Limiting dilution analysis of T cell unresponsiveness to mycobacteria in advanced disseminated tuberculosis

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Abstract. Peripheral blood mononuclear cells from patients with advanced disseminated tuberculosis (Dis-TB) do not respond to purified protein derivative (PPD) measured as cell proliferation, lymphokine production and interleukin (IL)-2 receptor (Tac antigen) expression. Limiting dilution analysis revealed "multi-hit" curves and low frequencies of PPD-reactive T cells in cultures of Dis-TB, and "single-hit" curves and high frequencies of PPD-reactive T cells in cultures of patients with localized form of pulmonary tuberculosis. Moreover, a strict relationship between Tac antigen expression and ability of exogenous IL-2 to enhance bulk culture cell proliferation was observed in Dis-TB patients.

Introduction

Acquired cellular immunity to many facultative intracellular bacteria is known to depend upon the generation of protective T lymphocytes, which activate host macrophages to control the growth of the infectious organism (Orme and Collins 1983; Rook et al. 1987). *Mycobacterium tuberculosis* is a classical intracellular pathogen which in some patients causes a persistent anergy or hyporesponsiveness to several antigens as judged by a skin test in vivo and by lymphocyte transformation in vitro (Bhatnagar et al. 1977; Lenzini et al. 1977; Chaparas 1982). The detailed mechanism of this unresponsiveness is unknown, although a defective macrophage function, absence of reactive T lymphocytes from the circulation and active suppression have been reported (Rook et al. 1976; Katz et al. 1979; Lombardi et al. 1986). Recently, it has been suggested that immunological unresponsiveness is a result of the recognition of antigen in the relative absence of

interleukin (IL)-2 (Malkovsky & Medawar 1984; Colizzi et al. 1986), and a lack of IL-2 production has been reported in both experimental (Hoffenbach et al. 1983) and human (Toossi et al. 1986) mycobacterial infections.

To extend the analysis of the anergy occurring in tuberculous patients, we have investigated (i) the purified protein derivative (PPD)-induced proliferative response and lymphokine production [IL-2 and interferon (IFN)] in bulk cultures of patients with disseminated (Dis-TB) and localized (Loc-TB) form of tuberculosis, (ii) the expression of IL-2 receptor (Tac antigen), and the effect of exogenous IL-2 on T cell proliferation and IFN synthesis, and (iii) the precursor frequency of PPD-reactive T cells using the limiting dilution analysis (LDA).

Materials and methods

Tuberculous patients

Peripheral blood mononuclear cells (PBMC) were collected from 25 patients with pulmonary tuberculosis and 15 healthy contacts. The diagnosis of tuberculosis was confirmed by the presence of acid-fast bacilli in sputum and by positive culture for *M. tuberculosis*. The extent of pulmonary disease was assessed from chest X-ray and patients were classified in two forms of pulmonary tuberculosis, i.e., advanced disseminated (Dis-TB) and cavitary localized (Loc-TB). Ten Dis-TB patients and 12 Loc-TB patients have been extensively studied in vitro.

Cell preparation

PBMC were isolated on a gradient of Lymphoprep (Nyeegaard, Oslo, Norway) and suspended at the concentration of 10^6 cells/ml in RPMI-1640 medium (Gibco) containing 10% autologous heat-inactivated serum, 2 mM L-glutamine (Eurobio, Paris) and 40 µg/ml gentamycin (Shering). For the LDA assay, T cells were partially enriched by nylon wool filtration.

LDA of PPD-reactive PBMC from TB patients and healthy individuals

Cultures were set up in flat-bottom Falcon microplates, each well containing varying numbers of nylon wool-enriched T cells (with a purity of 80%–90% of CD3-positive cells) as responders, mitomycin C-treated PBMC as accessory cells (10^4 cells/well) and PPD (10 µg/ml) in a total volume of 200 µl of complete culture RPMI-1640 medium with 10% human serum. After 11 days of incubation, cell proliferation in each microwell was evaluated as [3 H]thymidine ([3 H]) incorporation, and individual wells were scored as positive when the cpm exceeded the mean of the negative control (T cells alone) by at least 3 standard deviations, limiting the probability of false positivity to less than 0.14%. The analysis of the distribution of precursor cells was performed by plotting on the y axis the negative logarithm of the fraction of nonresponding cultures ($-\ln F_0$) and the cell input on the linear

scale on the x axis. The semilog plot has been used to estimate the frequency of precursor cells, because by interpolating at the level of 37% nonresponding cultures the size of the sample containing an average of one precursor cell can be estimated (Lefkovitz and Waldmann 1976).

Proliferation assay

PBMC were cultured (10^5 cells/well) in triplicate in flat-bottom microtiter plates (No. 3072, Falcon) in the presence or absence of an optimal dose of PPD (10 μ g/ml, Statens Seruminstitut, Copenhagen). A suboptimal dose of human recombinant (r) IL-2 (2 units/ml, kindly provided by Biogen SA, Geneva) was added at day 4 of culture. PBMC proliferation was evaluated as incorporation in the last 16-h pulsing with 0.5 μ Ci ($[^3\text{H}]\text{dThd}$, Amersham) and expressed as cpm.

Determination of Tac antigen expression

The expression of Tac antigen was evaluated at day 4 of culture by indirect immunofluorescence assay using anti-Tac monoclonal antibodies kindly provided by T. A. Waldmann (Leonard et al. 1982) and affinity-purified goat anti-mouse IgG, F(ab')₂-fluorescein isothiocyanate (Cappel Laboratories) as second reagent, as previously reported (Vismara et al. 1985).

IL-2 and IFN- γ production and assay

PBMC (10^6 cells/ml) were set up in 24-well plates (Nunc) in the same culture condition as described in the proliferation assay. The supernatants were collected at 48 h of culture and stored at -20°C . The presence of IL-2 and of IFN- γ in culture supernatants was measured biologically and the activity was expressed in laboratory units as previously reported (Piccolella et al. 1986). The IL-2 activity was tested in a standard microassay by using an IL-2-dependent T cell line (CTLL 10^4 /well) and evaluating the $[^3\text{H}]\text{dThd}$ incorporation in a 24-h culture experiment (Vismara et al. 1985). The IFN activity was characterized as γ type for its sensitivity to pH 2.0 and to anti-IFN- γ monospecific antiserum, kindly provided by Dr. M. P. Langford (UMTB, Galveston), and by its resistance to neutralization by anti-IFN- γ antibodies (Schering).

Results

Tuberculin skin test, proliferative response, and lymphokine (IL-2 and IFN- γ) production

Figure 1 illustrates the PBMC proliferative response to PPD (distribution and mean) and the tuberculin skin test of 10 patients with Dis-TB, 15 patients with

Fig. 1. Peripheral blood mononuclear cell (PBMC)-proliferative response and tuberculin skin reactivity in 10 Dis-TB patients, 15 Loc-TB patients and 15 healthy controls. Purified protein derivative (PPD) (1 unit) was given intradermally and the area of skin reaction evaluated 48 and 72 h later and expressed in mm (open symbol). PBMC (5×10^5 cells/well) were cultured for 5 days in the presence of PPD (closed symbol).

Loc-TB and 15 healthy contacts. Skin test in vivo correlated positively with the responsiveness in vitro, i.e., in the Dis-TB form the skin anergy was accompanied by a decreased cell proliferative response to PPD, whereas both Loc-TB patients and the healthy control group displayed high levels of both skin reactivity and cell proliferation. Healthy individuals, with a negative skin test and low proliferative response in vitro, were not included in this figure, although they were analyzed in the following experiments.

The proliferative response and the production of IL-2 and IFN- γ of patients and healthy individuals are shown in Table 1. A state of unresponsiveness or hyporesponsiveness was observed in all Dis-TB patients, whereas Loc-TB patients responded well to PPD. Healthy individuals may be divided into high responders and in low responders, considering the intensity of their proliferative response to PPD. The unresponsiveness in Dis-Tb patients appeared to be nonspecific, since their PBMC also did not respond to purified polysaccharide from *C. albicans*, whereas the low responsiveness to PPD of healthy individuals was specific as they responded normally to *C. albicans* antigens (data not shown).

Estimation of frequencies of PPD-reactive precursor cells

To investigate the frequency of PPD-reactive precursor cells, we have incubated various numbers of enriched T cells in the presence of PPD and scored the cellular growth as radioactive dThd incorporation. Figure 2 shows the results of a typical experiment in which the frequency of PPD-reactive cells in five Dis-TB (panel A) and four Loc-TB patients (panel C) was measured and compared with those of five low responders (panel B) and four high responders (panel D). The experimental values obtained from cultures of Loc-TB and healthy individuals (either low or high responders) fit straight lines passing through the origin, suggesting that a single population of proliferating T cell is being measured. The apparent frequency of PPD-reactive precursor cells ranged between 1/4,000 and 1/9,000 cells for the

Table 1. DNA synthesis, interleukin (IL)-2 and interferon (IFN) production of peripheral blood mononuclear cells (PBMC) from tuberculous patients and healthy controls

		³ H]dThd uptake (cpm)		IL-2 (U/ml)	IFN (U/ml)
		– PPD	+ PPD		
Dis-TB					
Patient:	1	186	2,376	0.5	< 3
	2	356	12,180	1.5	10
	3	240	5,704	0	30
	4	191	1,631	0	< 3
	5	289	2,703	ND	ND
	6	877	934	0	ND
Loc-TB					
Patient:	1	453	52,283	3.5	300
	2	288	45,600	9.5	1000
	3	1,095	54,257	ND	ND
	4	780	49,832	4.5	200
	5	412	63,609	3.5	300
	6	874	49,786	ND	ND
Healthy controls: high responders					
Individual:	1	852	88,941	9.0	600
	2	374	60,448	7.0	500
	3	580	73,984	6.0	400
	4	308	70,606	5.5	700
	5	240	54,551	20.0	600
	6	296	45,832	10.0	ND
	7	127	38,680	8.0	ND
Healthy controls: low responders					
Individual:	1	119	9,087	1.0	100
	2	80	1,720	0.5	300
	3	230	2,371	0	10
	4	1,007	2,202	0	50
	5	940	11,479	ND	ND
	6	660	2,060	ND	ND

PBMC from healthy individuals and patients with advanced disseminated tuberculosis (Dis-TB) and with the cavitary localized form of tuberculosis (Loc-TB) were cultured for 6 days with purified protein derivative (PPD) (10 µg/ml) and the radioactive thymidine (dThd) was added for the last 20 h of culture. The results are expressed as cpm. The standard deviation was less than 10%. The 48-h culture supernatants, collected from PPD-stimulated cultures, were tested for the presence of IL-2 and IFN-γ and expressed in unit/ml. ND = Not done

Loc-TB patients (panel C), between 1/5,000 and 1/19,000 for cultures of high responder healthy controls (panel D), and between 1/31,000 and 1/73,000 for cultures of healthy individuals selected for their low responsiveness to PPD (panel B) (Vismara et al. 1985). In contrast, the experimental values obtained in cultures from Dis-TB patients give a “multi-hit” curve, suggesting two possible explana-

Fig. 2A-D. Limiting dilution analysis of the PPD response of five Dis-TB (A), four Loc-TB patients (C), five low-responder (B), and four high-responder (D) healthy individuals. Each data point derives from 24-well microcultures. The lines shown were fitted by at least square method, the correlation coefficient r was calculated and the statistical significance P determined. In Loc-TB patients, and healthy high- and low-responder individuals, $r > 0.95$ ($P < 0.05$); and in Dis-TB, $r < 0.95$ ($P > 0.05$)

tions: the low amount of IL-2 present in culture is growth limiting at higher responder cell numbers; or two populations are limiting in the culture. The first possibility was excluded by LDA performed in the presence of exogenous IL-2 (20 U/ml) added at day 7 of cultures. In fact, the values did not differ from those obtained in cultures without the addition of the rIL-2 (data not shown). The correlation coefficient r was > 0.95 ($P < 0.05$) in Loc-TB and all healthy individuals, and r was < 0.95 ($P > 0.05$) in Dis-TB cultures. Thus, no precursor frequencies could be estimated in the Dis-TB cultures, although the data suggest that their frequencies are lower than that of Loc-TB and high-responder healthy individuals, and similar to that of low-responder healthy individuals.

Table 2. Expression of the Tac antigen and effect of recombinant IL-2 on PPD-driven cell proliferation and IFN production

Patients	Tac (%)	[³ H] dThd		IFN	
		— IL-2	+ IL-2	— IL-2	+ IL-2
Dis-TB					
5	10	2,703	18,073	ND	ND
6	0	934	2,100	ND	ND
7	18	277	7,247	10	30
8	15	13,804	66,507	ND	ND
9	19	5,349	14,299	10	30
10	0	1,823	3,218	< 3	10
Loc-TB					
7	40	44,430	73,181	ND	ND
8	35	26,053	51,979	200	200
9	33	51,830	105,852	100	300
10	30	32,547	46,171	30	300
11	38	54,266	59,855	30	300
12	ND	53,162	106,082	1000	3000

The percentage of cell expressing Tac antigen was evaluated by indirect immunofluorescence after 4 days of culture in the presence of PPD. Recombinant IL-2 (20 U/ml Biogen, Geneva) was added at day 4 of culture. PBMC proliferation and IFN- γ production have been evaluated as reported in the legend of Table 1

Expression of Tac antigen and effect of rIL-2 on cell proliferation and IFN- γ production

Although it is well known that IL-2 enhances PBMC proliferation and IFN synthesis in normal individuals (Piccolella et al. 1986), little information is available on the expression of IL-2 receptor and on the capacity of IL-2 to enhance the PBMC response of anergic tuberculous patients (Toossi et al. 1986).

Table 2 shows the expression of the Tac antigen, and the effect of exogenous IL-2 on cell proliferation and IFN synthesis. The percentage of cells expressing Tac antigen was lower in the PPD-stimulated Dis-TB PBMC in comparison with that observed in Loc-TB-stimulated cultures. Interestingly, PBMC from two Dis-TB patients (nos. 6 and 10) failed entirely to express the Tac antigen. The addition of IL-2 augmented the proliferation in PPD-stimulated cultures of Dis-TB patients 5 and 7-9, and of all Loc-TB patients. In contrast, no enhancing effect of IL-2 was observed with cells from Dis-TB patients 6 and 10. In the present model, IFN release was only marginally influenced by exogenous IL-2 and this confirms previous observations performed in PPD-stimulated cultures from healthy individuals (Piccolella et al. 1986), although in other systems IL-2 enhances the induction of IFN- γ (Pearlstein et al. 1983; Reem et al. 1984).

Discussion

This report investigates the mechanism of the immunological anergy observed in patients with Dis-TB. Although this anergy has been originally described as a negative tuberculin skin reaction in previously sensitized individuals, it has been recently demonstrated that these patients also display low PPD-driven T cell proliferation and IL-2 and IFN- γ production in vitro (Onwubalili et al. 1985; Toossi et al. 1986).

Therefore, the LDA was chosen to investigate the precursor frequency of PPD-reactive T cells in tuberculous and healthy individuals. It has been observed that PBMC responding to PPD showed a significant difference in the precursor cell frequencies between responder and nonresponder cultures. The single-hit curves found in Loc-TB and healthy individuals cultures indicated that a single cell type is involved in the proliferative response to PPD, while the multi-hit curves of Dis-TB patients strongly suggest that two populations are limiting in the cultures. Our estimate of the precursor frequencies in PPD-responder cultures are consistent with the studies of van Oers et al. (1978) in cultures of healthy individuals and of Brett et al. (1987) in cultures of BCG-vaccinated individuals.

The shape of the curves may reflect the ratio of T cells capable of proliferating and those regulating this response: for example, V-shaped curves have been demonstrated to result from the presence of suppressor cells in lepromatous leprosy (Kaplan et al. 1985) and tuberculosis (Onwubalili et al. 1985). We have previously reported that, in culture, PPD-activated PBMC release an antigen-nonspecific factor capable of inhibiting the proliferation of T lymphocytes (Lombardi et al. 1986). This factor is produced in large amounts by PBMC from anergic patients and mitomycin -C treatment does not abolish the capacity of T cells to release the inhibitory factor (Lombardi, unpublished results). Thus, the shape of the curves observed at low cell concentration in Dis-TB patients could be due to the fact that the few T cells present are easily suppressed by the inhibitory factor released by PBMC used as antigen-presenting cells, resulting in negative cultures; whereas at higher T cell concentration a balance between responding T cells and inhibitory factor may occur.

The defective expression of the IL-2 receptor (Tac antigen) in PPD-stimulated cultures from TB patients has been previously reported by Toossi et al. (1986) and it was accompanied by the failure of exogenous IL-2 to enhance T cell proliferation. This apparent contrast with our results could be due to the individual patients studied. In fact, cells responding to exogenous IL-2 in our study express higher levels of Tac antigen than that of cells reported in Toossi's experiments. Accordingly, Dis-TB patients 6 and 10, which failed to express Tac antigen responded very poorly to PPD in the presence of exogenous IL-2.

The immunological unresponsiveness in clinical situations has been mainly studied in lepromatous leprosy. These studies have given contrasting results. Some studies had clearly shown that the addition of exogenous IL-2 to T cell cultures is able to reverse leprosy unresponsiveness (Haregewoin et al. 1983; Kaplan et al. 1985; Barnass et al. 1986), while others had reported that T cell proliferation cannot be reconstituted by IL-2 (Mohaghehpour et al. 1985). More recently Brett et al. (1987), studying lepromatous leprosy patients, reported that LDA may reveal

M. leprae-reactive T cells not observed in bulk culture lymphocyte transformation, but the presence of exogenous IL-2 was strictly required.

Our present study documenting the presence of low numbers of PPD-reactive T cell precursors in Dis-TB patients, and the enhancing effect of IL-2 on the immunological hyporesponsiveness in patients expressing detectable levels of Tac antigen, strongly suggests a key role of IL-2 in increasing the anti-mycobacterial immunity. IL-2 may exert a differentiative effect on mycobacterial-specific cytotoxic T cells (Kaufmann et al. 1986) and/or activate the cytotoxicity of macrophages (Malkovsky et al. 1987). In conclusion, the possibility of using IL-2 to potentiate the effector mechanisms of cell-mediated immunity in some patients with advanced disseminated form of tuberculosis cannot be excluded (Iseman et al. 1986).

Acknowledgments. The authors thank D. Gueritore for his continuous encouragement, A. Luzzati for suggestion and advice for LDA, M. Zubiani for the microbiological investigations and A. Dolei, P. Pizzoli, E. Bafurdeci for IFN titration. Biogen Spa, Geneva, is acknowledged for the generous supply of rIL-2. This work was supported by funds from the WHO Vaccine Development Programme (IMM/TUB), and from the Italian Research Council (C.N.R.) grants 87.00628.52 and 87.1317.44.

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