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## Research Article

Reports by a number of investigators have described the thymus-derived (T)-cell dependence of immunoglobulin synthesis by pokeweed mitogen (PWM) stimulated human peripheral blood bone marrow-derived (B) cells. Because of the cooperative nature of this in vitro system, it was chosen for examination of the differential effects of low density lipoprotein inhibitor (LDL-In) on B- and T-cell functions. Supernates from 7-d cultures that contained either peripheral blood mononuclear cells (PBM) or combinations of isolated lymphocyte populations were assayed for immunoglobulin (Ig)G by competitive inhibition radio-immunoassay. LDL-In suppression of whole PBM IgG synthesis occurred at 5-20 µg protein/ml and was independent of PWM concentration. Maximal suppression required preincubation of cells with LDL-In before stimulation. Suppression was also observed when B cells alone were exposed for 24 h to LDL-In before PWM stimulation; these suppressed B cells were not rescued by normal T cells. Exposure of T cells alone to low doses of LDL-In for 24 h augmented, but high doses suppressed, IgG synthesis, suggesting a differential effect on T-helper vs T-suppressor cell populations. Independent LDL-In exposure of T-helper or T-suppressor cell enriched populations, separated by rosetting with IgG- or IgM-coated ox erythrocytes, identified the T-suppressor cell populations as the most sensitive of the lymphocyte populations tested. The sensitivities of lymphocyte subpopulations to LDL-In, relative to PBM, were 2.8, 1.2, and 0.3 for [...]

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# Differential Sensitivity of Lymphocyte Subpopulations to Suppression by Low Density Lipoprotein Inhibitor, an Immunoregulatory Human Serum Low Density Lipoprotein

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**A B S T R A C T** Reports by a number of investigators have described the thymus-derived (T)-cell dependence of immunoglobulin synthesis by pokeweed mitogen (PWM) stimulated human peripheral blood bone marrow-derived (B) cells. Because of the cooperative nature of this *in vitro* system, it was chosen for examination of the differential effects of low density lipoprotein inhibitor (LDL-In) on B- and T-cell functions. Supernates from 7-d cultures that contained either peripheral blood mononuclear cells (PBM) or combinations of isolated lymphocyte populations were assayed for immunoglobulin (Ig)G by competitive inhibition radioimmunoassay. LDL-In suppression of whole PBM IgG synthesis occurred at 5–20 µg protein/ml and was independent of PWM concentration. Maximal suppression required preincubation of cells with LDL-In before stimulation. Suppression was also observed when B cells alone were exposed for 24 h to LDL-In before PWM stimulation; these suppressed B cells were not rescued by normal T cells. Exposure of T cells alone to low doses of LDL-In for 24 h augmented, but high doses suppressed, IgG synthesis, suggesting a differential effect on T-helper vs T-suppressor cell populations. Independent LDL-In exposure of T-helper or T-suppressor cell enriched populations, separated by rosetting with IgG- or IgM-coated ox erythrocytes, identified the T-suppressor cell populations as the most sensitive of the lymphocyte populations tested. The sensitivities of lymphocyte subpopulations to LDL-In, relative to PBM, were 2.8, 1.2, and 0.3 for the T-sup-

pressor cells, B cells and T-helper cells, respectively. Thus, both B and T lymphocytes are sensitive to and can be regulated by LDL-In. In addition, the biologic activity observed when unseparated PBM are exposed to LDL-In appears to represent a composite of the sensitivity of each of the lymphocyte subpopulations.

## INTRODUCTION

Low density lipoprotein inhibitor (LDL-In)<sup>1</sup> in normal human serum was first identified by its capacity to suppress the early events involved in the proliferative response *in vitro* of human peripheral blood mononuclear cells (PBM) to lectins and allogeneic cells (1). Subsequent work has demonstrated that administration of LDL-In *in vivo* can inhibit the primary humoral immune response of mice to sheep erythrocytes (SRBC) (2) and the primary generation of thymus-derived (T) killer cell activity (3). Since the initial description of the phenotypic features of LDL-In mediated immunosuppression, we have attempted to elucidate the mechanism of action, and even more particularly, to identify the target cell(s). Two recent studies have (a) identified the target cell as the lymphocyte rather than the macrophage or monocyte (4), and (b) reported that lymphocytes bear a discrete surface receptor that is believed to mediate LDL-In modulation of their function (5). We have thus narrowed the possible target cells to lymphocyte populations. The present studies were designed to determine if both bone marrow-de-

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<sup>1</sup> Abbreviations used in this paper: EA, antibody-coated ox erythrocytes; LDL-In, low density lipoprotein inhibitor; nSRBC, neuramidase-treated sheep erythrocytes; PBM, peripheral blood mononuclear cells; PWM, pokeweed mitogen; SRBC, sheep erythrocytes; T<sub>γ</sub> cells, T-suppressor-enriched cells; T<sub>μ</sub> cells, T-helper-enriched cells, T<sub>t</sub> cells, total T-cell population.

rived (B) and T lymphocytes are sensitive to regulation by LDL-In.

Numerous investigators have agreed that T-cell help is needed if B cells are to synthesize immunoglobulin (Ig) after pokeweed mitogen (PWM) stimulation (6-9). This requirement for T cells is both specific, because other cell types, e.g., monocytes and fibroblasts are ineffective as substitutes for T cells (1), and direct, because monocytes are not required as a third-cell population (7, 9, 10). T cells do not require DNA synthesis to support Ig production (9) and this help is irradiation-resistant (6, 11). Moreover, cycloheximide treatment of the T cells to inhibit protein synthesis does not affect this helper function (8). Although Fauci et al. (7) reported that T-cell supernates could not substitute for T cells in the development of a plaque-forming cell response, Hirano et al. (12) demonstrated that cell-free supernates obtained from either PWM-stimulated PBM or T cells could replace the T-helper function when Ig production was monitored by radioimmunoassay. In either case, it appears that the T cell or a T-cell product must be present at the initiation of the cultures for maximum expression of the helper function(s) (12). Saxon et al. (9) reported that T lymphocytes demonstrated maximum helper effects when they were mixed with equal numbers of B cells, whereas larger T:B-cell ratios suppressed B-cell Ig synthesis. Thus, T cells not only are required for, but appear to regulate differentiation and Ig production by actively suppressing B-cell Ig synthesis at higher T:B-cell ratios (7, 10).

Human T cells have been shown to possess Fc receptors for IgG (13, 14) and IgM (15, 16), that are expressed by separate and distinct T-cell subpopulations (17). Functional analysis of these two subpopulations by Moretta and colleagues (11) demonstrated that the T cells expressing Fc receptors for IgM are helper cells, whereas those bearing Fc receptors for IgG are suppressor cells. Because these T-cell subpopulations as well as the B cells can now be separated and functionally assayed, this cooperative *in vitro* system of PWM-induced B-cell differentiation was chosen for examination of the differential effects of LDL-In exposure on these B- and T-cell functions.

## METHODS

**Isolation and culture of PBM.** PBM were isolated from heparinized venous blood as previously described (5). The cells were washed extensively at 37°C in complete media composed of RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.), 100 U/ml penicillin, 50 µg/ml streptomycin, 2 mM glutamine, and 10% fetal calf serum. Yields were 75% and constituted 83-90% lymphocytes, 10-15% monocytes, and <2% polymorphonuclear leukocytes. Viability was >97% in all cases. The cells were resuspended in complete media at the cell concentrations indicated, and 1.0-ml volumes were transferred to sterile 12 × 75-mm polypropylene tubes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). PWM (5-150 µg/ml)

(Grand Island Biological Co.) was added as indicated, and the cultures were incubated at 37°C for 7 d on a rocker platform (7 cycles/min) in 5% CO<sub>2</sub> in air at 100% humidity. 7 d later, the tubes were spun at 2,500 rpm for 15 min, and the supernates were collected and stored at -20°C until assayed for IgG by radioimmunoassay. In selected assays, the cultured cells (after removal of the supernates) were washed and resuspended in Puck's Saline G (Grand Island Biological Co.), for enumeration of viable cell recovery with Trypan blue.

**Separation of lymphocyte subpopulations.** PBM were separated into T and non-T populations with neuraminidase-treated SRBC (nSRBC) (19). Equal volumes of 5% nSRBC in Puck's Saline G and lymphocytes suspended to 1 × 10<sup>7</sup>/ml in complete media were mixed, incubated at 37°C for 10 min, and spun at 1,500 rpm for 5 min. After the cell pellets had been incubated at 23°C for 60 min, they were gently resuspended; 5.0-ml fractions were layered onto 3.0 ml of Ficoll-Hypaque (1.074 g/cm<sup>3</sup>; Ficoll, Pharmacia Fine Chemicals, Piscataway, N. J., Hypaque, Winthrop Laboratories, Sterling Drug Co., New York), and the gradients were centrifuged at 2,200 rpm for 15 min. Cells at the interface were removed, washed, resuspended in complete media, and were then designated as B cells. This preparation contained all nSRBC-rosette negative PBM including >99% of the monocytes and polymorphonuclear leukocytes originally present in the PBM preparation and <3% contaminating SRBC rosette positive T cells. The nSRBC rosette positive cells were collected at the bottom of the Ficoll-Hypaque gradient, and the nSRBC were lysed by incubation for 10 min at 37°C in 0.14 M NH<sub>4</sub>Cl<sub>2</sub>, 0.015 M Tris, pH 7.2, followed by washing and resuspension in complete media. All T-cell preparations contained >98% nSRBC rosette positive T cells.

Further subfractionations of the T-cell preparations were always performed after the T cells had been cultured overnight with complete media at 2 × 10<sup>6</sup>/ml in plastic tissue culture flasks (75 cm<sup>2</sup> growth area, Falcon Plastics). After culture the T cells were washed once in complete media, resuspended to 15 × 10<sup>6</sup>/ml, and mixed with an equal volume of 7.5% antibody-coated ox erythrocytes (EA)-IgG. 2-ml fractions of the cell suspension were immediately layered onto 3.0 ml of Ficoll-Hypaque. The gradients were incubated at 37°C for 10 min, then at room temperature for an additional 90 min, and finally spun at 2,200 rpm for 10 min. The EA(IgG) rosette positive cells recovered at the bottom of the gradient (T suppressor-enriched [T<sub>y</sub>] cells) were collected; the EA(IgG) were lysed with ammonium chloride-Tris buffer, and the cells were washed three times in complete media. The nonrosetted interface cells were washed once in complete media, resuspended to 15 × 10<sup>6</sup>/ml, mixed with an equal volume of 7.5% EA(IgM), and treated exactly as described for EA(IgG) rosettes except that the 90-min incubation of EA(IgM) and lymphocytes on the Ficoll-Hypaque was carried out at 4°C instead of at room temperature. The washed and resuspended EA(IgM) positive cells recovered at the bottom of the gradient (T helper enriched [T<sub>u</sub>] cells) were also finally resuspended in complete media.

**Preparation of EA(IgG) and EA(IgM).** Anti-ox erythrocytes were produced in rabbits immunized with ox erythrocyte stroma (20). Rabbit serum that contained predominantly IgM antibodies was generated by injecting rabbits intravenously with lyophilized ox stroma resuspended in saline. On days 1 through 5 the dose was 250 µg and, on days 6 through 11, 500 µg. On days 13 and 14 the sera were collected. IgG antibodies were obtained from multiple bleedings of a single rabbit hyperimmunized by the subcutaneous route. The IgM and IgG fractions were separated from the immune rabbit sera by (a) 50% ammonium sulfate precipitation, (b) ion ex-

change chromatography on DEAE cellulose (DE-52 Whatman Inc., Clifton, N. J.) in 0.02 M potassium phosphate, pH 6.5, followed by elution of IgM with a linear sodium chloride gradient, and (c) gel chromatography at pH 4.0 on ACA-34 (Bio-Rad Laboratories, Richmond, Calif.). The IgG was shown to be free of IgM, and the IgM free of IgG, by Ouchterlony analysis and immunoelectrophoresis. Ox erythrocytes (2%) (Colorado Serum Co., Denver, Colo.) were sensitized with IgG or IgM at final protein concentrations of 0.31 and 0.097 mg/ml, respectively. These concentrations were selected from hemagglutination and rosette assays as the lowest antibody dilution that resulted in no microscopically detectable hemagglutination of ox erythrocytes, and as yielding constant or plateau numbers of rosette forming lymphocytes.

**Isolation of LDL-In.** The multiple preparations of LDL-In used in these studies were purified more than 70,000-fold relative to serum proteins from pooled normal human serum by dextran sulfate precipitation and NaCl density gradient ultracentrifugation as previously described (1). LDL-In characteristically has a mean buoyant density that is somewhat lower than the peak density of total serum low density lipoprotein, and as currently isolated represents a subset of serum low density lipoprotein (5).

**Radioimmunoassay of IgG.** We used a competitive inhibition radioimmunoassay of the double antibody type (21). Standards or samples diluted from 1:3 to 1:27 in 330  $\mu$ l were diluted with 670  $\mu$ l of 0.14 M NaCl, 0.01 M sodium borate, pH 8.2, and  $^{125}$ I-IgG at 16 ng/ml (250  $\mu$ l). A similar volume of rabbit anti-IgG Fc specific only for human IgG was added at a concentration sufficient to bind 50–60% of the labeled IgG. Both of the latter were in sodium borate that contained 1:33 normal rabbit serum and were incubated for 18 h, after which 500  $\mu$ l of goat anti-rabbit IgG, absorbed with immobilized human IgG, were added at equivalence. After 5 h incubation, the tubes were centrifuged, and 1.0 ml of the supernate was collected and counted for  $^{125}$ I gamma emission. Results were calculated by reference to a standard curve. Sensitivity was to 10 ng/ml and precision was  $\pm 15\%$ . Fetal calf serum and the medium alone were negative as was the medium from PBM cultured 24 h in the absence of PWM.

## RESULTS

**PWM-stimulated IgG synthesis.** Unseparated PBM ( $5 \times 10^6$ /ml) stimulated with 25  $\mu$ g/ml PWM began to secrete detectable levels of IgG after 4 d of culture (Fig. 1). Between days 5 and 9, an average of 169 ng of IgG/d was released into the culture supernates. Control unstimulated PBM also secreted IgG; however, after 6 d of culture, the accumulation of IgG in the culture supernates was only minimally detectable. Between days 6 and 9, <18 ng/d was produced (Fig. 1).

The amount of accumulated IgG recovered in the supernates of these PBM cultures on day 7 depended upon cell density and the amount of PWM added. As illustrated in Fig. 2, optimum IgG secretion was obtained by stimulation of  $5 \times 10^5$  PBM/ml with 25  $\mu$ g/ml of PWM. Optimal stimulation at this PWM concentration occurred at all cell densities tested. However, as indicated, the magnitude of the response depended markedly upon cell concentration (Fig. 2).

During the course of this study, a number of normal lymphocyte donors were used, and it became apparent

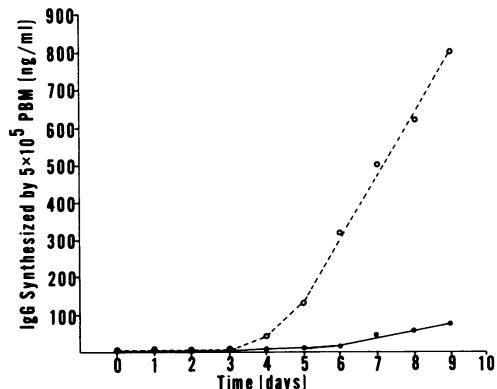


FIGURE 1 Temporal appearance of IgG in supernates of cultures of PWM-stimulated PBM. Replicate 1-ml cultures that contained  $5 \times 10^6$  PBM were established on day 0. (●), Control cultures, no PWM; (○), 25  $\mu$ g/ml PWM added on day 0. The ordinate indicates the total IgG accumulated.

that the amount of PWM required for optimum IgG synthesis was not the same for each individual (range, from 5 to 50  $\mu$ g/ml). However, although the amount of PWM required varied from individual to individual, it did not change appreciably over a period of time for the same individual (data not shown). PWM titrations were thus performed on all donors used in this study, and the amount of PWM used reflects optimum concentrations for that individual.

**Effect of LDL-In on PWM-stimulated PBM IgG secretion.** When PBM were incubated with LDL-In for 24 h before PWM stimulation and the culture supernates were assayed for IgG on day 7, secretion was suppressed (Fig. 3). The specific activity of this preparation of LDL-In for suppression of phytohemagglutinin-stimulated PBM (1, 4) was 27.2  $\mu$ g/ml (defined as the amount required for 50% inhibition of [ $^3$ H]thymidine uptake). Similar calculations performed

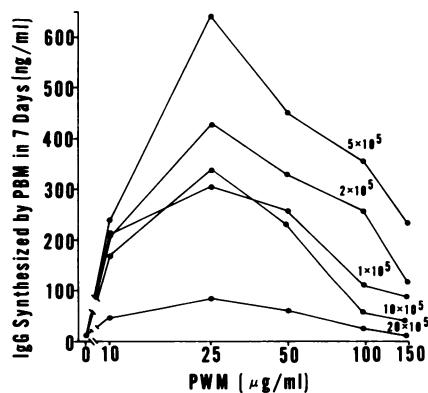


FIGURE 2 Effect of cell density and mitogen concentration on IgG synthesis and secretion by PWM-stimulated PBM. Numbers represent PBM cell concentrations per 1-ml culture.

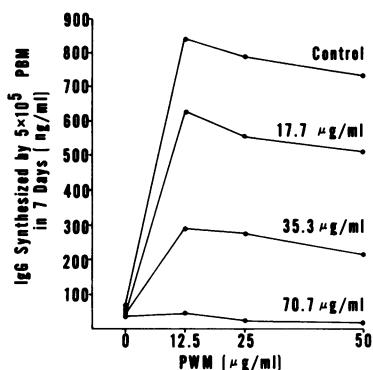


FIGURE 3 Influence of concentration of PWM on suppression of IgG synthesis by LDL-In (50% inhibition of PHA-stimulated [<sup>3</sup>H]thymidine uptake = 27.2  $\mu$ g/ml sp act). PBM ( $5 \times 10^5$ /ml) were precultured with LDL-In for 24 h before addition of PWM and the cultures were harvested 7 d later. 50% inhibition of IgG synthesis occurred at an LDL-In concentration of 27.7  $\mu$ g/ml.

on the data illustrated in Fig. 3 give a value of 27.7  $\mu$ g/ml for 50% inhibition of PWM-stimulated IgG synthesis. Thus, the inhibitory activity of LDL-In for [<sup>3</sup>H]thymidine uptake and for IgG synthesis appears to be equivalent. In addition, as reported previously (1), the suppressive activity appears to be independent of the mitogen concentration, at least within the limits tested.

The temporal aspects of the induction of suppression by LDL-In were explored by setting up a series of PBM cultures to which mitogen was added 24 h after initiation of the cultures. A single concentration of LDL-In sufficient to inhibit 70% when added 24 h before stimulation was then added at varying time periods from 24 h before PBM stimulation to 66 h afterward (Fig. 4). The suppressive activity of LDL-In clearly diminished when it was added at intervals shorter than 24 h before PWM. 24 h after stimulation, the PBM were

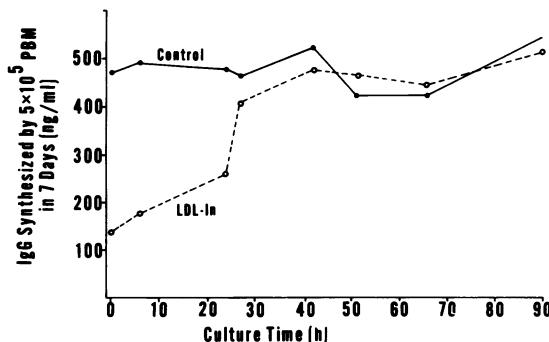


FIGURE 4 Temporal requirements for inhibition of PWM-stimulated PBM by LDL-In. The cultures were established at time 0 and LDL-In was added at times indicated. All cultures received PWM at 24 h and were harvested 7 d later. (●), Control cultures; (○), LDL-In, 40  $\mu$ g/ml.

completely refractive to the effects of LDL-In, and IgG synthesis and secretion were normal.

*T-cell dependence of PWM-stimulated IgG synthesis by B cells.* As reported (6–10), the synthesis of Ig by B cells in mitogen-stimulated lymphocyte cultures is dependent upon the presence of T cells. B lymphocytes ( $2.5 \times 10^5$ ) cultured alone with PWM did not secrete appreciable amounts of IgG into the supernate (Table I). In addition, the number of viable cells recovered after 7 d of culture represented only 52% of the starting cell number. However, when as few as  $2.5 \times 10^4$  T cells were added to the B cells before PWM stimulation (10% T cells), detectable supernatant IgG increased  $\approx 10$ -fold and the cell recovery increased to 132%, indicating that B-cell proliferation and further differentiation to antibody secreting cells are T-cell-dependent events. Further increases in IgG secretion accompanied increases in numbers of T cells (up to 37% T cells), after which IgG synthesis fell (Table I). This depression was interpreted to result from active suppression by T cells, which was verified by the results illustrated in Fig. 5. In this experiment, the total cell concentration was kept constant and only the ratio of T:B cells was varied. At two PWM concentrations, with ratios of T:B < 1, T help predominated, whereas at T:B ratios > 1, suppression predominated (Fig. 5).

*Independent exposure of T and B cells to LDL-In.* Because unbound LDL-In can be removed from cell cultures by washing after 24 h of incubation without loss of suppressive activity (4), B and T cells were separated and precultured for 24 h at 37°C in the presence of LDL-In. The cells were washed, then equal numbers of the B and T cells were mixed in culture and stimulated with PWM. When both B and T cells were exposed to LDL-In and then mixed, dose-dependent suppression was observed, with 50% inhibition by LDL-In occurring at 24.5  $\mu$ g/ml (Fig. 6). When B

TABLE I  
Effect of T cells on PWM-Stimulated B-Cell Proliferation and Differentiation\*

B cells	T cells	Viable cell recovery†	Supernatant IgG
No. $\times 10^5$		%	ng/ml
2.5	0	52	16
0	2.5	44	11
2.5	0.3	132	181
2.5	0.5	243	343
2.5	1.0	227	382
2.5	1.5	300	394
2.5	2.0	172	302
2.5	2.5	126	235

\* 1.0-ml cultures stimulated with 25  $\mu$ g/ml PWM on day 0 and assayed after 7 d.

† Trypan blue exclusion.

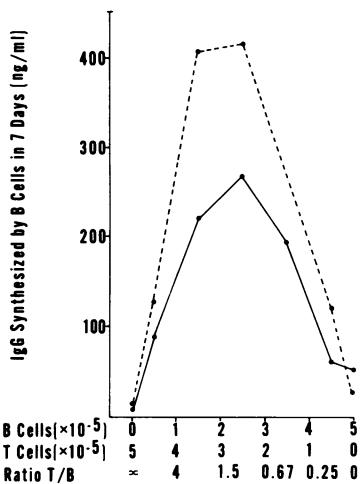


FIGURE 5 Effect of T:B cell ratios on PWM-stimulated IgG synthesis by B cells. All cultures contained  $5 \times 10^5$  total cells/ml, received either  $25 \mu\text{g}/\text{ml}$  (—) or  $50 \mu\text{g}/\text{ml}$  (---) PWM on day 0, and were harvested on day 7.

cells alone were exposed to LDL-In and added to untreated T cells, inhibition was somewhat similar and reached 50% at  $\approx 21 \mu\text{g}/\text{ml}$ . However, the results were quite different when LDL-In exposed T cells were added to untreated B cells; IgG synthesis was enhanced rather than suppressed (Fig. 6).

Two significant conclusions can be drawn from this experiment. First, B cells are clearly sensitive to the effects of LDL-In-mediated suppression. Thus, LDL-In appears to directly inhibit in vitro B-cell PWM-stim-

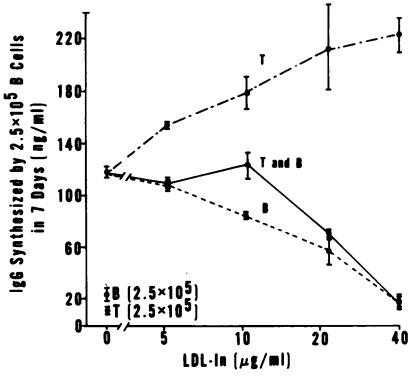


FIGURE 6 Effects of independent exposure of either B or T cells to LDL-In. Isolated B and T cells were preincubated with either lipoprotein buffer or LDL-In for 24 h at  $37^\circ\text{C}$ , at which time the unbound LDL-In was removed by washing.  $2.5 \times 10^5$  B cells were added to  $2.5 \times 10^5$  T cells in 1-ml cultures and  $50 \mu\text{g}/\text{ml}$  PWM were added. Cultures were harvested on day 7. Control cultures that contained only B cells or T cells are shown in the lower left. (---), Cultures that contained LDL-In exposed T cells and nonexposed B cells; (—), LDL-In exposed T cells and B cells; (—), LDL-In exposed B cells and nonexposed T cells. Brackets represent the SD.

ulated IgG secretion. Supporting evidence for the fact that B-cell suppression appears to be absolute can be seen in Table II. To determine if B cells exposed to LDL-In for 24 h could be rescued from suppression, increasing numbers of untreated T cells ( $5 \times 10^4$ – $5 \times 10^5$ ) were added to  $2.5 \times 10^5$  LDL-In-exposed and washed B cells. As expected, increasing T:B-cell ratios resulted in increased IgG production, whereas still higher ratios resulted in decreased IgG production. Moreover, this change occurred in control B as well as in LDL-In-suppressed B cell cultures (Table II), suggesting that LDL-In-suppressed B cells can not be rescued by normal T cells. However, the degree to which B-cell activity is suppressed by a given concentration of LDL-In is dependent upon the magnitude of the Ig synthetic response, which is clearly T cell-dependent.

The second conclusion that can be drawn from the data in Fig. 6 is that LDL-In is also capable of altering functional aspects of the T-cell population. Functional T-cell subsets have been described in this system on the basis of their ability to either help or suppress B-cell IgG synthesis (11). The observation that exposure of T cells alone to LDL-In results in the enhanced appearance of IgG can be explained in two ways if one assumes that the helper and suppressor T-cell subpopulations have differential sensitivities to LDL-In mediated immunoregulation. That is either (a) a suppressor T-cell population is selectively inhibited, or (b) a helper T-cell population is selectively stimulated.

To distinguish whether the suppressor T-cell function was inhibited or the helper T-cell function was stimulated in the foregoing experiment, we separated these T-cell populations on the basis of surface receptors that mediate rosette formation with either IgM or IgG antibody bound to ox erythrocytes (EA[IgM] and EA[IgG], respectively). In initial experiments, increasing numbers of the isolated T-cell fractions were added to  $2.5 \times 10^5$  B cells in the presence of  $50 \mu\text{g}/\text{ml}$  PWM (Fig. 7). When total T-cell population ( $T_t$  cells) were added, a typical dose-response curve was obtained with enhancement of IgG production occurring at low numbers of  $T_t$  cells, and suppression predominating in the presence of greater numbers of  $T_t$  cells. In contrast,  $T_\mu$  cells fully supported IgG synthesis, even at high T-cell concentrations.  $T_\gamma$  cells lacked the capacity to support B-cell IgG synthesis at any cell concentration tested (Fig. 7). T cells devoid of both IgM and IgG Fc receptors exhibited only slight support of IgG synthesis.

**Independent exposure of  $T_\mu$  and  $T_\gamma$  cells to LDL-In.** To test whether LDL-In activated or stimulated a T-helper cell population, we incubated isolated  $T_\mu$  cells ( $5 \times 10^5/\text{ml}$ ) with increasing concentrations of LDL-In for 24 h. After being washed,  $7 \times 10^4$  of these cells were cultured with  $2 \times 10^5$  unexposed B cells and  $50 \mu\text{g}/\text{ml}$

TABLE II  
Can T Cells Rescue LDL-In Suppressed B Cells?

B-cell treatment	Number of T cells added to $2.5 \times 10^5$ B cells					
	0	$0.5 \times 10^6$	$1 \times 10^6$	$1.5 \times 10^6$	$2.5 \times 10^6$	$5 \times 10^6$
Buffer control, ng IgG/ml*	44±15	201±12	290±1	427±36	164±4	102±35
LDL-In (7.5 $\mu$ g/ml)† ng IgG/ml	72±17	171±40	204±61	245±57	129±57	90±34
Percent inhibition, %	0	15	30	43	21	12
LDL-In (15 $\mu$ g/ml)† ng IgG/ml	66±14	129±26	160±17	151±22	110±33	79±26
Percent inhibition, %	0	36	45	65	33	23

\* After 7 d of culture.

† 24 h incubation at 37°C in the presence of LDL-In followed by washing before establishment of 1-ml cultures and the addition of 50  $\mu$ g/ml PWM.

ml PWM. In the absence of suppressor cells and LDL-In, the culture supernates contained a mean of 353 ng/ml of IgG (Fig. 8).  $T_\mu$  cells exposed to LDL-In did not significantly enhance this response at any of the concentrations tested. To the contrary, high concentrations of LDL-In ( $>50 \mu$ g/ml) decreased IgG synthesis, suggesting suppression of helper cell function. The concentration of LDL-In required for 50% inhibition of helper cell function was estimated to be 95  $\mu$ g/ml.

To test the possibility that LDL-In suppresses T-suppressor cell function, isolated  $T_\gamma$  cells ( $5 \times 10^6$ /ml) were incubated with increasing concentrations of LDL-In, washed, and then  $2 \times 10^5$  of these  $T_\gamma$  cells were cultured with  $2 \times 10^5$  unexposed B cells and

$7 \times 10^4$  unexposed  $T_\mu$  cells in the presence of 50  $\mu$ g PWM. In the absence of LDL-In, active suppressor cell function could be demonstrated by the reduced amount of IgG present in these culture supernates, e.g., 113 ng/ml with  $T_\gamma$  cells in contrast to 353 ng/ml without  $T_\gamma$  cells (Fig. 8). Exposure of  $T_\mu$  cells to as

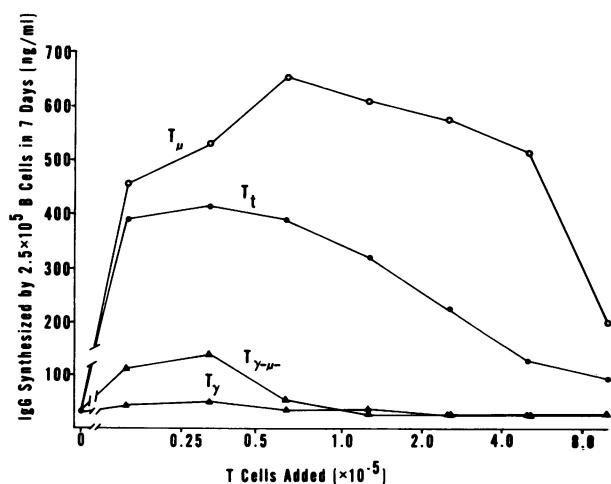


FIGURE 7 Functional capacity of T-cell subpopulations to support B-cell PWM-stimulated IgG synthesis. The T-cell subpopulations isolated on the basis of Fc $\gamma$  or Fc $\mu$  receptor expression were added to  $2.5 \times 10^5$  B cells, stimulated with 50  $\mu$ g/ml PWM in 1.0 ml cultures, and the cultures were harvested on day 7.  $T_{\gamma-\mu}$ , T suppressor and T helper-depleted T cells.

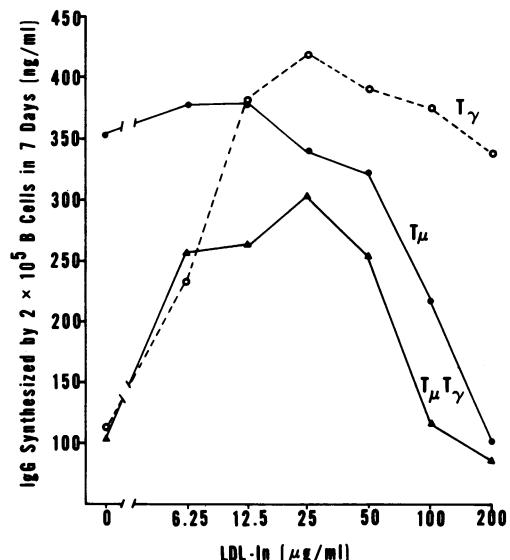


FIGURE 8 Effect of independent exposure of  $T_\mu$  and  $T_\gamma$  cells to LDL-In on support of PWM-stimulated IgG synthesis and secretion by B cells. T-cell populations ( $5 \times 10^6$ /ml) were incubated with either buffer or LDL-In for 24 h at 37°C at which time the free LDL-In was removed by washing. (●), 1-ml cultures that contained  $2 \times 10^5$  nonexposed B cells plus  $7 \times 10^4$  LDL-In-treated  $T_\mu$  cells; (○), 1-ml cultures that contained  $2 \times 10^5$  nonexposed B cells,  $7 \times 10^4$  nonexposed  $T_\mu$  cells, and  $2 \times 10^5$  LDL-In-treated  $T_\gamma$  cells; (▲), 1-ml cultures that contained  $2 \times 10^5$  nonexposed B cells,  $7 \times 10^4$  LDL-In-treated  $T_\mu$  cells, and  $2 \times 10^5$  LDL-In-treated  $T_\gamma$  cells. All cultures received 50  $\mu$ g/ml PWM and were harvested on day 7 for analysis of secreted IgG.

little as 6  $\mu\text{g}$  LDL-In/ml significantly inhibited this suppressor function, as evidenced by enhanced B cell IgG synthesis. Maximum inhibition of T<sub>γ</sub>-cell function occurred at LDL-In concentrations of  $\approx 25 \mu\text{g}/\text{ml}$ , and 50% inhibition occurred with LDL-In at 9  $\mu\text{g}/\text{ml}$ . Thus, LDL-In attenuated T suppressor cell function. When T<sub>μ</sub> and T<sub>γ</sub> cells were exposed to LDL-In independently and then co-cultured with unexposed B cells (Fig. 8), the amount of IgG obtained reflected the composite result of T<sub>γ</sub>-cell inhibition at LDL-In concentrations of 6–25  $\mu\text{g}/\text{ml}$  and T<sub>μ</sub>-cell inhibition at LDL-In concentrations of 50–200  $\mu\text{g}/\text{ml}$ .

## DISCUSSION

The response of human PBM to PWM was monitored by quantitating the amount of supernatant IgG recovered after 7 d of culture. We chose to limit our quantitation of Ig production in this study to IgG because (a) the amount synthesized and released is readily detectable by radioimmunoassay, (b) no Ig class switch has been reported yet in this system (6, 9, 10, 12, 22), (c) although the total amount of each Ig class secreted differs from individual to individual, all normal individuals possess cells capable of secreting each class of Ig in response to PWM, and (d) these cells appear simultaneously and their numbers increase in a parallel fashion (22).

It is apparent from the present studies that PWM-induced PBM IgG synthesis is sensitive to and can be regulated by LDL-In. Preparations of LDL-In used in this study are active at concentrations (27  $\mu\text{g}/\text{ml}$ ) that are well within the physiologic range of the estimated serum concentrations of LDL-In (5). Optimal expression of functional suppression by LDL-In requires at least 24 h of exposure before mitogen stimulation, suggesting that LDL-In suppresses early inductive event(s) that must precede the proliferative signal to a resting lymphocyte. Even though shorter preincubation periods result in decreased but detectable levels of suppression, a more complex process than simple competitive or steric blocking of the cell surface is suggested. Further evidence for a complex metabolic or physiologic regulation of triggering rather than of protein synthesis per se is suggested by the inability of LDL-In to inhibit ongoing IgG synthesis by committed PBM when added 20 h or more after PWM stimulation (Fig. 4).

Exposure of lymphocytes to LDL-In for 24 h, followed by extensive washing to remove unbound LDL-In before PWM-stimulation, results in comparable inhibition indicating that free LDL-In need not be available in the cultures at the time of stimulation to maintain a stable suppressed state. We demonstrated previously that (a) the rate of exchange of free and lymphocyte receptor bound LDL-In at 4°C is negligible (b) the binding of LDL-In to lymphocytes at 37°C is

not saturable, and (c) LDL-In does not accumulate at the cell surface (5). Taken together, these data suggest that at 37°C during the preincubation period, there is a progressive linear association of LDL-In with the lymphocyte, which is facilitated by metabolism-dependent internalization of the surface-bound molecule (5). Because of this distinctive characteristic of LDL-In-mediated suppression, we independently exposed selected subpopulations of lymphocytes to various concentrations of LDL-In for 24 h at 37°C, washed and remixed the lymphocyte subpopulations, added mitogen, and then assayed cell function. No carry-over or leakage of biologically active LDL-In from treated to untreated cells was observed. For example, in the B- vs. T-cell studies (Fig. 6), T-cell exposure resulted in enhanced IgG synthesis. If soluble LDL-In had been carried over, suppression of the B cells would have resulted. Therefore, this approach to identifying the LDL-In sensitive lymphocytes appears valid.

Independent exposure of the B cells alone to LDL-In indicated that LDL-In is capable of suppressing the B-cell population. The relative sensitivity of B cells compared with that of total PBM (summarized in Table III) suggests that, when total PBM are exposed to LDL-In, predominantly B-cell suppression is the factor being measured. The fact that B cells were not rescued by increasing numbers of unexposed T cells suggests that the suppression of B-cell function is absolute, at least within this *in vitro* assay and time frame. The rescue experiments were also performed with isolated T<sub>μ</sub> cells (data not shown) with similar results, indicating that the presence of the T<sub>γ</sub> cell in the T<sub>γ</sub>-cell population did not interfere with the rescue attempts.

An interesting observation in these rescue experiments is that the extent of B-cell suppression obtained with a given concentration of LDL-In depended upon the magnitude of the response (e.g., number of T cells present). Because the only variable in this experiment was the number of T cells and because these T

TABLE III  
*Differential Sensitivity of Lymphocyte Subpopulations to LDL-In Exposure\**

Lymphocyte	Specific inhibitory activity	Relative sensitivity
$\mu\text{g}/\text{ml}^{\dagger}$		
Total PBM	25	1.0
B	21	1.2
T helper	95	0.3
T suppressor	9	2.8

\* 24 h, 37°C.

† Amount of LDL-In (micrograms protein per milliliter) required for 50% inhibition of cell function.

cells were added to the B cells after removal of free LDL-In, the results suggest that LDL-In exposure has revealed the existence of heterogeneity in the B-cell population, at least relative to the secretion of IgG in response to PWM. If one assumes that the amount of IgG secreted per B cell is not constant but differs with the degree of differentiation to the plasma cell, then those B cells that are less sensitive to limiting amounts of LDL-In should produce more IgG than those that are sensitive to LDL-In. It would then follow that these B cells are less responsive to T-cell help or suppression. An explanation that could account for this assumption is that B-cell heterogeneity is a function of the stage of differentiation of the B cells at the time of exposure to LDL-In. Thus, those B cells that were suppressed by limiting amounts of LDL-In were at an earlier stage of B-cell differentiation characterized by limited IgG secretion and maximum T-cell sensitivity, whereas the more mature plasmacytic cells were characterized by more abundant IgG synthesis and minimal sensitivity to T-cell regulation.

The observation that LDL-In treatment of T cells alone resulted in enhanced IgG production by untreated B cells suggested that T cells have differential sensitivities to LDL-In. Siegal and Siegal (23) reported similar enhancement by mitomycin C treatment or x irradiation of the T cells on the differentiation of human plasmacytoid cells. Lipsky et al. (24), who described increased suppressor T-cell activity after in vitro culture of freshly isolated T cells, reported that this augmentation of suppressor cell function could be prevented if DNA synthesis was inhibited during the "aging" process by mitomycin C treatment or the continued presence of hydroxyurea. The phenotypic expression of T-cell corticosteroid treatment described by Fauci et al. (25), very closely parallels that of LDL-In treatment with regard to B-cell Ig synthesis, inasmuch as enhancement of the PWM-induced plaque-forming cell response was seen only when physiologic concentrations of hydrocortisone were added within the first 24 h of culture (25). However, one important difference is that the hydrocortisone had to be present in the cultures at the time of PWM stimulation. LDL-In produced a relatively stable state of suppression that persisted even after its removal from the cultures, whereas hydrocortisone treatment produced a reversible suppressed state that was absolutely dependent upon the continued presence of the drug (25).

Selective exposure of isolated  $T_{\mu}$  or  $T_{\gamma}$  cells to LDL-In enabled us to conclude that the enhanced IgG production observed with LDL-In exposure of the  $T_{\gamma}$ -cell population resulted from the selective inhibition of  $T_{\gamma}$ -cell function rather than from activation of  $T_{\mu}$ -cell function. It is important to note that in all cell functions so far examined in this and other systems (1-4),

the biological result of LDL-In exposure has been selective suppression rather than activation of cell function.

When the concentration of LDL-In required to inhibit 50% of cell function is calculated for each of the lymphocyte subpopulations, their relative sensitivity to LDL-In exposure can be compared (Table III). The similar values we found (PBM and B cells were 25 and 21  $\mu$ g/ml, respectively) suggest that the inhibition that is observed with exposure of unseparated PBM is in fact B-cell suppression. Keightley et al. (6) and Hirano et al. (12) reported that B-cell division is required for IgG production. Supposedly, LDL-In could act by inhibiting this requisite proliferative event.

On the other hand, functional suppression of the  $T_{\mu}$ -cell population required threefold more LDL-In than other cells (Table III). Although LDL-In did not detectably kill or decrease cell numbers, LDL-In could conceivably act on the  $T_{\mu}$  cell at the higher concentrations required by interfering with cell contact or release of presynthesized soluble mediator(s). Because T-cell help does not require protein and DNA synthesis (9, 23), inhibition of cell proliferation should not affect T helper cells, which may account for their relative insensitivity to LDL-In suppression. In either case, this virtual insensitivity of  $T_{\mu}$  cells to LDL-In probably results from their separation of function and proliferation.

The  $T_{\gamma}$  cells were 3 times more sensitive to functional inhibition by LDL-In than B cells and almost 10 times more than  $T_{\mu}$  cells, making it the most sensitive of the lymphocyte subpopulations tested. The fact that  $T_{\gamma}$ -cell function is radiosensitive and requires cell division (11) again points to inhibition of cell proliferation as a probable mode of LDL-In action. At this point, one can only speculate on the reasons for the exquisite sensitivity of the  $T_{\gamma}$  cells to LDL-In regulation described in this report. However, current experiments suggest that the administration of low doses of LDL-In *in vivo* may result also in selective inhibition of suppressor cell function.

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