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Nonspecific Recruitment of Lymphocytes in Purified Protein Derivative-Induced Lymphocyte Proliferative Response of Patients with Tuberculosis

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Peripheral blood lymphocytes (PBL) from tuberculosis patients were studied for their in vitro proliferative response stimulated with purified protein derivative (PPD)-tuberculin. Studies were designed to characterize the lymphocytes involved in the PPD-induced proliferation. PPD-responsive lymphocytes were eliminated in PBL by the procedure of cultivation of PBL with PPD in the presence of 5-bromodeoxyuridine and light illumination of the cultured cells. These PBL which lost PPD reactivity were no longer able to proliferate to PPD stimulation but were still capable of proliferation in the presence of both PPD and X-irradiated, autologous fresh PBL or upon addition of culture fluids from PPD-stimulated PBL. In addition, these nonspecifically activated lymphocytes released a soluble factor into the culture fluids which inhibited the migration of leukocytes. It was likely that large numbers of nonspecific T cells were induced to proliferate as a result of the presentation of specific T cells with the antigen PPD. It is suggested that a similar recruitment of lymphocytes by PPD-stimulated T cells takes place in vivo during the establishment of tuberculosis or antituberculous immunity or both.

The mechanism of generation of effector lymphocytes is a central question in the understanding of the cell-mediated immune response such as tuberculin-induced delayed-type hypersensitivity in man. The cell-mediated immune response appears to result from the specific recognition of an antigen by T cells, and it is probable that the effector cells are the clonal descendants of the sensitized lymphocytes that recognize the antigen. Upon stimulation with purified protein derivative (PPD)-tuberculin in vitro, T lymphocytes from patients with tuberculosis proliferate, and in this study, T-cell antigen specificity has been inferred on the basis of a direct correlation between antigen addition and proliferation. However, it is not clear whether observed proliferative response exclusively reflects the proliferation of PPD-specific T cells.

In man, stimulation of T cells from tuberculosis patients with PPD in vitro leads to very high levels of [^{3}H]thymidine ([^{3}H]TdR) uptake, as high as that induced with the polyclonal T-cell activator, concanavalin A (ConA). We have also observed that levels of lymphocytes forming autorosettes were strongly enhanced when pleural fluid lymphocytes from patients with tuberculous pleurisy were stimulated in vitro with PPD (13). Autorosetting lymphocytes, lymphocytes rosetting with autologous human erythrocytes, are reported to belong to activated T lymphocytes (10). Since the frequency of antigen-sensitive T lymphocytes even in primed donors may be no greater than 1 in 10^3, these results strongly suggest that a substantial degree of recruitment of nonspecific lymphocytes to proliferate occurs as the result of stimulation of a few antigen-sensitive T cells. This recruitment of normal nonspecific T cells by primed T cells may be operating in the development of cell-mediated immunity and augmenting the response.

To test this prediction in a human system, we attempted to analyze the PPD-induced proliferative response of peripheral blood lymphocytes (PBL) obtained from tuberculin-positive individuals. Negative selection of PPD-reactive lymphocytes was performed by culturing the lymphocytes in the presence of both PPD and 5-bromo-2-deoxyuridine (BUdR). Our results show that lymphocytes which were depleted of PPD reactivity by negative selection could no longer proliferate to PPD stimulation but could proliferate vigorously in the presence of both PPD and X-irradiated autologous lymphocytes or even by the stimulation of culture fluids of PPD-stimulated PBL and that these nonspecifically activated lymphocytes did elaborate a lymphokine, leukocyte migration inhibition factor (LMIF), in their culture supernatants.

MATERIALS AND METHODS

Subjects. PBL were obtained from patients with tuberculosis or from tuberculin skin test-positive healthy individuals. Six patients with tuberculosis studied were inpatients in our hospital. Tuberculin skin test-positive healthy donors were doctors and workers in our hospital. PBL from these donors gave high proliferative responses to PPD stimulation in vitro. The proliferation assay revealed no significant differences in responsiveness to PPD stimulation between PBL from patients and PBL from healthy volunteers. Therefore, in studies where a large number of lymphocytes were required, healthy volunteers were employed as lymphocyte donors. PBL were separated by centrifugation over a cushion of Ficoll-Hypaque as described in the previous report (14). Purified T cells were prepared by first rosetting with sheep erythrocytes, followed by isolation with Ficoll-Hypaque sedimentation. The cell fraction rich in T cells that rosetted with sheep erythrocytes was obtained after lysis of erythrocytes in hypotonic solution and then filtered on a nylon wood column. A 10-ml plastic syringe filled with 300 mg of nylon wool was autoclaved and rinsed with 10 ml of warmed medium supplemented with 10% heat-inactivated fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.) before use. Cell suspensions were applied on the column, incubated at 37°C for 1 h in 5% CO_2 in air, and then slowly eluted (1 ml/min), washed twice with RPMI 1640 medium, and suspended in the appropriate medium. More than 95% of the purified T cells thus prepared rosetted with sheep erythrocytes. Adherent cells were obtained by recovering with a rubber policeman the cells which were not E-rosetting and which were adherent to the plastic petri dish (Falcon Plastics, Oxnard, Calif.; no. 3002).

In vitro proliferative response of lymphocytes. A 0.2-ml sample of lymphocyte suspension (10^6/ml in RPMI 1640 medium containing 10% human serum, 100 μg of streptomycin per ml, and 100 U of penicillin per ml) was cultured in flat-bottomed tissue culture plates (Microtest II; Falcon no. 3042) at 37°C in an atmosphere of 5% CO_2 in air. The amount of PPD used was 50 μg/ml, the optimum amount for the proliferation of PBL. The PPD was kindly donated by S. Fujii, Institute for Microbial Diseases, Osaka University. PBL were cultured in triplicate for various days as indicated. At the end of the culture period, 0.2 μCi of [^{3}H]TdR was added to each well. After 18 h, cells were harvested on a glass filter with a semiautomated cell harvester (Laboscience Co. Ltd., Tokyo), and the radioactivity was counted in a Packard Tri-Carb scintillation counter.

PPD-induced production of lymphocyte culture supernatants. A 1-ml amount of PBL suspension (2×10^6/ml in RPMI 1640 medium supplemented with 1% fetal calf serum) was incubated for 48 h in polystyrene culture tubes (12 by 75 mm; Falcon no. 2054) in the presence of PPD (50 μg/ml) at 37°C in 5% CO_2 in air. At the end of incubation, supernatants were collected and centrifuged at 1,500 rpm for 5 min to remove cells. Control cultures were incubated without PPD and reconstituted with PPD at the end of 48 h of incubation. Culture supernatants were millipore filtered (0.45 μm; Millipore Corp., Bedford, Mass.) and kept frozen at −70°C.

Negative selection of PPD-reactive lymphocytes. To eliminate PPD-reactive lymphocytes in PBL, a negative selection procedure using BUdR (P-L Biochemical, Inc., Milwaukee, Wis.) and light treatment was employed (8). Two million PBL suspended in 1 ml of culture medium supplemented with 5×10^{-5} M 2-mercaptoethanol and 10% autologous plasma were placed in polystyrene culture tubes (12 by 75 mm) and cultured in the presence of both PPD (50 μg/ml) and BUdR (2 μg/ml) at 37°C in 5% CO_2 in air. On day 4 of the culture period, cells were illuminated for 120 min by a fluorescent light source to eliminate the PPD-induced DNA-synthesizing cells that proliferated during the culture period. Cells were washed once with medium and used in the nonspecific proliferation study as described below.

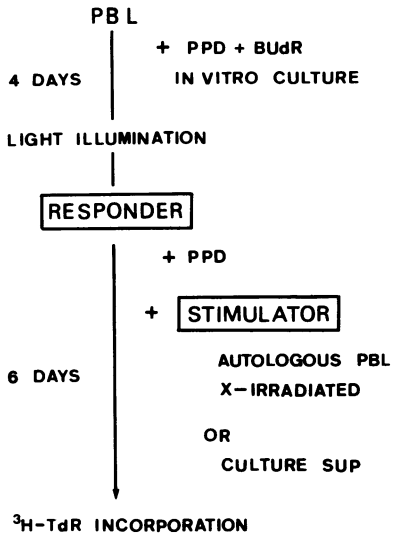

FIG. 1. Schematic diagram of experimental procedure.

Nonspecific proliferation of PBL which have no PPD reactivity. Freshly obtained PBL were irradiated with 2,600 roentgens (R) from an X-ray source and employed as stimulators. They were mixed in various proportions with non-irradiated autologous PBL (responders) in which the PPD-reactive lymphocytes had been previously depleted by the method of BUdR treatment as described above. In typical experiments, 2×10^5 responder cells were cultured together with equal numbers of irradiated stimulator cells in the presence or absence of PPD (50 μg/ml). The rest of the proliferation assay was performed as described above. In the proliferation experiments where responder cells were stimulated with PPD-induced lymphocyte culture supernatants, the cells were cultured in the presence of various concentrations of culture supernatants instead of stimulator cells. A schematic diagram of the procedure is shown in Fig. 1.

Assay of LMIF. An indirect agar plate assay for the inhibitory factor, originally described by Clausen in 1976 (2), was employed in this study. Cord blood obtained from full-term, delivered, healthy babies was used as the source of leukocytes. Mononuclear cells were first removed by the Ficoll-Hypaque sedimentation technique, and leukocytes were prepared from mononuclear cell-depleted cord blood sedimented for

TABLE 1. Negative selection of PPD-reactive cells in PBL by BUdR and light treatment

Treatment of PBL[a]	[^{3}H]TdR incorporation (cpm)[b] with		
	No stimulation	PPD	ConA
None	796 ± 69	8,645 ± 278	8,536 ± 499
PPD	8,232 ± 19	12,104 ± 887	32,081 ± 2,081
PPD + BUdR	185 ± 11	469 ± 45	5,379 ± 396
Fresh PBL, non-irradiated	448 ± 36	4,997 ± 1,382	8,269 ± 841

[a] PBL were precultured in vitro with or without PPD (50 μg/ml) and in the presence or absence of BUdR (2 μg/ml) for 4 days. At the end of the culture period, harvested cells were light illuminated.

[b] Cells were cultured in vitro for 5 more days with PPD (50 μg/ml) or ConA (5 μg/ml), and the counts per minute of [^{3}H]TdR incorporated during the last 18 h were counted. The values given are the mean counts per minute of triplicate determinations ± the standard error of the mean.

30 min at 37°C with 5% dextran (molecular weight, 250,000 to 300,000; Wako Pure Chemicals Co. Ltd., Osaka) in saline solution in the proportion 8:1. The leukocyte-rich plasma was centrifuged, and cells were washed after treatment with hypotonic solution to lyse erythrocytes. Leukocytes were finally resuspended in culture medium supplemented with 5% fetal calf serum to give a final cell concentration of 4×10^8/ml. Equal volumes of culture supernatants to be assayed were added to the cells. Ten-microliter portions containing 2×10^6 leukocytes were placed in holes (3.0-mm diameter) in the agar medium which consisted of 1.2% agar and 20% horse serum in single-strength RPMI 1640 medium. The migration inhibitory effect of a supernatant was expressed as a migration index which indicated the ratio between the average migration area of leukocytes resuspended in supernatant from stimulated cultures and that of leukocytes resuspended in supernatant from the corresponding control cultures.

RESULTS

Negative selection of PPD-reactive lymphocytes in PBL. Depletion of PPD-reactive lymphocytes was carried out by preculturing PBL with PPD in the presence of BUdR and by illuminating the cultured cells at the termination of the culture period. After varying experimental culture conditions using PBL from six tuberculosis patients and three healthy donors, the following procedure was employed as optimum. Cells suspended in RPMI 1640 culture medium (2×10^6/ml) supplemented with 5×10^{-5} M 2-mercaptoethanol and 10% human AB serum were incubated in the presence of 50 μg of PPD and 2.0 μg of BUdR per ml in a Falcon plastic tube (Falcon no. 2054) at 37°C in 5% CO_2 in air. On day 4, the cultured PBL were illuminated for 120 min with a fluorescent light source, washed, and resuspended in the fresh medium. Samples of cells were again stimulated in vitro with PPD (50 μg/ml) or ConA (5 μg/ml), and the proliferative response was assessed on days 3 and 6 by measuring the incorporation of [^{3}H]TdR during the last 18 h. A representative result is shown in Table 1. PBL precultured with PPD in the presence of BUdR and exposed to light lost their ability to respond to stimulation with PPD. The responsiveness of the PBL to ConA stimulation, however, remained intact although it was slightly decreased by treatment with BUdR. The highest response observed in ConA-stimulated PBL which were precultured with PPD and without BUdR (second column) was probably due to the summation of responses stimulated with ConA and PPD. PBL devoid of PPD reactivity by these criteria were used as responders in the nonspecific proliferation experiments.

Nonspecific proliferation of PBL which contain no PPD-reactive cells in the presence of PPD and autologous fresh PBL. To investigate whether PBL from tuberculin-positive individuals stimulated with PPD could induce proliferation of bystander lymphocytes, we performed cell mixture experiments. Suspensions of PBL depleted

TABLE 2. Nonspecific proliferative response of PBL

Treatment of responder cells[a]	[^{3}H]TdR incorporation (cpm)[b]	
	3 Days	6 Days
Expt 1		
None	539 ± 41	88 ± 10
PPD	602 ± 81	111 ± 21
Stimulator[c]	6,128 ± 38	5,950 ± 780
PPD + stimulator	14,575 ± 1,742	14,889 ± 1,289
Expt 2		
None	105 ± 47	74 ± 8
PPD	330 ± 30	330 ± 31
Stimulator	16,566 ± 875	5,493 ± 735
PPD + stimulator	19,618 ± 518	17,498 ± 1,735[d]

[a] Responder cells (2×10^6/ml) were cocultured with equal numbers of stimulator cells in the presence or absence of PPD (50 μg/ml) for 3 and 6 days in vitro, and the amount of [^{3}H]TdR incorporated during the last 18 h was counted. The responder cells were PBL that had been precultured in vitro with PPD and BUdR, followed by light illumination.

[b] Mean counts per minute [^{3}H]TdR incorporated into cells of triplicate determinations ± the standard error of the mean.

[c] Fresh PBL, irradiated with 2,600 R.

[d] In the absence of responder cells, stimulator cells with PPD gave a result of 1,779 ± 98.

TABLE 3. Nonspecific proliferative response of PBL

Treatment	[^{3}H]TdR incorporation (cpm)[a]	
	No PPD	PPD
Responder cell[b]		
None	539 ± 41	602 ± 81
Unseparated stimulator[c]	6,128 ± 380	14,575 ± 1,742
T cell-enriched stimulator	6,926 ± 135	13,500 ± 101
B cell-enriched stimulator	1,558 ± 59	3,521 ± 260
Stimulator cell alone		
Irradiated	87 ± 2	1,022 ± 65
Non-irradiated	242 ± 51	6,526 ± 153

[a] Mean counts per minute [^{3}H]TdR incorporated into cells of triplicate determinations ± the standard error of the mean.

[b] PBL precultured with PPD and BUdR, followed by light illumination. Responder cells (2×10^6/ml) were cocultured with equal numbers of stimulator cells in the presence or absence of PPD (50 μg/ml) for 6 days in vitro, and the amount of [^{3}H]TdR incorporated during the last 18 h was counted.

[c] Stimulator cells are fresh PBL irradiated with 2,600 R.

of PPD-reactive cells by PPD and BUdR treatment (responders) were mixed with fresh, autologous PBL that had been irradiated with 2,600 R (stimulators). The proliferation of responder cells, as detected by [^{3}H]TdR incorporation, is shown in Table 2. Responder cells failed to proliferate after presentation with PPD (responder + PPD). However, when irradiated, fresh autologous PBL were mixed in a proportion of 1:1 with responders in the presence of PPD (responder + PPD + stimulator), extensive proliferation was detected as assessed by [^{3}H]TdR incorporation. By the addition of irradiated, fresh PBL (stimulator), even in the absence of added PPD, responder cells showed a significant degree of proliferation. This might be due to the existence of small amounts of PPD remaining in the responder cell suspension which was not washable after treatment with PPD and BUdR. Another possibility was, as discussed later, the autologous mixed lymphocyte reaction. Stimulator cells (irradiated with 2,600 R) alone showed no significant response, irrespective of the addition of PPD. Table 3 shows that nylon wool column-purified T cells, but not B cells, were able to stimulate responder lymphocytes into proliferation when presented with PPD. PBL devoid of PPD reactivity (responder) lacked stimulatory activity either in the presence or absence of PPD. These experiments demonstrated that PPD-specific T cells in the presence of PPD could recruit a significant number of nonspecific lymphocytes into proliferation.

A time course study of this nonspecific proliferative response has been carried out, and the

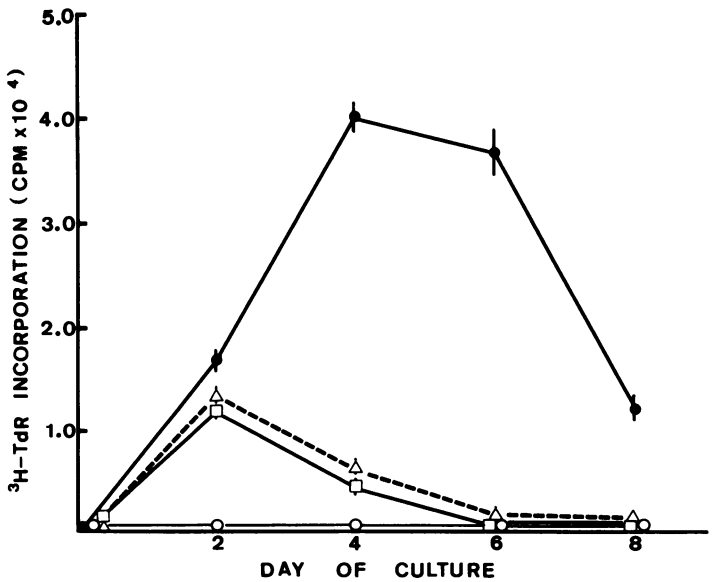

FIG. 2. Kinetics of nonspecific proliferative response of PBL. Responder cells (2×10^6/ml; PPD and BUdR treated) were cultured in vitro with equal numbers of stimulator cells (autologous fresh PBL, irradiated with 2,600 R) and with or without added PPD (50 μg/ml). The counts per minute (cpm) of [^{3}H]TdR incorporated during the last 18 h were counted. Mean levels ± the standard error of the mean are shown. Symbols: ●, responder + stimulator + PPD; △, responder + PPD; □, responder alone; ○, stimulator + PPD.

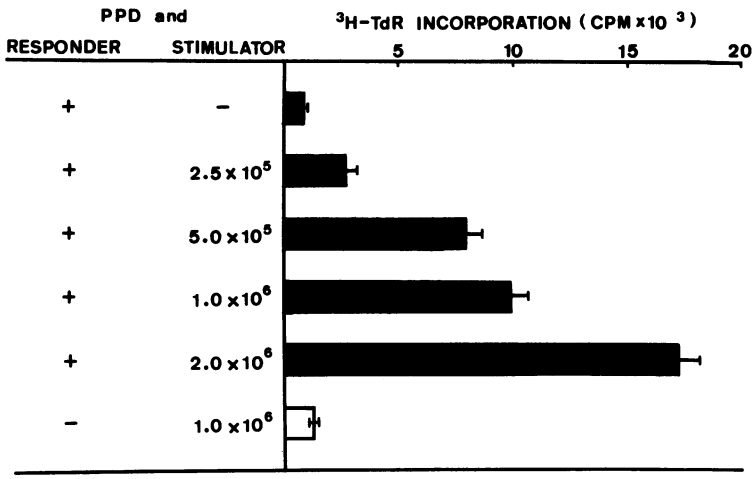

FIG. 3. Nonspecific proliferative response of PBL. Responder cells (10^6/ml; PPD and BUdR treated) were cocultured in vitro for 6 days with varying doses of stimulator cells (autologous fresh PBL, irradiated with 2,600 R) in the presence of PPD (50 μg/ml).

results are shown in Fig. 2. Maximum incorporation of [^{3}H]TdR into the responder cells in the presence of X-irradiated stimulators and PPD was observed between days 4 and 6 of culture.

Figure 3 shows the effect of varying doses of stimulator cells on the proliferative response of responders in the presence of 50 μg of PPD per ml. The culture period was 5 days. The proliferation was observed to follow a dose-response curve.

Nonspecific proliferation of PBL by stimulation with PPD-induced culture supernatants of fresh PBL. Table 4 shows that a PPD-induced culture supernatant of fresh PBL could substitute for X-irradiated PBL (stimulator) in the stimulation of responder cells. Supernatants were obtained from X-irradiated, fresh PBL cultured together with PPD (50 μg/ml) for 24 or 48 h. Responders which were depleted of PPD-reactive cells (2×10^6/ml) were mixed with equal volumes of supernatants and cultured in vitro for 5 days. The incorporation of [^{3}H]TdR during the last 18 h was measured and presented as counts per minute. As a control, culture supernatants obtained from PBL incubated without PPD were reconstituted with PPD at the harvest. As shown in Table 4, increased degrees of proliferative response of responder cells were observed when the cells were cultured in vitro in the presence of PPD-induced culture supernatant, whereas no response was observed at all when the cells were cultured in the presence of control culture supernatant. PPD was required in the production of active culture supernatant. Whether PPD was also required in the step of proliferation of responder cells could not be determined by these experiments although responders did not proliferate at all in the presence of PPD alone.

From the results in Table 3, T cells appear to be the producer of active supernatants. The results in Table 5 demonstrate that T cells did produce active supernatant and that adherent cells are required for T cells to produce active supernatant upon stimulation with PPD.

LMIF assay with culture supernatant obtained from nonspecifically activated lymphocytes. Responder PBL depleted of PPD reactivity by PPD

TABLE 4. Nonspecific proliferative response of PBL stimulated with culture supernatants

Treatment of responder cells[a]	[^{3}H]TdR incorporation (cpm)[b]
None	260 ± 74
PPD	635 ± 29
PPD + stimulator[c]	35,234 ± 2,279
Stimulator	17,044 ± 1,728
Sup [PBL-48][d]	437 ± 75
Sup [PBL+PPD-24][e]	2,313 ± 295
Sup [PBL+PPD-48] ×1[f]	7,165 ± 845
Sup [PBL+PPD-48] ×2	4,156 ± 121
Sup [PBL+PPD-48] ×4	573 ± 72

[a] Responder cells are PBL that were precultured in vitro with PPD and BUdR, followed by light illumination. They were mixed with equal volumes of diluted or nondiluted culture supernatants and cultured in vitro for 5 days.

[b] Mean counts per minute of [^{3}H]TdR incorporated into cells of triplicate determinations ± the standard error of the mean.

[c] Fresh PBL, irradiated with 2,600 R.

[d] Supernatants of a 48-h culture of irradiated PBL, reconstituted with PPD at harvest.

[e] PPD-induced 24-h culture supernatants of irradiated fresh PBL.

[f] PPD-induced 48-h culture supernatants of irradiated fresh PBL, diluted one time.

TABLE 5. Adherent cells requirement for PPD-induced soluble factor production

Treatment of responder cells[a]	[^{3}H]TdR incorporation (cpm)[b]
None	1,182 ± 199
PPD	2,045 ± 48
Sup [T][c]	835 ± 360
Sup [T+PPD][c]	2,368 ± 266
Sup [T+Ad][c]	1,535 ± 282
Sup [T+Ad+PPD][c]	13,469 ± 489

[a] PBL precultured in vitro with PPD and BUdR, followed by light illumination.

[b] Mean counts per minute of [^{3}H]TdR incorporated into cells of triplicate determinations ± the standard error of the mean.

[c] Culture supernatant of irradiated T cells in the presence or absence of irradiated adherent cells (Ad; 10% of T cells) and with or without PPD (50 μg/ml).

and BUdR treatment were pulse-stimulated for 24 h with culture supernatant of PPD-stimulated PBL, washed three times with medium, reconstituted with fresh medium, and cultured further for 48 h at 37°C in 5% CO_2 in air. At the end of the culture period, supernatants were harvested after centrifugation for 10 min at 2,500 rpm, millipore filtered, and subjected to the LMIF assay as described above. A representative result is shown in Table 6. PBL precultured in the presence of PPD and BUdR gave no PPD-induced proliferation or LMIF production. The PBL depleted of PPD reactivity, however, when pulse-stimulated with PPD-induced active supernatant (PPD-induced 48-h culture supernatant of X-irradiated, autologous PBL), showed vigorous proliferation as well as LMIF production.

DISCUSSION

The present study showed that in the specific antigen PPD-induced T-cell proliferative response, a substantial recruitment of nonspecific cells to proliferate occurred as the result of stimulation of a few activated, antigen PPD-sensitive T cells and that this nonspecific proliferation could be mediated by the cell-free supernatant of PPD-stimulated lymphocytes. In addition, the biologically active soluble factor, LMIF (a lymphokine), which reflects the in vivo delayed-type hypersensitivity, was elaborated from the nonspecifically activated lymphocytes.

Antigen- or nonspecific-mitogen-stimulated T-cell proliferation required antigen-presenting or accessory cells. In our study, BUdR-treated responder cells alone proliferated well to the ConA stimulation (Table 1). The addition of X-irradiated autologous lymphocytes (stimulator), therefore, does not represent the effect of addition of antigen-presenting or accessory cells. Moreover, the T cell-enriched fraction is a much better stimulator than the non-E-rosetting (B cells or accessory cells or both) fraction as shown in Table 3.

Stimulation of T lymphocytes with soluble antigen induces their proliferation, and proliferation assays have been useful in characterizing the requirement for the activation of T cells. However, the responding T-cell population involved has not been clearly delineated. In antigen-primed animals, the frequency of antigen-specific proliferating T cells is probably less than 1 in 10^3, whereas the percentage of cells undergoing blast transformation in cultures after antigen-specific stimulation may be as high as 19% (12). Using autologous rosette formation, we previously reported that as high as 20% of T lymphocytes were activated when pleural fluid lymphocytes from patients with tuberculous pleurisy were cultured in vitro for 6 days in the presence of PPD-tuberculin (13). These results, taken together, strongly suggest that a substantial degree of recruitment of nonspecific cells occurred as the result of stimulation of a few activated, antigen-sensitive T cells.

Our findings are analogous to those of Augus-

TABLE 6. Production of LMIF by nonspecifically stimulated PBL[a]

Treatment of PBL	Stimulation with PPD		Stimulation with PPD-induced factor[b]	
	Proliferation (cpm)	LMIF (%)[c]	Proliferation (cpm)	LMIF (%)
Fresh	12,315 ± 671	62.7		
PPD for 4 days	25,004 ± 204	71.7		
PPD + BUdR for 4 days	1,894 ± 169	92.3	24,699 ± 334[d]	76.0[d]

[a] Freshly obtained PBL or PBL cultured in the presence of PPD or BUdR or both were cultured for 6 days with PPD or PPD-induced factor. Proliferation was assessed by measuring the [^{3}H]TdR incorporated during the last 18 h. LMIF activity was estimated in 48-h culture supernatants stimulated with PPD or pulse-stimulated with PPD-induced factor.

[b] A 48-h PPD-induced culture supernatant of fresh, irradiated PBL.

[c] Percent migration, calculated with respect to the migration of cells in the presence of culture medium alone.

[d] Results obtained with culture supernatant after pulse-stimulation with non-PPD-induced control factor and reconstitution with PPD were 3,516 ± 287 cpm and 93.3%.

tin and co-workers (1) who demonstrated, using murine splenocytes, that large numbers of T cells that were not antigen specific were induced to proliferate as a result of the presentation of antigen-specific T cells with antigen, and they referred to this phenomenon as trans-stimulation. In our present study, we showed that although PPD-specific T cells were required, they did not have to proliferate to induce nonspecific lymphocyte proliferation. In addition, we also showed that culture supernatants of PPD-stimulated lymphocytes were capable of inducing this nonspecific lymphocyte proliferation. Philip et al. (9) recently have demonstrated a heat-labile, low-molecular-weight (<5,000) lymphokine in the PPD-induced culture supernatants of tuberculin-sensitive guinea pig peritoneal exudate cells which stimulated migration inhibition factor production by nonsensitive lymphocytes. Recent studies have indicated that initiation and maintenance of T-cell proliferation are mediated by a soluble T-cell growth factor (IL-2) released from lectin- or antigen-activated T lymphocytes (4, 7) and not from antigens or mitogens themselves. This report, together with other previous findings, suggests that IL-2 may play a physiological role in the generation of the in vitro T cell-mediated immune response. T cells are required for IL-2 production, and activity is elicited only upon mitogen or antigen stimulation of T cells. IL-2 selects for and maintains exponential proliferation of T cells. IL-2, however, does not induce growth in resting T cells but provides the proliferative stimulus only after mitogen or antigen activation. Activated T cells preferentially absorb IL-2 activity (11). In our study, culture supernatant of irradiated PBL stimulated by PPD-tuberculin provided a remarkable proliferation of autologous PBL which had been previously depleted of PPD-reactive cells by the treatment with BUdR. Treatment of a population of antigen-stimulated lymphocytes with BUdR and light eliminates the dividing cells since cells undergoing PPD-induced proliferation incorporate BUdR into their DNA and are subsequently damaged and eliminated when exposed to light. In fact, such treated PBL could not respond to the in vitro stimulation with PPD but did respond to stimulation with X-irradiated PBL in the presence of PPD or to the stimulation of culture supernatants. Whether cells treated with PPD and BUdR retained their ability to respond to IL-2 remains to be elucidated. There are several observations which indicated that normal IL-2-responsive cells belonged to a subset of T cells distinct from IL-2-producing T cells. It is possible that pretreatment of lymphocytes with PPD and BUdR only eliminates the subset of PPD-activated T cells that release IL-2 and does not eliminate activated T cells bearing IL-2 receptors since we have observed that lymphocytes precultured with PPD and BUdR, but not with BUdR only, proliferate upon stimulation with partially purified IL-2 (unpublished data). Geha et al. (3), on the other hand, have reported that lymphocyte mitogenic factor is in the culture supernatants of antigen-stimulated T cells and acts on either normal T or B lymphocytes.

The autologous MLR should be taken into consideration as a further cause of nonspecific T-cell stimulation by PPD in coculture experiments. The autologous MLR is the proliferative response of T lymphocytes when cultured with autologous non-T lymphocytes. Ia-like antigens on the surface are required for the non-T lymphocytes to stimulate autologous T cells (6). Indiveri et al. (5) reported phytohemagglutinin-activated human T cells acquired Ia-like antigens having structural properties similar to those of B lymphocyte-derived Ia-like antigens and that these phytohemagglutinin-activated T cells stimulated a unidirectional allogeneic MLR. Therefore, it is conceivable that T cells activated by specific antigen, such as PPD in our study, develop Ia antigens on their surfaces and become capable of stimulating other T cells nonspecifically. Autologous MLR, however, does not seem to play a major role in the lymphocyte activation in our present study. In our study, as shown in Table 3, T cells and not B cells (including macrophages) were good stimulators for nonspecific lymphocyte proliferation. Furthermore, our study shows that nonspecific lymphocyte recruitment could be mediated by cell-free culture supernatants and did not necessarily require cell-to-cell interaction which is essential in the autologous MLR.

In summary, our data indicate that a substantial degree of recruitment occurred as the result of stimulation of activated PPD-sensitive T cells in the PPD-induced T-cell response of human tuberculous patients. We suggest that [^{3}H]TdR incorporation is not a direct reflection of antigen-specific T-cell proliferation, but rather reflects the growth of a heterogeneous T-cell population in which the PPD-specific T cells are a minority. It is likely that similar recruitment of lymphocytes by PPD-stimulated T cells takes place in the cell-mediated immune response in vivo, probably during the establishment of tuberculosis or antituberculous immunity or both. Our observation that nonspecifically activated lymphocytes did produce active migration inhibition factor is intriguing in this regard. Our preliminary kinetic study on PPD-induced factor production by lymphocytes from tuberculous patients shows that culture supernatants harvested after a 24-h culture period contained both lymphocyte mitogenic activity and LMIF activi-

ty, whereas culture supernatants harvested after a 72-h culture period contained mostly LMIF activity.

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