# THE IN VITRO EFFECTS OF *BORDETELLA PERTUSSIS* LYMPHOCYTOSIS-PROMOTING FACTOR ON MURINE LYMPHOCYTES

## IV. Generation, Characterization, and Specificity of Cytotoxic Lymphocytes*

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The lymphocytosis-promoting factor (LPF)[1] of phase I *Bordetella pertussis* induces lymphocytosis in a variety of experimental animals (1–3). The marked increase in recirculating lymphocytes is a result of a redistribution of cells from lymphoid tissue to the blood (4–6) which, in part, is related to the inability of cells to home from the blood into lymph nodes at a normal rate (7, 8). In addition to induction of lymphocytosis, purified LPF also causes sensitization of mice to the lethal effects of histamine and unresponsiveness to the hyperglycemic effect of epinephrine (9).

Although in vivo lymphocytosis is not associated with lymphocyte proliferation, LPF is a potent mitogen for both murine (10, 11) and human (12) lymphocytes in vitro. LPF is clearly a T-cell mitogen for mouse lymphocytes, but the proliferative response requires the presence of an accessory cell-surface immunoglobulin ($sIg^+$) cell (13). In this paper, LPF is shown also to induce cytotoxic effector lymphocytes (CEL) in vitro that are active against both syngeneic and allogeneic tumor cells, as well as allogeneic normal cells.

## Materials and Methods

*Mice.* Female CBA/J, BALB/cJ, DBA/2J, and C57BL/6J mice, 8–12 wk old, were obtained from The Jackson Laboratory, Bar Harbor, Maine or from The Jackson Laboratory stock bred in this department.

*Lymphocytosis-Promoting Factor (LPF), Concanavalin A (Con A), and Phytohemagglutinin-P (PHA).* LPF was isolated from culture supernatant fluids of phase I *B. pertussis* strain NIH 114 by previously described methods (9). Con A was obtained from Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J. and PHA (PHA-P) from Difco Laboratories, Detroit, Mich.

*Lymphoid Cell Suspensions.* Unless otherwise indicated, the medium used throughout was

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[1] *Abbreviations used in this paper:* ATS, rabbit anti-mouse thymocyte serum; BSA, bovine serum albumin; CEL, cytotoxic effector lymphocytes; CMC, cell-mediated cytotoxicity; Con A, concanavalin A; FCS, fetal calf serum; LPF, lymphocytosis-promoting factor isolated from *Bordetella pertussis*; LPS, lipopolysaccharide endotoxin; PHA, phytohemagglutinin-P; RPMI-1640, Roswell Park Memorial Institute medium-1640; sIg, cell-surface immunoglobulin.

Roswell Park Memorial Institute medium-1640 (RPMI-1640) (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 5% heat-inactivated fetal calf serum (FCS) (Reheis Chemical Co., Div. of Armour Pharmaceuticals, Chicago, Ill.), 100 U/ml penicillin, and 100 μg/ml streptomycin. Mice were killed by cervical dislocation. Spleen and peripheral lymph nodes (inguinal and axillary) were aseptically removed and teased with forceps in medium. Tissue fragments were allowed to sediment and the supernate containing single cells was aspirated. Cell suspensions were centrifuged, washed once, and resuspended in fresh medium. Viability was assessed with the trypan blue dye exclusion test, and only preparations with >90% cell viability were utilized.

*Preparation of LPF Effector Cells.* In all experiments, $4 \times 10^6$ viable spleen or peripheral lymph node cells/ml were mixed with an equal volume of medium containing 4 μg/ml of LPF and 2.0-ml aliquots were incubated in tubes at 37°C in a humid atmosphere of 5% $CO_2$ in air. After 72 h, the cells were harvested, washed once, and resuspended in fresh medium.

*Peritoneal Macrophages.* Mice were killed by cervical dislocation and the abdominal skin reflected. 2 ml of RPMI-1640 with 20% FCS was injected intraperitoneally and the fluid aspirated with a Pasteur pipette. The peritoneal exudate cells were washed once and viability was determined by the trypan blue dye exclusion test. The cells were resuspended to a concentration of $2 \times 10^6$/ml and 25-ml aliquots were dispensed to 100-mm Petri dishes. After incubation in a 5% $CO_2$ incubator at 37°C for 60 min, the nonadherent cells were removed and the adherent cells were washed once in 10 ml of RPMI-1640-20% FCS. The washing was repeated after an additional 60 min of incubation. At the end of a 20–24-h incubation period, the adherent cells were scraped from the glass surface with a rubber policeman.

*LPS Blast Cells.* $2 \times 10^6$ spleen cells were cultured with 10 μg of LPS (*Salmonella typhosa*, Difco Laboratories) in a final volume of 1 ml in a 5% $CO_2$ incubator at 37°C for 48 h. The cells were then harvested and washed in fresh medium. Cells were resuspended in RPMI-1640 with 10% bovine serum albumin (BSA) at a concentration of $50–70 \times 10^6$ cells/ml. 1 ml of the cell suspension was layered on 4 ml of RPMI-1640-23% BSA and after centrifugation at 13,000 *g* for 30 min at 4°C, the lipopolysaccharide endotoxin (LPS) blast cells were collected at the interface.

*Separation of Lymphocytes.* Spleen cells, stimulated with LPF for 72 h, were separated in discontinuous BSA density gradients using the methods of Raidt et al. (14) and Adler et al. (15). 35.1% BSA (Path-o-cyte 4, Miles Laboratories Inc., Miles Research Products, Elkhart, Ind.) was diluted appropriately in RPMI-1640 and 1.7-ml layers of 29 and 23% BSA were placed in cellulose nitrate tubes. $150–200 \times 10^6$ cells from LPF-stimulated cultures in 1.6 ml of 10% BSA were placed on top of the gradient. The tubes were then centrifuged at 13,000 *g* for 30 min at 4°C. Discrete bands of cells were collected at the interfaces, washed once, and viability determined by the trypan blue exclusion test.

*Determination of DNA Synthesis.* Methods previously described in detail were employed (10). Briefly, triplicate cultures in microtiter wells containing $0.5 \times 10^6$ cells and an optimal concentration of mitogen in 0.25 ml of medium were harvested on glass-fiber filter paper after an 8-h pulse with 2.0 μCi of $^3$H-thymidine, sp. act. 6.0 Ci/mM (Schwarz/Mann Div. Becton, Dickinson, & Co., Orangeburg, N. Y.). The dried filters were placed in 5 ml of an omnifluor-toluene scintillation mixture (New England Nuclear, Boston, Mass.) and counted. Results are expressed as counts per minute ± standard deviation.

*Target Cells.* P815, a DBA/2 mastocytoma; EL4, a C57Bl/b lymphoma; L1210, DBA/2 lymphoma; PU-5 and S49, BALB/c lymphomas; and J744, a BALB/c macrophage tumor were maintained in medium. After a single wash with fresh medium, $5 \times 10^6$ tumor cells were labeled with 200 Ci of $Na_2CrO_4$ (Amersham Corp., Arlington Heights, Ill.) in a 1.0-ml vol for 1 h at 37°C. The target cells were then washed four times with fresh medium. Normal macrophages and LPS blast cells to be used as target cells, were labeled with $^{51}$Cr in the same fashion.

*Cytotoxic Assays.* $4 \times 10^4$ labeled target cells in 0.2 ml of medium were added to 0.25 ml of an appropriate number of effector cells in triplicate. After 5 h at 37°C, 2.0 ml of medium was added to each tube and the tubes centrifuged at 250 *g* for 10 min. 1 ml of the supernate was removed, and assayed for $^{51}$Cr release in an Intertechnique (Fairfield, N. J.) gamma counter. The percent specific lysis was calculated according to the formula:

$$\frac{^{51}\text{Cr release in the presence of stimulated lymphocytes} - {}^{51}\text{Cr release in the presence of control cells}}{\text{maximum } {}^{51}\text{Cr release} - {}^{51}\text{Cr release in the presence of control cells}} \times 100.$$

The maximum $^{51}$Cr release from target cells was determined by rapidly freezing and thawing $4 \times 10^4$ labeled target cells four times.

*Mitomycin C Treatment.* Mitomycin C (Sigma Chemical Co., St. Louis, Mo.) was diluted to 1 mg/ml in medium immediately before use. 0.25 ml was added per 10 ml of lymphocyte suspension containing $20 \times 10^6$ cells. The suspensions were then incubated at 37°C for 1 h and the cells washed three times before use.

*Thy-1.2 Antiserum and Rabbit Anti-Mouse Thymocyte Serum (ATS).* T cells were lysed by treatment with Thy-1.2 antiserum plus guinea pig complement. The sera were prepared and characterized as described previously (16). Thy-1.2 antiserum and normal AKR serum were heat inactivated at 56°C for 30 min before use. ATS was obtained from Microbiological Associates, Walkersville, Md. ATS and control normal rabbit serum (NRS) were heat inactivated at 56°C for 30 min before use.

## Results

*Induction of Cytotoxic Activity in Spleen and PLN Cultures by LPF.* Initial experiments were performed to test whether spleen or PLN of CBA mice, incubated with LPF for 72 h, demonstrated a cytotoxic effect against allogeneic P815 mastocytoma cells. The dose of LPF chosen was the same as that which produced an optimal mitogenic effect, 2 μg/ml of LPF per $2 \times 10^6$ cells/ml; this dose was subsequently shown also to generate maximal production of cytotoxic cells. Mitogen was not added to the mixture of cells in the cytotoxic assay. Fig. 1 presents the results of a typical experiment and it can be seen that both the stimulated spleen cells and PLN demonstrated cytotoxic activities over a broad range of effector:target cell ratios.

The relationship between the time-course of proliferation as measured by [$^3$H]-thymidine uptake, and the generation of cytotoxic cells in spleen cell cultures containing LPF was next examined. It can be seen in Fig. 2 that whereas incorporation of [$^3$H]thymidine was ≅78% of maximum at 48 h, cytotoxicity was virtually absent. Maximal [$^3$H]thymidine incorporation was at 72 h and now the cytotoxic activity was marked. At 96 h, the proliferative response had decreased to 44% of the peak value, but cytotoxic activity remained elevated. Thus, there was a 24-h time lag between the development of the proliferative response and expression of cytotoxicity; furthermore, cytotoxic activity, once reaching a peak at 72 h, remained elevated whereas $^3$H-thymidine incorporation decreased.

It was shown that the cytotoxic effects were not a result of soluble cytotoxins released by LPF-activated cells nor by LPF itself because neither culture supernate nor LPF, in amounts (0.5–1.0 μg) vastly exceeding that which could be carried over in free form by the washed cells, caused $^{51}$Cr release from P815 cells.

*Effect of Mitomycin C on the Generation and Activity of Cytotoxic Cells.* Although the times of peak proliferation and maximal cytotoxic activity were the same (72 h), it was not clear whether DNA synthesis was required for production of effector cells. As shown in Table I, pretreatment of CBA spleen cells with mitomycin C, an inhibitor of DNA synthesis, inhibited the induction of cytotoxic cells by LPF thereby indicating that proliferation was required. In contrast, when cells, stimulated by LPF for 72 h, were treated with mitomycin C, they still exerted full cytotoxic activity.

*Characterization of the LPF-Induced Cytotoxic Cells.* To determine the nature of the

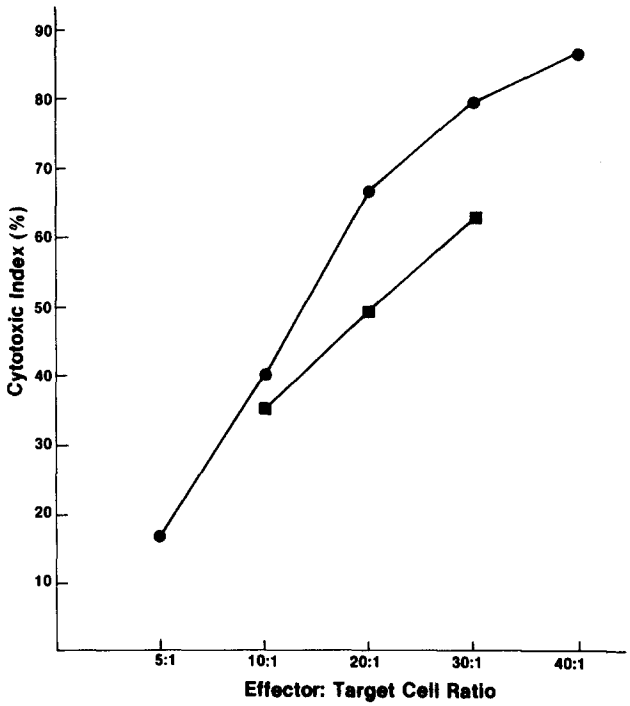


FIG. 1. Cytotoxicity of LPF-induced effector cells against $^{51}$Cr-P815. CBA spleen cells (●) and lymph node cells (■) at concentrations of 2 × $10^6$/ml were cultured with 2 μg/ml of LPF for 72 h and assayed for cytotoxic activity. Data are expressed as mean percent lysis of triplicate assays after 5 h.

cytotoxic effector cell, CBA spleen cells, previously incubated for 72 h with LPF, were separated on a discontinuous BSA gradient, and the cytotoxic activity of the fractions was examined. The gradient was designed to separate LPF-stimulated spleen cells into low density transformed or blast-like cells and high density small lymphocytes. Cells collected from the less dense fraction, consisting of over 80% transformed cells with the electron microscopical appearance of T-cell blasts, showed enhanced target cell lysis at all effector:target cell ratios tested, compared to the unseparated spleen cells (Fig. 3). However, cells from the high density fraction, which were predominately small lymphocytes, expressed little or no cytotoxic effect. Thus, the cytotoxic effector cells were blast like and not small lymphocytes.

*Inhibition of LPF-Induced Cytotoxicity by ATS and Thy-1.2 Antiserum.* Previous work demonstrated that the cells proliferating in response to LPF are T cells (11). Because blast-like cells were shown to be the effector cells, it was of interest to determine conclusively whether these cytotoxic cells were transformed T lymphocytes. Kirchner and Blaese (17) reported that preincubation of PHA and Con A-induced cytotoxic effector cells with anti-thymocyte serum inhibited the lysis of target cells. It can be seen in Fig. 4 that the addition of different dilutions of ATS to the mixture composed of LPF-stimulated CBA spleen cells and P815 cells decreased the amount of target cell lysis. More than 50% inhibition was obtained with a 1:100 dilution of the antiserum and with higher concentration, cytotoxicity was virtually abolished. NRS had no effect.

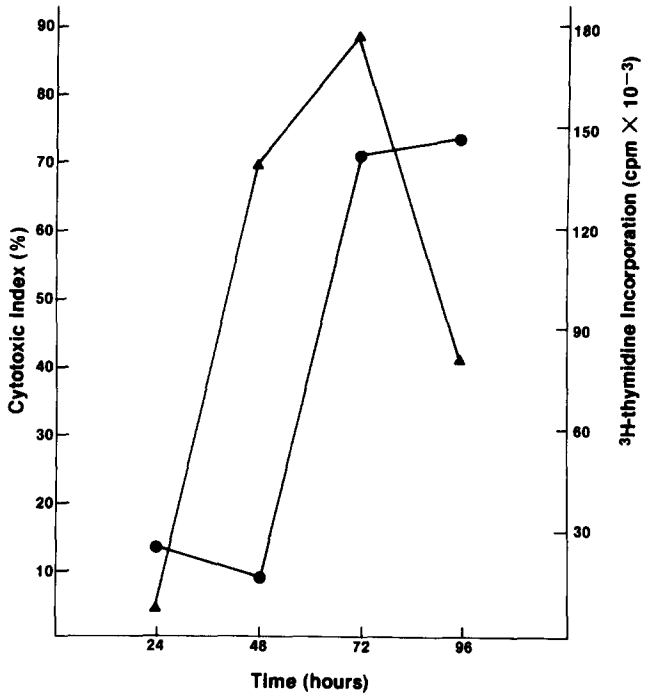


FIG. 2. Relationship between the time-course of the proliferative response (▲) and the generation of cytotoxic effector cells (●) induced by LPF. Stimulation of $0.5 \times 10^6$ CBA spleen cells by 0.5 μg LPF in microtiter wells was measured by the incorporation of [$^3$H]thymidine and compared with cytotoxicity at an effector:target cell ratio of 30:1 against $^{51}$Cr-P815 cells at various time periods.

As also shown in Fig. 4, when CBA spleen cells, cultured with LPF for 72 h, were treated with different dilutions of Thy-1.2 antiserum plus complement, cytotoxicity was diminished. Normal mouse serum plus complement had no effect. Collectively, the data show that the LPF-induced cytotoxic cells, like the proliferating cells, are T cells.

*Comparison of Activity of Cytotoxic Cells Induced by LPF, Con A, and PHA.* It has previously been shown that full activity of cytotoxic effector lymphocytes (CEL), generated by Con A or PHA, requires the addition of mitogen to the cytotoxic assay (18, 19). In contrast, expression of cytotoxicity by LPF-generated CEL does not require exogenous mitogen. The results present in Table II demonstrate this difference. After a 72-h incubation of spleen cells with optimal amounts of mitogen, the cells were tested for cytotoxicity against P815. In the case of CBA, BALB/c, and DBA/2 spleen cells cultured with LPF, marked specific lysis occurred, whereas both Con A- and PHA-stimulated cells produced only minimal target cell lysis. However, because LPF can be demonstrated on the surface of the effector cells by immunofluorescent techniques using monospecific LPF antiserum, it is possible that the cell-surface LPF subserves the function of additional mitogen in the cytotoxic assay. Indeed, it was shown that the cytotoxic activity of Con A-stimulated spleen cells could be markedly enhanced by adding LPF, as well as Con A or PHA, to the cytotoxic assay system (Fig. 5).

TABLE I

*Effect of Mitomycin C on the Generation of Cytotoxic Cells by LPF*

| Mitomycin treatment | [$^3$H]thymidine incorporation | Cytotoxic index | |
|---|---|---|---|
| | | 15:1* | 30:1* |
| | | % | |
| None | 32,115 ± 1,253.8 | 71 | 82 |
| Prestimulation | 261 ± 24.5 | 1 | 0 |
| Poststimulation | ND‡ | 61 | 83 |

CBA spleen cells were treated with mitomycin C before culturing with LPF (prestimulation) or after culturing for 72 h and before mixing with $^{51}$Cr-P815 cells (poststimulation). Incorporation of $^3$H-thymidine was also measured at 72 h.

* Effector: target cell ratio.

‡ Not determined.

*Specificity of Cytotoxic Lymphocytes Induced by LPF.* In Table II it is seen that not only spleen cells from allogeneic CBA (H-2$^k$) mice, but also spleen cells from syngeneic BALB/c and DBA/2 mice (both H-2$^d$) formed CEL active against P815 (H-2$^d$) mastocytoma cells when stimulated by LPF. Thus, the effector cells were active against both allogeneic and syngeneic tumor cells with respect to the H-2 haplotype. Furthermore, LPF-induced effector cells in CBA spleen cell cultures were active against a variety of allogeneic tumor cells (Table III). The extent of lysis varied with the cell type, and in the case of EL4, it was shown that these cells are highly susceptible to lysis by cytotoxic cells generated in a mixed lymphocyte reaction, even though relatively low cytotoxicity occurred with LPF-induced cells. Thus, the susceptibility of allogeneic tumor cells to lysis by LPF-activated cytotoxic cells is not directly correlated with the major histocompatibility locus.

Having demonstrated that LPF-induced killer cells were effective against both syngeneic and allogeneic tumor cells, their effect on syngeneic and allogeneic normal cells was examined. For these experiments the effector cells were derived from BALB/c (H-2$^d$) spleen cell cultures. Target cells were the syngeneic tumor macrophage cell-line J774, normal syngeneic BALB/c macrophages, and allogeneic C57Bl/6 (H-2$^b$) macrophages. LPF-stimulated BALB/c spleen cells were also tested against blast cells from BALB/c and CBA (H-2$^k$) mice which had been induced by culturing spleen cells with LPS.

The results of these experiments are shown in Figs. 6 and 7 and demonstrate that LPF-induced cytotoxic cells are active against allogeneic, but not syngeneic, normal cells although they kill both allogeneic and syngeneic tumor cells.

## Discussion

The results of experiments presented herein show clearly that the lymphocytosis-promoting factor of *B. pertussis*, in addition to being an in vitro T-cell mitogen for mouse spleen cell or PLN cultures, causes the generation of cytotoxic effector lymphocytes in cultures of these cells. The effector cells are of low density and are blast like in appearance under the light microscope and have the electron microscopical features of T-cell lymphoblasts. Their effector function is blocked by ATS, and, more importantly, the cytotoxic cells are lysed by anti-Thy 1 antiserum and complement.

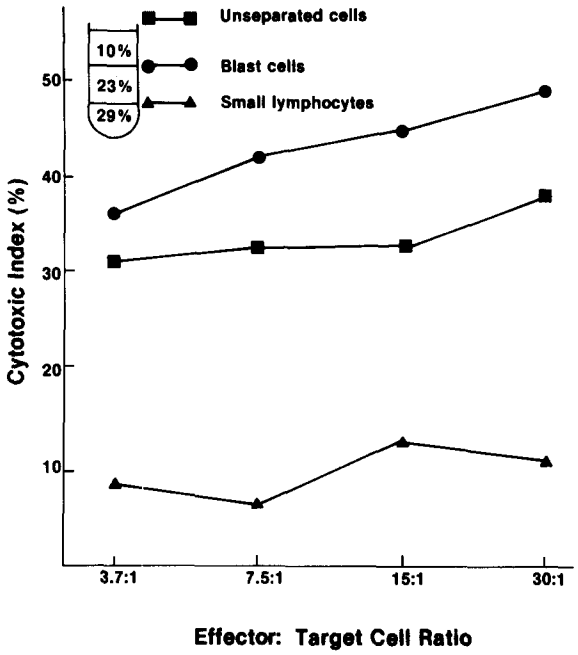


FIG. 3. Cytotoxic activity of cell fractions isolated on a discontinuous BSA gradient. CBA spleen cells were cultured with LPF for 72 h. Unseparated stimulated spleen cells (■), stimulated low density cells (●) and stimulated high density cells (▲) were assayed for cytotoxicity against $^{51}$Cr-P815 cells at various effector:target cell ratios.

Thus the cytotoxic cells are T-cells. These findings parallel those of Clark (20) who demonstrated that blast cells are the CEL induced by Con A. Soluble factors did not appear to be involved in cytotoxicity because neither culture supernatant fluids of LPF-stimulated cells nor LPF itself were toxic for target cells.

Preincubation of spleen cells with mitomycin C before incubation with LPF, blocked the generation of cytotoxic cells and, as in the case of the production of alloimmune effector T cells (21), indicates that proliferation of at least one cell population is a prerequisite. (However, the expression of cellular cytotoxic activity does not require DNA synthesis because mitomycin C did not block the activity of LPF-stimulated cells.) In preliminary experiments, it also appears that the generation of CEL by LPF, like the proliferative T-cell response, is completely dependent upon the presence of a nonproliferating accessory sIg$^+$ cell (B cell) (13). Interestingly, the kinetics of maximal $^3$H-thymidine incorporation and the generation of cytotoxic cells were different. The 24-h time lag between the appearance of proliferation and CEL suggests that one or more populations of T cells might be involved in LPF-induced cytotoxicity; unpublished studies, A. S. Kong, have indicated that the precursors of proliferating cells and of cytotoxic effector cells have similar radiation sensitivity.

None of the above findings conclusively shows that the cells undergoing DNA synthesis are the same cells that become effector cells. For example, T-cell interactions may take place. Evidence of T-T collaboration in the generation of cytotoxic activity is well documented (22, 23), although the mechanism of interaction remains unknown.

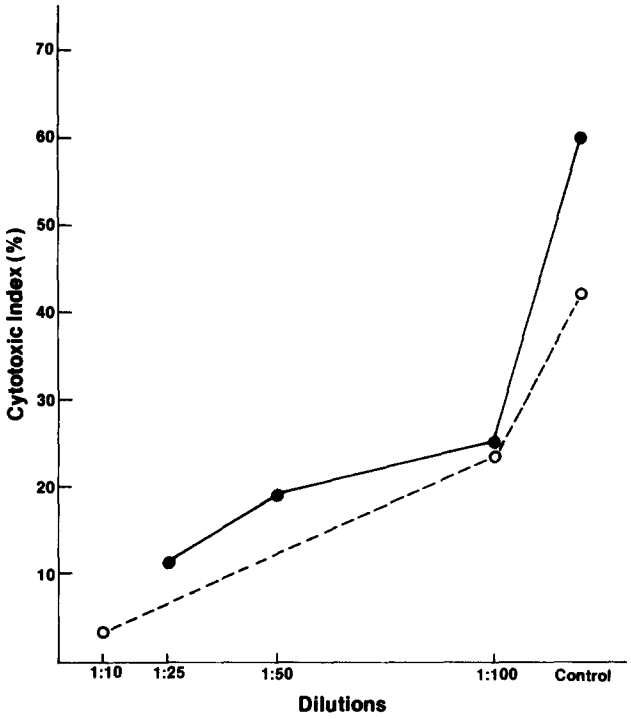


FIG. 4. Effect of anti-thymocyte serum and Thy-1.2 antiserum plus complement on LPF-induced effector cells. CBA spleen cells at a concentration of $2 \times 10^6$ cells/ml were cultured with 2 μg/ml of LPF. Cytotoxicity against $^{51}$Cr-P815 was determined at an effector:target cell ratio of 30:1 in the presence of various dilutions of ATS or normal rabbit serum (1:25) (●). In another experiment the effector cells were treated with different dilutions of Thy-1.2 antiserum or normal AKR serum (1:10) plus complement (○). The treated cells were washed and cytotoxic activity assessed at an effector:target cell ratio of 30:1.

In the alloimmune system, cells of the T-helper population (ly $1^+$) have been clearly distinguished from cells of the T-killer population (Ly $2^+3^+$) on the basis of their cell-surface phenotype (24, 25). Recently, Pilarski et al. (26) have shown that thymocytes alone stimulated by Con A did not exert cytotoxicity, but when cocultured with a population of radio-resistant spleen cells expressed a high level of cytotoxicity. Thus, helper T-cells may play a role in Con A-induced cytotoxicity.

Previous work (10) demonstrated that LPF was mitogenic for T lymphocytes from adult spleen, PLN, and cortisone-treated thymus, but not for normal thymus or bone marrow cells. Tissue distribution studies indicate that the LPF-induced cytotoxicity follows a similar pattern as the mitogenic response and no significant cytotoxicity was shown by thymocytes cultured with LPF (A. S. Kong, unpublished data). There are three obvious possibilities for the lack of LPF-induced cell mediated cytotoxicity (CMC) of thymocyte cultures: (*a*) there are not enough B cells present to exert a helper function for T-cell proliferation; (*b*) there is an insufficient percentage of T cells in normal thymus that are capable of responding to LPF; and (*c*) the thymus may be rich in cells that suppress the response. Preliminary experiments indicate that addition of B cells does not enhance the mitogenic response of thymocytes and it is therefore unlikely that the absence of helper cells solely accounts for unresponsiveness.

TABLE II

*The Cytotoxic Effect of Mitogen-Stimulated Allogeneic and Syngeneic Spleen Cells on $^{51}Cr$-Mastocytoma Cells*

| Spleen cell source and H-2 haplotype | Mitogen | Cytotoxic index* | Proliferative response |
|---|---|---|---|
| | | % | |
| CBA/J (H-$2^k$) | LPF | 78.0 | 132,028 ± 5,434 |
| | Con A | 23.5 | 135,834 ± 5,829 |
| | PHA | 9.4 | 94,747 ± 615 |
| BALB/c (H-$2^d$) | LPF | 90.6 | 92,257 ± 4,348 |
| | Con A | 5.8 | 134,713 ± 5,355 |
| | PHF | 12.7 | 65,920 ± 288 |
| DBA/2 (H-$2^d$) | LPF | 63.3 | 75,869 ± 5,710 |
| | Con A | 7.1 | 102,927 ± 1,446 |
| | PHA | 0 | 1,286 ± 403 |

Spleen cells were cultured for 72 h with an optimal concentration of mitogen and the proliferative response and generation of CEL assayed.

* Effector: target cell ratio of 30:1.

Although there are conflicting reports as to the requirement for added mitogen in the cytotoxic assay for maximal CMC of PHA- or Con A-stimulated cultures, in our studies the requirement in the case of Con A was apparent (Fig. 5). Moreover, in the absence of added mitogen, Con A-stimulated cells demonstrated far more CMC against allogeneic tumor cell target cells than against syngeneic tumor cells (Table II). The CMC of PHA-stimulated cells was too low in all cases to permit detection of differences related to H-2 specificity. The ability of added lectin to alter the specificity of effector T cells is also seen with alloimmune T cells which normally show exquisite specificity against cells bearing the alloantigens used for immunization (27). In the presence of added Con A or PHA, specificity is lost and syngeneic tumor target cells may be lysed (28, 29). The role of added lectins in CMC reactions is not clear. Initially it was thought that they served solely to attach the effector cells to the target cells under circumstances in which the effector cells could not bind tightly to the target cells, but Asherson et al. (19) have demonstrated that only mitogenic agglutinins (e.g. PHA and Con A) but not nonmitogenic agglutinins, such as axinellor and wheat germ agglutinin, promote CMC. Hence, binding of cytotoxic T cells to target cells is not sufficient to cause lysis. Rubens and Henney (30), propose that the role of Con A in lectin-dependent CMC is twofold: (*a*) to provide approximation of effector and target cells; and (*b*) to activate the effector cells.

LPF-activated cytotoxic T cells differed from those activated by Con A or PHA. First, extraneous mitogen was not required for maximal lysis of target cells to take place and second, both allogeneic and syngeneic tumor cell targets were equally susceptible. Because LPF-stimulated cells contain surface LPF, the bound material might subserve the function of added mitogen. In preliminary experiments it has been found that addition of monospecific LPF antiserum to the CMC assay blocks the cytotoxic reaction; however, this may be a result of steric hinderance of neighboring cell-surface receptors rather than the specific binding of the antiserum to LPF. When

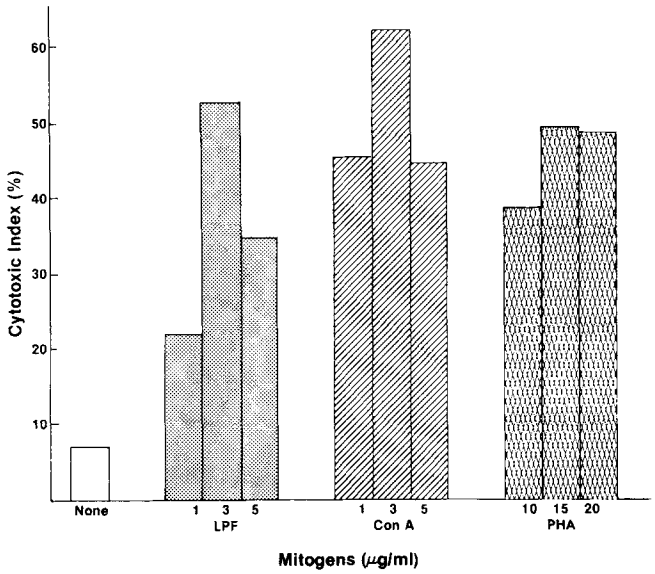


FIG. 5. Cytotoxic activity of Con A-induced effector cells in the presence and absence of different T-cell mitogens. CBA spleen cells were cultured with Con A for 72 h and assayed for cytotoxicity against P815 cells at an effector:target ratio of 30:1. The cytotoxic assay was performed without mitogen or with LPF, Con A, or PHA added at the concentrations shown.

LPF is added to Con A-stimulated cells in the CMC assay, cytotoxicity is enhanced in the same fashion as the enhanced activity observed when Con A or PHA are added. These results suggest, but by no means prove, that the cell-bound LPF on LPF-induced CEL plays a critical role in CMC and experiments are in progress to determine whether the cells retain the capacity to lyse target cells after LPF is removed from their surface.

LPF-activated cells lyse tumor cells of both allogeneic and syngeneic origin whereas Con A-induced cytotoxic cells, in the absence of added mitogen, demonstrate activity against allogeneic but not syngeneic tumor target cells (Table II, and reference 31). The relative nonspecificity of the LPF-induced cells showing CMC against tumor cells is not seen when normal cells are utilized as targets. Thus, the CEL are active against both syngeneic tumor and normal allogeneic macrophages but not against normal syngeneic macrophages. Moreover, the effector cells will kill allogeneic but not syngeneic LPS blast cells. These findings are similar to those recently reported by Bevan et al. (32) for Con A-induced cytotoxic cells which exhibit CMC in the absence of added mitogen for normal cells with an allogeneic haplotype but not for syngeneic normal cells.

The target site(s) for LPF-induced cytotoxic T-cells has not been elucidated, but in the case of syngeneic tumor cells it is surely not the major histocompatibility complex because syngeneic normal cells are not susceptible. In this regard, Lundak and Raidt (33) reported that DBA/2 spleen cells can be sensitized in vitro to DBA/2 mastocytoma cells with the production of cells exhibiting CMC against the P815 mastocytoma. It will be of great importance to determine the specific point of recognition of LPF-

TABLE III

*The Cytotoxic Effect of LPF-Stimulated CBA Spleen Cells on Various Allogeneic Tumor Cells*

| Tumor | H-2 | Histologic type | Cytotoxic index | |
|---|---|---|---|---|
| | | | 15:1 | 30:1 |
| | | | % | |
| P815 | d | Mastocytoma | 78 | 88 |
| PU-5 | d | Lymphoma | 60 | 65 |
| S49 | d | Lymphoma | 49 | 62 |
| L1210 | d | Leukemia | 40 | 46 |
| J774 | d | Macrophage | 76 | 81 |
| EL4 | b | Lymphoma | 28 | 34 |

CBA spleen cells were incubated with LPF for 72 h and then assayed for cytotoxicity at effector: target cell ratios of 15:1 and 30:1.

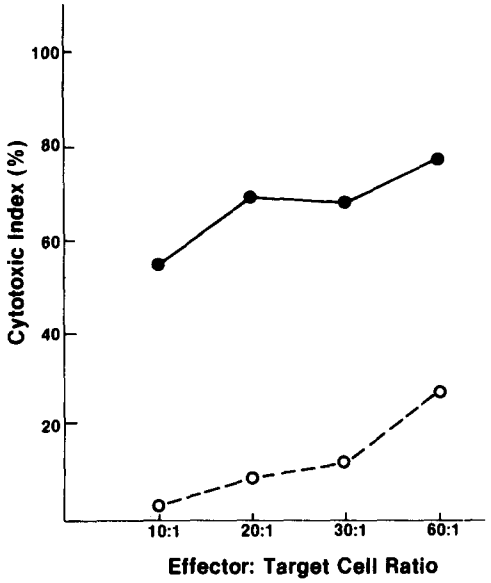


FIG. 6. Cytotoxicity of LPF-induced effector cells against syngeneic tumor and normal cells. BALB/c spleen cells were cultured with LPF for 72 h and assayed for cytotoxic effect against J774 BALB/c tumor macrophages (●) and normal BALB/c macrophages (○).

induced CEL and whether there are different CEL populations exhibiting different specificities, or a limited number of populations with multiple specificities.

## Summary

Cytotoxic effector lymphocytes were induced in cultures of mouse spleen or lymph node cells by lymphocytosis promoting factor (LPF). The LPF-activated cytotoxic cells: (*a*) were not generated unless proliferation occurred; (*b*) sedimented in the lighter density fraction of a bovine serum albumin gradient; (*c*) were large, blast-like cells; and (*d*) were lysed by Thy-1.2 antiserum plus complement and, therefore, were T cells. Neither LPF alone nor supernates from stimulated cultures were cytotoxic.

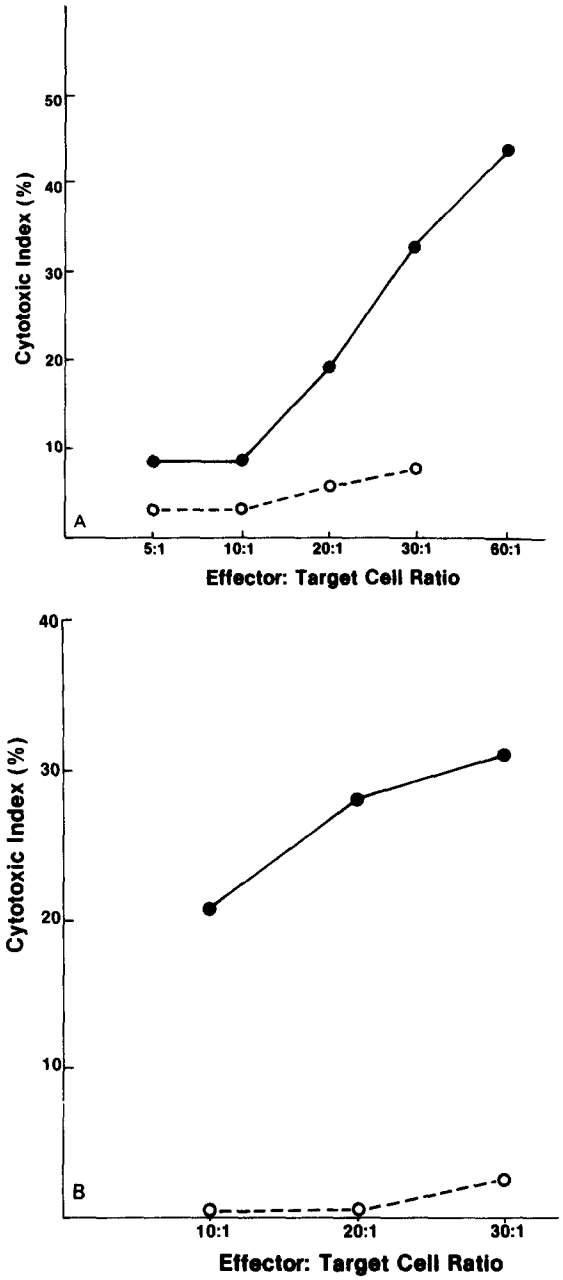


FIG. 7. Cytotoxicity of LPF-induced effector cells against allogeneic and syngeneic normal cells. BALB/c (H-$2^d$) spleen cells were cultured with LPF for 72 h and assayed for cytotoxic activity against (A) BALB/c macrophages (○) and C57Bl/6 H-$2^b$) macrophages (●); and (B) BALB/c LPS blasts (○) and CBA (H-$2^k$) LPS blasts (●).

Unlike the situation with concanavalin A and phytohemagglutinin P, LPF-stimulated cytotoxic effector lymphocytes required no further addition of mitogen for maximal cytotoxicity. The effector cells displayed specificity, destroying only allogeneic but not syngeneic normal cells; in the case of tumor cells, both allogeneic and syngeneic

cells were lysed in the absence of added mitogen. The reason for differential cytotoxicity toward syngeneic tumor and normal cells is not clear but may have some relevance to in vivo tumor rejection initiated by *Bordetella pertussis*.

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