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Analysis of T cell subsets by monoclonal antibodies in patients with tuberculosis after *in vitro* stimulation with purified protein derivative of tuberculin

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SUMMARY

By using OKT monoclonal antibodies; OKT3(pan T), OKT4(inducer/helper), OKT8 (suppressor/cytotoxic) and OKIa1, T lymphocyte subsets were examined in lymphocytes of patients with tuberculosis both before and after *in vitro* stimulation with purified protein derivative of tuberculin (PPD). In freshly obtained lymphocytes samples before culture, a significantly high T4/T8 ratio in pleural fluid lymphocytes (PFL) from patients with tuberculous pleurisy was observed as compared with either their PBL, or the PBL from healthy controls. In addition, PFL from patients with tuberculous pleurisy showed increased numbers of E rosetting (E-RFC), $OKT3^+$ and $OKT4^+$ cells as compared with their PBL. A low T4/T8 ratio was also observed in PBL of patients with advanced, refractory tuberculosis. After stimulation with PPD *in vitro*, the T4/T8 ratio increased further in PFL as well as in PBL from patients with newly diagnosed, fresh tubeculosis. Investigation of fractionated T lymphocyte subsets revealed that PPD-induced proliferating lymphocytes belonged to $T4^+$ and not $T8^+$ lymphocytes. Ia antigen bearing T lymphocytes (Ia-T) were increased in all lymphocyte groups studied after *in vitro* stimulation with PPD. In particular, a remarkable increase was observed when PFL were stimulated *in vitro* with PPD. Our results suggest that the clinical features of tuberculosis reflect the immunological activity of T lymphocyte subsets in this disease.

Keywords T cell subsets tuberculosis monoclonal antibodies

INTRODUCTION

In tuberculosis, the T cell-mediated immune response plays an important role in the pathogenesis of the disease and also in protective immunity against the bacillus. The tuberculin skin test has been well known as the prototype of delayed type hypersensitivity in man. Variation in the host response to tubercle bacillus may result in the various clinical manifestations of tuberculosis, but there has been comparatively little immunological research in patients with the disease. Our laboratory has been involved in the clinical and immunological studies of tuberculosis: We have reported previously that activation of lymphocytes *in vitro* with tuberculin PPD of tuberculous patients provided T lymphocytes with a receptor for the Fc portion of IgG (Tsuyuguchi *et al.*, 1980) and a receptor for autologous human erythrocytes (Tsuyuguchi, Shiratsuchi & Fujiwara, 1982a), respectively, depending on the clinical features of types of tuberculosis studied. Recently, specific monoclonal antibodies (MoAb; OKT and Leu series) reactive with different antigenic determinants

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present on human T lymphocytes (Engleman *et al.*, 1981; Gatenby, Kotzin & Engleman, 1981; Reinherz *et al.*, 1979a) have been developed and used widely for the enumeration of T lymphocytes and T lymphocyte subsets in various diseases (Bach & Bach, 1981; Jackson *et al.*, 1982; Phan-Dinh-Tuy *et al.*, 1981; Raeman *et al.*, 1981) or in relation to ageing (Mascart-Lemone *et al.*, 1982; Nagel, Chrest & Adler, 1981).

In the present study, we have characterized the T lymphocyte subsets using OKT MoAb in patients with tuberculosis both before and after *in vitro* stimulation with purified protein derivative of tuberculin (PPD).

MATERIALS AND METHODS

Patients. Twenty-two patients with active pulmonary tuberculous, and 10 patients (seven males and three females, 19–64 years old, mean 36 years) with tuberculosis pleurisy with effusion were studied. All patients studied were in-patients in our hospital. Patients with pulmonary tuberculosis were divided into two groups; 12 patients (six males and six females, 3–78 years old, mean 39 years) with newly diagnosed tuberculosis, and 10 patients (eight males and two females, 22–75 years old, mean 53 years) with advanced, refractory tuberculosis. Patients with newly diagnosed tuberculosis were those diagnosed as having pulmonary tuberculosis for the first time by chest X-ray and by demonstration of acid fast bacilli in sputum. Patients with advanced, refractory tuberculosis were those who had been hospitalized for several years without improvement. Cultures of sputum specimen from patients with advanced, refractory tuberculosis were continuously positive for acid fast bacilli resistant to anti-tuberculous drugs. Diagnosis of tuberculous pleurisy was confirmed by the histological findings of tuberculosis in the biopsied specimen. Fourteen healthy volunteers (seven males and seven females, 23–72 years old, mean 46 years) who were all positive for the tuberculin skin test, served as control subjects.

Lymphocyte preparation. Peripheral blood lymphocytes (PBL) were isolated from heparinized venous blood by the Ficoll-Hypaque sedimentation method. Pleural fluid lymphocytes (PFL) were obtained from heparinized pleural fluid from patients with tuberculous pleurisy by the same method as for PBL. Blood and pleural fluid samples were collected for study on the same day.

MoAb. OKT3(T3), OKT4(T4), OKT8(T8) and OKIa1 MoAb were purchased from Ortho Pharmaceutical, New Jersey, USA. OKT3 has been shown to react with the entire human peripheral T lymphocytes (Kung *et al.*, 1979). OKT4 has been shown to define the inducer/helper T lymphocytes (Morimoto *et al.*, 1982; Reinherz *et al.*, 1979b, 1980a), whereas OKT8 defines the reciprocal suppressor/cytotoxic population (Morimoto *et al.*, 1982; Reinherz *et al.*, 1980b). OKIa1 was shown to react with the human Ia like antigen (HLA-DR) framework (Reinherz *et al.*, 1979c).

Isolation of T lymphocyte subsets. To fractionate T lymphocytes into T4 and T8 depleted subpopulations, negative cell selection with MoAb and complement was used. First, PBL were divided into T and non-T lymphocyte fractions by the method of rosette formation with sheep red blood cells (SRBC) with subsequent separation by Ficoll-Hypaque centrifugation. T lymphocyte rich fraction that rosetted with SRBC was obtained after lysis of erythrocytes in 0·8% ammonium chloride (NH_4Cl). Purified T lymphocytes were suspended at a concentration of 20×10^6 cells/ml in RPMI 1640 medium containing 5% heat-inactivated fetal calf serum (FCS; M.A. Bioproducts, Maryland, USA), together with anti-T4 or anti-T8 at a final dilution of 1:100. After 60 min incubation at 30°C, fresh rabbit serum was added at a final dilution of 1:5 and incubated another 60 min at 37°C in a shaking water bath. After incubation at 37°C, cells were washed three times with RPMI 1640 medium and resuspended in fresh medium. T4 or T8 depleted T cell fraction thus prepared contained less than 5% of $T8^+$ or $T4^+$ lymphocytes, respectively.

Lymphocyte culture and detection of T lymphocyte subsets. Lymphocytes were cultured at a density of 2×10^6 cells per ml in culture tube (Falcon, No. 2054) with 10 μg/ml of PPD for various periods at 37°C in 5% CO_2 in air. Purified protein derivative of tuberculin(PPD) was kindly donated by Dr Fujii, Institute for Microbial Diseases, Osaka University, Japan. The culture medium used was RPMI 1640 supplemented with 10% FCS, 100 u of penicillin per ml and 100 μg of streptomycin per ml. At the end of the culture period, the cells were washed three times with medium, resuspended

in RPMI 1640 containing 10% FCS, and subjected to assay for T lymphocyte subsets. One hundred microlitres of cell suspension containing $2–5 \times 10^5$ cells was mixed with 2·5 μl of MoAb and incubated for 30 min in an ice bath with agitation every 10 min. The cells were washed after incubation and then 100 μl of FITC conjugated rabbit anti-mouse IgG(Miles-Yeda Ltd., Rehovot, Israel) were added at a final dilution of 1 : 16. After a further 30 min incubation on ice, the cells were washed and resuspended in one drop of FCS, and the number of positive cells was assessed using a fluorescence microscope. Ia antigen positive T lymphocytes (Ia-T) were determined by counting OKIa1 positive lymphocytes which rosetted with SRBC.

In vitro *assay of proliferative response. In vitro* proliferative response was measured as described previously (Tsuyuguchi *et al.*, 1982b). In brief, 2×10^5 lymphocytes in 0·2 ml of RPMI 1640 medium supplemented with 10% heat-inactivated pooled human sera were placed in flat bottomed microtitre plate (Falcon No. 3072) with 10 μg/ml of PPD. For the functional study with fractionated T lymphocytes, $T4^+$ or $T8^+$ lymphocytes enriched T cell fraction obtained as described above, was cultured *in vitro* with PPD for the indicated culture period. The E rosette depleted, 2,600 rad irradiated non-T fraction was cultured together at a cell concentration of one-tenth of the T cells as the source of accessory cells. The plate was incubated for 6 days in humidified 5% CO_2 in air at 37°C. At the end of the culture period, ^{3}H-thymidine (^{3}H-TdR) was added to each well (0·2 μCi/well). After 18 h, the cells were harvested on glass fibre filters using a semiautomatic cell harvester (Laboscience Co. Ltd., Tokyo, Japan) and radioactivity was counted in a Packard Tricarb Scintillation counter. Each determination was performed in triplicate and the data were expressed as mean counts per minute (ct/min).

Statistical analysis. The significance of the difference between groups was calculated by Student's *t*-test.

RESULTS

T lymphocyte profile in patients with tuberculosis

The distribution of T lymphocyte subsets in freshly obtained lymphocytes before culture is shown in Table 1. With regard to T4 and T8 subsets, significant differences were observed in patients with tuberculous pleurisy. PFL were mostly E rosetting, OKT3 positive T cells, and these cells were mostly T4 positive. The T4/T8 ratio in PFL was, therefore, significantly higher in the pleural fluid ($P < 0·01$) when compared to PBL of controls, in contrast to the low T4/T8 ratio in their PBL. The T4/T8 ratio in PBL of patients with advanced, refractory tuberculosis was also significantly lower

Table 1. T lymphocyte profile in patients with tuberculosis

Subjects studied	E-RFC (%)	T lymphocyte subsets				
		OKT3 (%)	OKT4 (%)	OKT8 (%)	T4/T8 ratio	Ia-T (%)
Pulmonary tuberculosis, PBL						
Newly diagnosed ($n=12$)	70·2±9·1	63·1±7·8	44·7±6·8	23·5±5·7	2·0±0·6	4·2±2·2
Advanced ($n=9$)	74·1±10·0	59·4±10·4	37·1±8·5	29·5±4·9	1·4±0·3*†	5·4±5·5
Tuberculous pleurisy						
PFL ($n=10$)	89·0±3·9‡	86·4±4·5‡	64·2±6·7‡	20·3±5·7	3·4±1·1†‡	11·7±11·1
PBL ($n=9$)	68·0±8·4	56·1±8·3	37·8±9·0	24·1±5·1	1·7±0·6	4·3±2·0
Healthy controls, PBL ($n=14$)	74·3±9·1	64·2±9·0	46·7±6·4	23·2±5·2	2·1±0·6	3·2±2·3

* $P < 0·02$ compared to newly diagnosed tuberculosis patients.
† $P < 0·01$ compared to healthy controls.
‡ $P < 0·001$ compared to their PBL.

($P < 0{\cdot}01$) than the PBL of controls, because of the increased percentage of $T8^+$ lymphocytes. With regard to Ia-T(Ia antigen bearing, E rosetting) cells, no significant differences were seen between the lymphocyte groups examined.

The kinetics of distributions of T lymphocyte subsets after PPD stimulation

PFL from tuberculous pleurisy patients had high responsiveness to *in vitro* PPD stimulation (Fujiwara *et al.*, 1982). The kinetic curves of the changes of T cell subsets in PFL after *in vitro* PPD stimulation were shown in Fig. 1. The number of $T4^+$ lymphocytes as well as Ia-T is significantly increased after PPD stimulation. The peak response occurred after 6 days of culture. On the contrary, the number of $T8^+$ lymphocytes was decreased after PPD stimulation.

The distribution of $T4^+$ and $T8^+$ lymphocytes after in vitro *PPD stimulation*

Lymphocytes were cultured *in vitro* in the presence or absence of PPD for 6 days and T cell subsets were examined. The optimum culture period of 6 days was determined from the kinetic curves as described above. The results were expressed as the ratio of T4 to T8 positive cells (T4/T8) in lymphocytes culture in the presence or absence of PPD. As shown in Fig. 2, the T4/T8 ratio was significantly higher in PBL from patients with newly diagnosed tuberculosis ($P < 0{\cdot}02$) and in PFL ($P < 0{\cdot}01$) after PPD stimulation when compared with control cultures without PPD. On the contrary, the T4/T8 ratio in PBL from patients with tuberculous pleurisy or advanced pulmonary tuberculosis was decreased because of increased percentage of $T8^+$ lymphocytes in these PBL after PPD stimulation.

Increase of Ia-T lymphocytes after PPD stimulation in vitro

Activated T lymphocytes have been reported to express Ia like antigen on their surface (Ko *et al.*, 1979). By using monoclonal OKIa1 antibody and E rosetting method, we detected Ia-T after *in vitro* stimulation with PPD for 6 days and the result is shown in Fig. 3. A significant increase of percentage of Ia-T was observed in all lymphocyte groups examined except for PBL from patients with advanced, refractory tuberculosis. The percentages of Ia-T in total T cells in cultures in the absence and presence of PPD were; $9{\cdot}1 \pm 4{\cdot}9$ and $16{\cdot}0 \pm 12{\cdot}4$($0{\cdot}10 < P < 0{\cdot}20$) in PBL from advanced tuberculosis patients, $7{\cdot}7 \pm 3{\cdot}2$ and $18{\cdot}4 \pm 6{\cdot}5$($P < 0{\cdot}001$) in PBL from newly diagnosed tuberculosis

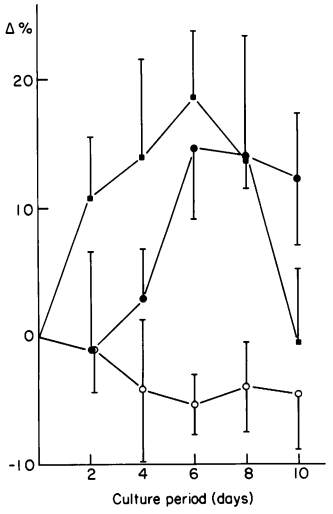

Fig. 1. Kinetics of PPD-induced appearance of T cell subsets (○ = OKT8; ● = OKT4; ■ = Ia-T) in PFL. PFL (2×10^6/ml) were cultured in the presence or absence of PPD (10 μg/ml). Results were expressed as Δ% of positive cells: % of positive cells in culture in the presence of PPD subtracted by % of positive cells in culture in the absence of PPD. Each determination was the mean of five subjects ± s.d.

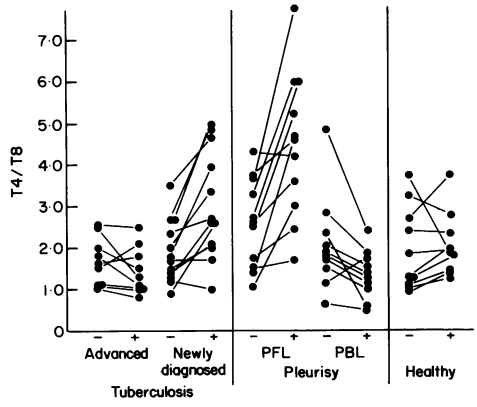

Fig. 2. The changes of $T4^+/T8^+$ ratio by PPD stimulation. Lymphocytes were cultured *in vitro* for 6 days in the presence (+) or absence (−) of PPD. $T4^+$ and $T8^+$ cells were determined by the immunofluorescence technique. Each point connected by a line indicates the data from the same donors.

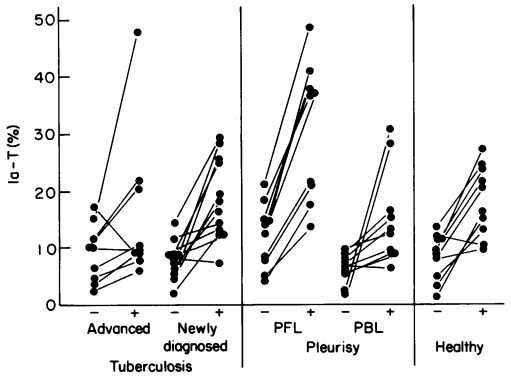

Fig. 3. The increase of Ia-T by PPD stimulation. Lymphocytes were cultured *in vitro* for 6 days in the presence (+) or absence (−) of PPD. Ia-T was defined as OKIa1 positive, E rosetting cells. Data were expressed as percentage positive cells in total T cells.

patients, $14{\cdot}1 \pm 8{\cdot}0$ and $34{\cdot}9 \pm 16{\cdot}0 (P < 0{\cdot}01)$ in PFL, $6{\cdot}2 \pm 2{\cdot}3$ and $15{\cdot}0 \pm 7{\cdot}8 (P < 0{\cdot}01)$ in PBL from pleurisy, and $8{\cdot}5 \pm 3{\cdot}9$ and $18{\cdot}4 \pm 5{\cdot}6 (P < 0{\cdot}001)$ in PBL from healthy controls. A remarkable increase of Ia-T was observed when PFL were stimulated *in vitro* with PPD.

Functional study with isolated $T4^+$ and $T8^+$ lymphocytes

In order to study whether $T4^+$ and/or $T8^+$ lymphocytes were responsible in *in vitro* PPD-induced proliferation, fractionated T lymphocytes prepared from tuberculin positive PBL were employed for experiments. For the isolation of T lymphocyte subpopulations, negative selection was performed using T4 or T8 MoAb and rabbit complement. By the method described in the Materials and Methods, T4 or T8 depleted T lymphocyte fractions were obtained from a tuberculin skin test positive healthy donor and cultured in the presence or absence of PPD for 6 days. The E rosette depleted, irradiated non-T fraction was added to each fraction as the source of accessory cells. The proliferative response with these T lymphocyte fractions were assessed and the data were expressed as ^{3}H-TdR incorporation (ct/min). The percentage of Ia-T in culture was also examined after PPD stimulation in these T4 and T8 depleted T lymphocyte fractions. As shown in Table 2, the T lymphocyte fraction depleted of T4 gave neither proliferative response nor increase in percentage of Ia-T after PPD stimulation. On the contrary, the T lymphocyte fraction depleted of T8 as well as unfractionated T lymphocytes gave a highly proliferative response by the stimulation with PPD.

Table 2. Functional study with isolated lymphocyte subsets

	PPD (10 μg/ml)	Expt. 1 ^{3}H-TdR (ct/min)	Expt. 1 Ia-T (%)	Expt. 2 ^{3}H-TdR (ct/min)	Expt. 2 Ia-T (%)
PBL	−	315	6·7	1,419	8·4
	+	11,327	17·9	5,595	24·7
Unfractionated T+non-T	−	1,248	4·4	756	11·4
	+	32,996	7·8	6,518	19·3
T8 depleted T+non-T	−	1,620	4·3	1,572	8·8
	+	16,809	20·0	5,974	15·3
T4 depleted T+non-T	−	104	6·5	232	ND*
	+	295	4·8	397	ND*

Fractionated or unfractionated T lymphocytes (2×10^6) were cultured *in vitro* for 6 days with or without PPD in the presence of 2,600 rad irradiated non-T lymphocytes. Proliferation was assessed by counting the radioactivity of ^{3}H-TdR incorporated during the last 18 h of culture.

* Not determined.

The percentage of Ia-T in these fractions was also increased after PPD stimulation. These results indicate that the PPD reactive and proliferating lymphocytes were $T4^+$ and Ia antigen positive T cells.

DISCUSSION

Patients with tuberculosis show a wide spectrum of immunological as well as clinical characteristics, ranging from an immunologically hyper-reactive state to an almost totally unresponsive state. It is not uncommon for patients with advanced, refractory tuberculosis to show depressed skin reactions to tuberculin PPD injection (tuberculin anergy), although they are continuously in contact with tubercule bacilli. We have previously reported that when PBL from patients with advanced, refractory tuberculosis were stimulated *in vitro* with PPD, the number of IgG Fc receptor bearing T cells (FcR+ T) increased and that these FcR+ T cells suppressed the PPD-induced proliferative response of lymphocytes (Tsuyuguchi *et al.*, 1980). This is consistent with the results of our present study which showed that the number of $T8^+$ lymphocytes (suppressor T) increased when PBL from patients with advanced, refractory tuberculosis were stimulated *in vitro* with PPD. The question as to whether FcR is expressed on the surface of $T8^+$ lymphocytes or not, is now under investigation.

Tuberculous pleurisy presents an interesting phenomenon from an immunological viewpoint. As reported previously, the PFL from pleurisy patients were highly responsive to *in vitro* PPD stimulation, whereas their PBL showed poor responses (Ellner, 1978a, 1978b; Fujiwara *et al.*, 1982). In the present study, as shown in Table 2, PPD-induced proliferating lymphocytes belonged to $T4^+$ lymphocytes. Evidence that the number of $T4^+$ and not $T8^+$ lymphocytes were increased (increased T4/T8 ratio) in PFL after *in vitro* stimulation with PPD could explain the high proliferative responses observed with PFL to PPD stimulation. We have reported previously that the poor response of PBL from pleurisy patients was reversed by the elimination of adherent cells from PBL which suggests the existence of suppressor macrophages (Fujiwara *et al.*, 1982). The existence of suppressor macrophages in PBL from tuberculous patients with low tuberculin response was shown by Ellner (1978a, 1978b). OKT8 MoAb detects suppressor T lymphocytes (Morimoto *et al.*, 1982, Reinherz *et al.*, 1980b). Suppressor macrophages reported by us and by Ellner might be generated by the action of $T8^+$ lymphocytes which increased in PBL from tuberculous pleurisy patients after PPD stimulation, or vice versa. $T8^+$ lymphocytes which were generated in PBL by PPD stimulation

play a suppressive role and may provide some tuberculous patients with unresponsiveness to tuberculin PPD. In this context, it is intriguing that patients with tuberculous pleurisy or advanced, refractory tuberculosis not uncommonly reveal decreased or even negative skin reactions to tuberculin PPD.

Ia like antigen is shown to be expressed on the activated T lymphocytes (Ko *et al.*, 1979) as well as on the surface of B lymphocytes and monocytes. Ia antigens, in man, are expressed by both $T4^+$ and $T4^-$ subsets in the mixed lymphocyte culture (Reinherz *et al.*, 1979c). In contrast, only the inducer ($T4^+$) T lymphocyte population which proliferates maximally to soluble antigen expresses Ia antigens after activation by tetanus toxoid (Reinherz *et al.*, 1981). This is consistent with our present observations of PFL that remarkable increases in Ia antigen bearing and $T4^+$ T lymphocytes occurred and high proliferative responses were generated when PFL were cultured *in vitro* in the presence of PPD.

As shown in the present study, tuberculosis exhibits various immunological as well as clinical features. The analysis of T lymphocyte subsets in tuberculosis, therefore, provides a new insight into the understanding of immunological mechanisms operating in the pathogenesis of tuberculosis.

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