unstimulated (medium alone) cultures. The counts per minute of triplicate sample wells varied less than 10% from the sample mean. Values in parentheses indicate mean stimulation index (S.I.) ± standard error of the mean from indicated number of donors. S.I. = mean cpm per well for stimulated cultures/mean cpm per well of unstimulated cultures.

We examined whether high or low PFC and proliferative responses induced by chlamydiae in vitro might be due to prior exposure to the bacteria. We therefore compared serum endpoint antichlamydial antibody titers (4) of individual donors with their PFC or proliferative responses. Using the indirect immunofluorescence technique, no significant correlation was seen between endpoint serum antichlamydial antibody titers and number of PFC ($r = 0.217$; $P > 0.1$) or DNA synthesis ($r = 0.283$; $P > 0.1$). Thus, the magnitude of either the PFC or proliferative response induced by *C. trachomatis* in vitro is not necessarily due to previous exposure to the bacteria.

DISCUSSION

Perturbation of the immune system by *C. trachomatis* has been well studied but incompletely characterized. Persistent infiltrates of lymphocytes and macrophages have been found in the conjunctiva and cornea during trachoma (25), and lymphoid hyperplasia is a significant component of LGV (39). Antibodies against chlamydiae and their surface components have been detected in tears, genital secretions, and serum of infected individuals (25, 37). During early infancy, a period normally associated with immune hyporesponsiveness, chlamydial pneumonia is associated with pronounced hyperglobulinemia (4, 44) and excessive numbers of B lymphocytes and plasma cells in the peripheral blood (Levitt et al., in press). This latter finding prompted the present study.

Our results demonstrate that *C. trachomatis* can induce B-cell differentiation to immunoglobulin-secreting plasmacytes when added to cultures of PBL. These bacteria stimulate B-cell proliferation and, in the presence of T lymphocytes, secretion of large quantities of immunoglobulins. In this manner, chlamydiae behave similarly to the extracellular bacteria *S. aureus* Cowan I (46). Few of the antibodies secreted are directed against the bacteria themselves.

The peak mitogenic response to chlamydiae preceded maximum immunoglobulin secretion in vitro by 2 days. This mitogenic effect occurs predominantly in the B-cell fraction. Such circumstances usually suggest the necessity of DNA synthesis for B-cell differentiation (11). Although certain polyclonal activators do induce B-lymphocyte proliferation (3, 46), others stimulate substantial differentiation with minimal thymidine incorporation (1, 34). With the methods we employed, we could not demonstrate whether DNA synthesis by B cells was necessary for plasma cell differentiation. However, it seems reasonable that *C. trachomatis* could expand numbers of B lymphocytes that are capable of receiving signals from T cells and develop into PFC.

A small, yet significant, number of PFC occurred in B-cell fractions stimulated with *C. trachomatis*. This T-cell-independent development of immunoglobulin-secreting cells could result from proliferation of a B-cell subpopulation that

TABLE 7. Proliferative responses of mononuclear cell subpopulations separated with a fluorescence-activated cell sorter

Cell fraction[a]	[3H]thymidine incorporation (Kcpm)[b]					
	No stimulant		LGV		PWM	
	MB_3	RW_3	1	2	1	2
PBL	ND[c]	0.4 ± 0.1	ND	2.8 ± 0.2	ND	12.9 ± 0.4
B cell[d]	1.0 ± 0.1	1.2 ± 0.6	6.4 ± 0.1	11.7 ± 0.3	2.1 ± 0.3	2.1 ± 0.3
B cell[e]	ND	0.9 ± 0.1	ND	7.5 ± 0.7	ND	2.0 ± 0.1
T cell	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	3.6 ± 0.3	1.3 ± 0.1
μ^-	1.0 ± 0.1	1.0 ± 0.1	2.5 ± 0.1	2.4 ± 0.1	3.2 ± 0.1	1.8 ± 0.1
μ^+	0.3 ± 0.0	0.7 ± 0.1	9.0 ± 1.1	15.8 ± 1.7	0.8 ± 0.0	0.8 ± 0.0
T + μ^- 1:1[f]	0.8 ± 0.0	ND	2.0 ± 0.0	0.9 ± 0.0	4.6 ± 0.0	ND
T + μ^+ 1:1[f]	0.3 ± 0.0	ND	3.9 ± 0.1	7.6 ± 0.2	3.9 ± 0.3	ND

[a] B-cell fractions from monocyte-depleted PBL were separated with a FACS-II cell sorter into μ^+ cells and μ^- cells. PBL indicates unseparated cells, B cell indicates B-cell-enriched fraction (unsorted), T cell indicates T-cell-enriched fraction, and μ^+ and μ^- indicate μ^+ and μ^- subpopulations of the B-cell fraction, respectively.

[b] Cell fractions were cultured at 0.5×10^6 cells per ml in triplicate wells of 96-well microtiter plates (0.2 ml per well, final volume). Cells were labeled for 18 h with [3H]thymidine on day 2. Data are given as mean cpm ± standard error of the mean. Final dilutions of stimulants are indicated in Table 2.

[c] ND, Not done.

[d] Unstained B-cell fraction.

[e] B-cell fraction stained with anti-human μ but not sorted.

[f] T cells were added to either μ^- or μ^+ cells at a ratio of 1:1 and a final cell concentration of 0.5×10^6 cells per ml (0.2 ml per well).

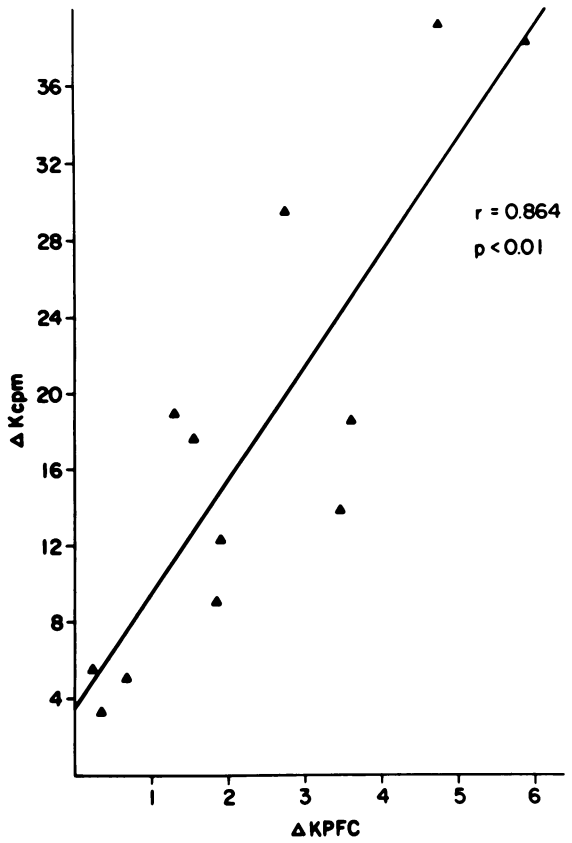

FIG. 2. Significant correlation between the PFC response of human PBL to *C. trachomatis* and the proliferative response. The PFC response (see Table 3) and the proliferative response (see Table 6) from each volunteer (n = 12) were compared. The line represents the calculated linear regression.

is already secreting immunoglobulin or B cells recently activated by other means (12). The number of PFC in *C. trachomatis*-stimulated cultures was, however, enhanced by the addition of autologous T cells. The optimal T to B cell ratio for generation of PFC was 7:3 for both bacterial polyclonal activators (*S. aureus* and *C. trachomatis*). In contrast, a lower ratio of T to B cells was necessary for maximum development of PFC in PWM-stimulated cultures. The difference in optimum T to B cell ratios between PWM cultures versus *S. aureus*- and *C. trachomatis*-stimulated cultures could be due to the induction of suppressor T cells in the former cultures which would reduce the PFC response (46).

The predominant heavy-chain isotype produced by *C. trachomatis*-stimulated PBL is IgM, similar to findings with *Escherichia coli* lipopolysaccharide (LPS) (29). Two other polyclonal activators, *S. aureus* and PWM, produce greater numbers of IgG PFC. Such differences may reflect the subpopulations of B lymphocytes receptive to *C. trachomatis* stimulation, the character of T-cell help provided to different B cells, or merely the temporal relationship between IgM and IgG synthesis in cultures stimulated with certain polyclonal activators. Continuation of cultures past 6 to 8 days may permit a second wave of IgG–B-lymphocyte differentiation after reexposure to *C. trachomatis*.

The infectious process and multiple forms of chlamydiae during their life cycle have been well studied (38). Our results demonstrate that this infectious process is not necessary for polyclonal activation to occur. It seems more likely that chlamydiae interact with B cells directly (perhaps binding to membrane immunoglobulins [32] in a manner analogous to *S. aureus* protein A [36]) before the induction of proliferation. This situation, along with our finding of T-cell involvement in the generation of immunoglobulin-secreting cells, supports a two-signal model of B-cell differentiation into plasma cells, as previously postulated for both murine and human systems (7, 12, 40). Proliferation of B cells interacting directly with *C. trachomatis* and production of nonspecific T-cell helper molecules (9, 40) would then allow terminal differentiation of large numbers of plasma cells.

We were unable to detect antibodies in vitro against the stimulating L-2 serotype of *C. trachomatis*. These results may be due to suboptimal culture conditions involving concentration of antigen, length of culture, or cell density, as seen in other studies (21, 29, 46). It is also possible that precursor B cells capable of secreting antichlamydial antibodies are present in very low frequencies in peripheral blood. To determine whether different mechanisms exist for polyclonal versus specific activation by *C. trachomatis*, it would be important to examine responses induced by chlamydiae using cells from other lymphoid organs (e.g., spleen, tonsils, lymph nodes) which contain larger percentages and possibly a greater diversity of B lymphocytes.

Our data show that whereas a highly significant correlation exists between proliferative and PFC responses induced by *C. trachomatis* in a single individual, these parameters have no relationship to a person's serum anti-L_2 antibody titer. These results agree partially with the findings of Hanna et al. (15), indicating that in an individual, antichlamydial antibody titers do not aid in predicting the results of proliferative responses. In contrast to their study, we could find no correlation between serum antichlamydial antibodies and thymidine incorporation in our population analyzed as a whole. In addition, Brunham and colleagues (8) suggested that *C. trachomatis* was not mitogenic for PBL from sexually inexperienced, seronegative adults. No prior infectious histories were obtained from our volunteers; however, sera from several individuals were assayed multiple times for antibodies against chlamydiae with identical results. It is unclear why our findings differ from the results of Brunham et al. in this regard; possible explanations include different culture and assay systems, sample size, and study population.

Since the outer membrane of *C. trachomatis* is similar to that of gram-negative bacteria, it seemed possible that LPS, a component of gram-negative bacterial cell walls, was responsible for lymphocyte activation in this system, similar to murine studies using *E. coli* LPS (2, 17). This hypothesis appears unlikely because (i) lipid A has not been clearly demonstrated in chlamydiae (10; L. F. Guymon, P. B. Wyrick, and C. H. Davis, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, B62, p. 33); (ii) the 2-keto-3-deoxyoctanoic acid found on *C. trachomatis* by Dhir et al. (10) differed significantly from similar molecules found on *Salmonella enteritides* LPS; (iii) human PBL proliferate poorly in response to LPS under our culture conditions (29); (iv) LPS is heat stable (boiling for 10 min), whereas stimulatory effects of *C. trachomatis* were completely abolished by boiling for 5 min (J. Bard and D. Levitt, Fed. Proc. **42**:864, 1983).

Several microorganisms or their products can induce polyclonal immunoglobulin secretion in vitro. In each instance, adherent cells or monocytes inhibit maximum responses (3, 20). We have also found this situation to exist in

C. trachomatis-stimulated cultures, which may be due to ingestion or infection of the microorganism (limiting the number of stimulatory particles) or secretion of inhibitory molecules (e.g., prostaglandins) (45). Chlamydiae infect mouse peritoneal macrophages (22, 48), so precedence for the former possibility exists.

Cell wall components of certain bacteria (e.g., streptococci) may persist for long periods in macrophages. This situation has been associated with modulation of host immune responses and may contribute to the pathogenesis of bacterial-induced chronic diseases (42). Since noninfectious *C. trachomatis* can perturb the human immune system in vitro, the persistence of elevated immunoglobulins against chlamydiae in sera of people months and even years after initial exposure may result from ongoing reexposure to sequestered, slowly released bacteria. Our system will allow a closer examination of the means by which these intracellular bacteria interact with the human immune system.

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