T-cell Subpopulations in Tuberculosis and the Effects of Rifampicin*

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SUMMARY T-cell subpopulations were evaluated by several recent methods in 38 tuberculosis patients (24 active and 10 quiescent cases of pulmonary tuberculosis; two of miliary and two of active extrapulmonary tuberculosis) before and during rifampicin (RMP) treatment.

There was a significant reduction in the total number of T cells (E-RFC and OKT3+ cells) and of helper T cells (OKT4+) coinciding with an increase in the number of suppressor T cells when the 38 tuberculosis patients were compared with 21 healthy control subjects. When the changes of T-cell subpopulations in groups of subjects and patients with different clinical forms of the disease were analysed, these changes could be clearly shown with both sets of assays (receptor assays and monoclonal antibody assays) among those with the active pulmonary form of tuberculosis while similar changes could be demonstrated only by one or the other assay among those with the other forms of the disease.

The effects of one month of RMP treatment on these parameters were much more obvious among the clinically active patients than the quiescent patients, i.e. a recovery of total T cells from a low pre-treatment to a near normal level accompanied by a significant reduction in the number of suppressor cells (OKT8+). In fact, among quiescent patients the number of suppressor cells (as TG) appeared to rise further with RMP treatment.

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In animal models, cellular immunity has been demonstrated to play an important and dominant role in the killing of tubercle bacilli. However, during mycobacterial infection in humans, different levels of cellular immunity and humoral immunity have been demonstrated to correlate with clinical forms.[1-3] In addition, the levels of cellular immunity, tuberculin hypersensitivity,[1-6] the purified protein derivatives (PPD)-induced proliferative response of peripheral blood lymphocytes[6,7] and the number of T cells[1,4,7,8] have been observed regularly to decrease. Also, T-cell depletion is accompanied by an increase in T cells with receptors for Fc of IgG (TG).[8]

On the other hand, rifampicin (RMP), a semi-synthetic antibiotic derived from *Streptomyces mediterranei* has been reported to suppress skin reactivity to tuberculin, circulating T cells and *in vitro* lymphocyte blastogenic response to phytohaemagglutinin (PHA) or PPD.[5,9-11] However, those parameters were reported by other investigators to be unaffected during RMP treatment.[7,12,13]

In the present studies, various means were used to detect T cells and T-cell subpopulations among tuberculosis patients with different clinical forms of the disease and at five forthnightly intervals, i.e. pretreatment, during treatment, at termination of one month of RMP treatment and two samples subsequently. Used for thier purpose were spontaneous sheep erythrocytes (SRBC) rosetting by T cells (E-FRC) and the receptor for Fc of IgG or TG, which acted as a suppressor of polyclonal B-cell differentiation induced by pokeweed mitogen (PWM)[14,15] and monoclonal antibodies (MoAb) in OKT series: OKT3 (T cell), OKT4 (helper T cell) and OKT8 (suppressor T cell).[16-20] The correlation coefficient among the different parameters used for detecting the T cells and T-cell subpopulations were also evaluated.

MATERIALS AND METHODS

Human subjects

Included among the 38 patients studied were 24 with active pulmonary tuberculosis and 10 with quies-

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cent pulmonary tuberculosis, two with miliary tuberculosis and two with active extra-pulmonary tuberculosis. These were classified according to their bacteriological, histological and radiographical expressions as well as clinical manifestations. Most of the cases with active disease had acid-fast bacilli in their sputa or clinical specimens; these were demonstrated by direct smear and/or culture. All 24 patients (13 males and 11 females ranging in age from 22 to 67 years) had clinical and radiographic signs of tuberculosis activity. The 10 quiescent pulmonary patients (8 males and 2 females ranging in age from 20 to 75 years) had been treated with non-rifampicin conventional chemotherapy for over six months and their sputa had been cleared of acid-fast bacilli. They were clinically stable and asymptomatic; also their lesions were radiographically stable. The four remaining patients included a 21-year-old woman with cervical lymphadenitis, a 67-year-old woman with peritonitis and two women (one 25 years old; the other 30 years old) with miliary tuberculosis.

Twenty of the 38 patients also participated in a study on the effects of RMP treatment. Six males and five females (ranging in age from 24 to 67 years) had active tuberculosis and seven males and two females (ranging in age from 21 to 75 years) had quiescent tuberculosis.

The 21 healthy controls (17 males and 4 females ranging in age from 19 to 48 years) were regular blood bank donors.

Collection of blood specimens

About 10-15 ml of venous blood from the healthy donors and the patients were collected into heparin-coated syringes upon their admission to the studies. For those treated with RMP, four additional blood samples were obtained at fortnightly intervals following initiation of RMP.

Chemotherapeutic regimens

All patients were given isoniazid (300 mg daily); ethambutol (800 mg daily) with or without streptomycin (750-1,000 mg daily). For the RMP-treated patients, doses of isoniazid (300 mg daily) and RMP (600 mg daily) were given during the 30-day study period; thereafter, conventional regimens were resumed to finish the course.

Mononuclear cell separation

Mononuclear cells (MNC) were separated from the heparinised peripheral blood by a method slightly modified from that described by Boyum.[21] In brief, 10 parts of a 1:2 dilution of heparinised blood in phosphate buffer saline (PBS), were gently layered onto four parts of a Ficoll-Hypaque density solution (1.077 g/ml), then centrifuged for 30 minutes at 300 × g at 25°C. Mononuclear cells at the plasma:Ficoll-Hypaque interface were collected and washed twice with rRPMI (RPMI 1640 with 10 mM HEPES, pH 7.4). The MNCs were then counted and divided into two parts, one for rosetting studies, the other for indirect immunofluorescent studies. The MNCs were centrifuged and resuspended in rRPMI and iRPMI (rRPMI supplemented with 5% inactivated calf serum, pH 7.2) respectively to give a final cell concentration of 5×10^6 /ml.

Detection of T cells and T-cell subpopulations

Two techniques, rosetting and indirect immunofluorescence, were employed.

A. Rosetting technique. Lymphocytes with receptors for spontaneous binding to SRBCs (E-RFCs) and E-RFCs with receptors for Fc of IgG were estimated by rosetting with neuraminidase-treated SRBCs and IgG-coated ox erythrocytes (ORBCs) respectively. Neuraminidase-treated SRBC rosetting was performed as previously described by Weiner *et al.*[22] In brief, the mixture of 0.1 ml of 1 U/ml neuraminidase (neuraminidase type IV from *Clostridium perfringens*, Sigma Chemical, St. Louis, MO, U.S.A.) and 0.5 ml of 5% SRBCs in HBSS, pH 6.5, was incubated at 37°C for one hour, washed twice and resuspended in rRPMI (1% SRBCs). An equal volume of MNCs (5×10^6 cells/ml) was added and incubated for 10 minutes in a 4°C ice-water bath; the cells were then pelleted by centrifugation at 200 × g for five minutes. Following gentle resuspension of the pellet, the suspension was placed on a haemocytometer and a total of 300 cells were counted for rosette formation. The cells with three or more SRBCs were counted as E-RFCs. These cells were also diluted with 3% acetic acid to determine the proportion of lymphocytes among the MNCs. E-RFCs were then recorded as a percentage of total lymphocytes. Then these E-RFCs were separated from non-rosetting cells by two cycles of Ficoll-Hypaque sedimentation. The pellet was harvested and resuspended in one volume of a hypotonic sodium chloride solution (1.497 g/litre) for 30 seconds in order to lyse the SRBCs before two volumes of hypertonic sodium chloride solution (12.75 g/litre) were added to achieve isotonicity. After washing twice with rRPMI, the 5×10^6 cells/ml suspension was adjusted for further use in the detection of TG. The resulting suspension contained more than 98% E-RFCs as determined by re-rosetting before the lysis procedure. For TG, enumeration rosette formation between the purified E-RFCs and IgG-coated ORBCs was performed as described by Moretta *et al.*[14,23] Briefly, IgG-coated ORBCs were prepared by mixing equal volumes of 2% ORBCs and a subagglutinating dilution of purified rabbit IgG anti-ORBC. After 90 minutes, incubation at room temperature, an equal volume of purified E-RFCs (5×10^6 cells/ml) was added and the mixture pelleted by centrifugation at 200 × g for five minutes. Following 15 minutes' incubation in a 4°C ice-water bath, the pellet was gently resuspended and counted for TG rosettes in the same manner as above for counting E-RFCs. The number

was recorded as a percentage of total E-RFCs.

B. Indirect Immunofluorescent technique. T cells, helper T cells and suppressor T cells were also detected by using MoAb of the OKT series (OKT3, OKT4 and OKT8 from Orthro Pharmaceuticals, Raritan, NJ, U.S.A.). As mentioned in Orthro Pharmaceuticals' leaflets describing Orthroclone MoAb, 5 μl each of the reconstituted MoAb solutions were added to 200 μl of 5×10^6 cells/ml of MNC suspension. After 30 minutes' incubation in a 4°C ice-water bath with agitation every 10 minutes, the cells were washed twice with iRPMI. After centrifugation, the pellet was resuspended with about 100 μl iRPMI. Then 100 μl of FITC-conjugated goat anti-mouse Ig (Cappel Laboratories, Gochranville, U.S.A.), previously diluted to an appropriate concentration by titration using OKT3, were added to the cell suspension and further incubated for 30 minutes at 4°C with agitation every 10 minutes. The cells were washed another two times with 0.01 M of PBS, pH 7.2. The pellet was resuspended with about two drops of 30% (v/v) glycerine in PBS; a slide prepared from it was examined immediately under a fluorescent microscope. At least 300 cells were counted as positive. They appeared as cells with coarse granular fluorescence. The cell suspension was also diluted with 3% acetic acid and couted to determine the purity of the lymphocytes. Then the positive cells were recorded as a percentage of total lymphocytes for OKT3 and as a percentage of total OKT3 for OKT4 and OKT8.

Statistical analysis

The student's t-test for independent samples was used in assessing whether there was a significant difference between the means of the groups compared. With regard to the RMP-treated patients' data, the student's t-test for related samples was used in analysing the difference of the means among the five time intervals.

RESULTS

As compared with the 21 control subjects, the 38 tuberculosis patients displayed a reduction in the number of total T cells (both E-RFC and OKT3+ cells) and of helper T cells (OKT4+). These reductions were accompanied by an increase in the number of suppressor T cells (both TG and OKT8+ cells) (data not shown). When the changes of these parameters in the groups of patients with different clinical forms of the disease were analysed, a reduction in the number of E-RFCs in the patients with active pulmonary ($p < .001$) and quiescent pulmonary ($p < .001$) tuberculosis was shown (Fig. 1) while the number of OKT3+ cells was reduced in the cases with active pulmonary tuberculosis ($p < .05$) and in the group with the maliary and extra-pulmonary ($p < .01$) forms (Fig. 2). In addition, a significant reduction in the number of OKT3+ cells was also detected in the miliary and extrapulmonary form ($p < .05$) when these were compared with the quiescent form. The reduction of T-cell number was accompanied by decreases in helper T cells (OKT4+) among the patients with active pulmonary ($p < .001$) and quiescent pulmonary ($p < .01$) forms (Fig. 3). Moreover, significant increases in the number of TG cells were detected in the cases with quiescent pulmonary ($p < .001$), active pulmonary ($p < .001$) and miliary and extra-pulmonary ($p < .001$) tuberculosis when compared with normal subjects, and in the cases with active pulmonary ($p < .05$) and with miliary and extra-pulmonary ($p < .05$) tuberculosis when compared with the quiescent form (Fig. 4). In comparison with TG, OKT8+ cells were shown to increase only in patients with active pulmonary tuberculosis ($p < .05$) when compared with normal subjects (Fig. 5). No significant differences were detected among the other groups. (In Figures 1 to 5, the bars denote mean and standard deviations).

The effects of RMP treatment on

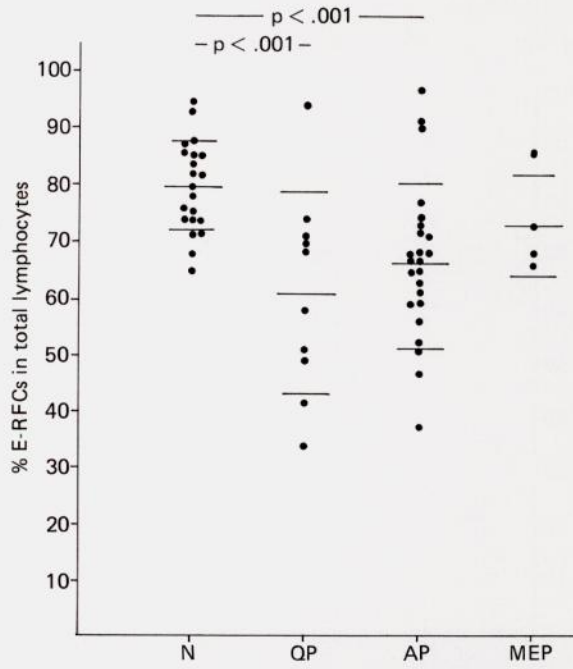

Fig. 1 The percentages of E-RFCs in total lymphocytes in normal subjects (N), quiescent pulmonary (QP), active pulmonary (AP) and miliary and extra-pulmonary (MEP) tuberculosis patients. The statistical significance of the difference between each group to each of the rest was analysed, but p values are shown only for those comparisons with a significant difference.

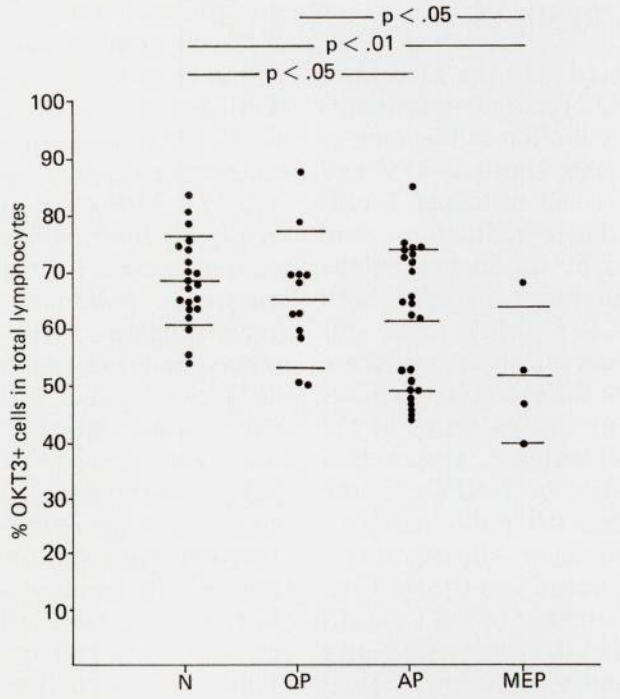

Fig. 2 The percentages of OKT3+ cells in total lymphocytes in normal subjects (N), quiescent pulmonary (QP), active pulmonary (AP) and miliary and extra-pulmonary (MEP) tuberculosis patients. The statistical significance of the difference between each group to each of the rest was analysed, but p values are shown only for those comparisons with a significant difference.

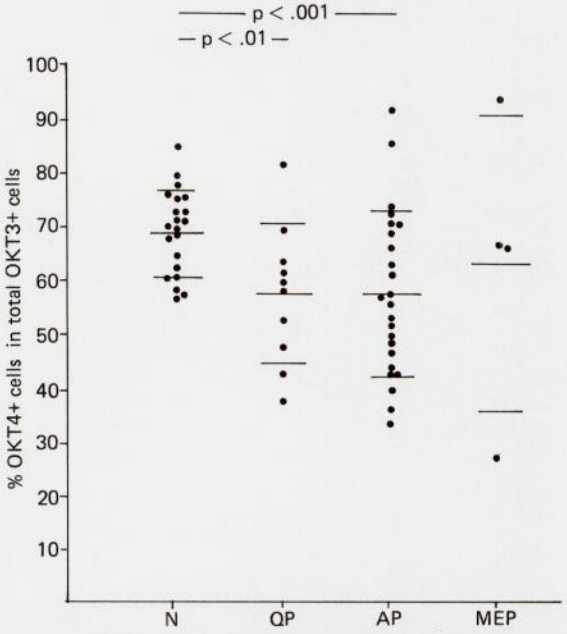

Fig. 3 The percentages of OKT4+ cells in total OKT3+ cells in normal subjects (N), quiescent pulmonary (QP), active pulmonary (AP) and miliary and extra-pulmonary (MEP) tuberculosis patients. The Statistical significance of the difference between each group to each of the rest was analysed, but p values are shown only for those comparisons with a significant difference.

those parmameters of T cells and T-cell subpopulations were assessed in nine quiescent and 11 active tuberculosis patients using blood samples that were collected at five fortnightly intervals: once before RMP administration (week 0), twice during the month of RMP administration (weeks 2 and 4) and another two times after withdrawal of RMP (weeks 6 and 8). As summarised in Table 1, in the group with active tuberculosis, there was a decrease in the number of E-RFCs at the sixth week ($p<.001$) and at the eighth week ($p<.05$) with an increase in the number of OKT3+ cells at the fourth week ($p<.05$) accompanied by decreases in the number of OK4+ cells at the second ($p<.05$), fourth ($p<.05$) and sixth ($p<.05$) weeks with a decrease in the number of OKT8+ cells at the fourth week when compared with the values of week 0. With regard to the patients with quiescent tuberculosis, there were decreases in the number of OKT3+ cells at the second ($p < .05$). and the sixth ($p < .05$) weeks with increases in TG at the second ($p < .05$) and the fourth ($p < .05$) weeks.

The correlation between E-RFC/lymphocytes and OKT3+/lymphocytes and between TG/E-RFCs and OKT8+/OKT3+ cells were evaluated. As shown in Figures 6 and 7, their correlation coefficient values (r) were 0.367 and −0.017 respectively.

DISCUSSION

The results of the present studies show a singificant reduction in the number of total T cells among all three groups of tuberculosis patients (Figs. 1 and 2), which is consistent with earlier reports that the cell-mediated immune response is depressed during mycobacterial infection in man and animals.[7,8,24-29] Among the three clinical categories, the immune response of the active pulmonary group of tuberculosis patients appeared to be more consistently depressed as reflected by the finding that a significant difference could be shown by each of

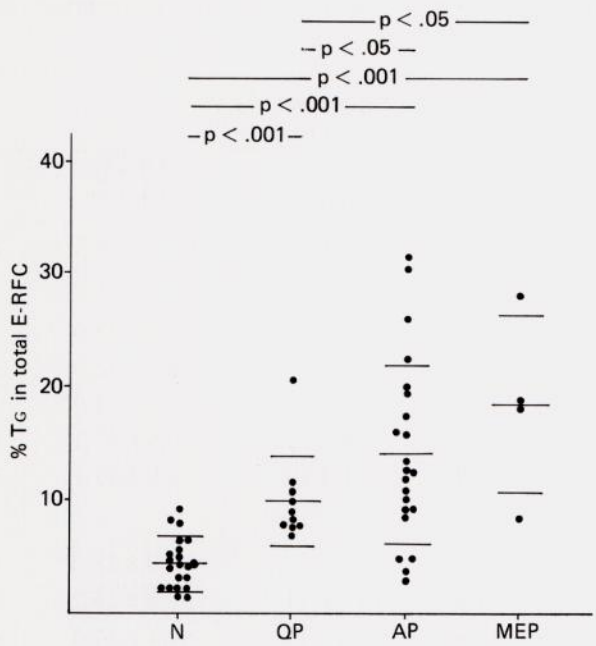

Fig. 4 The percentages of TG lymphocytes in total E-RFC in normal subjects (N), quiescent pulmonary (QP), active pulmonary (AP) and miliary and extra-pulmonary (MEP) tuberculosis patients. The statistical significance of the difference between each group to each of the rest was analysed, but p values are shown only for those comparisons with a significant difference.

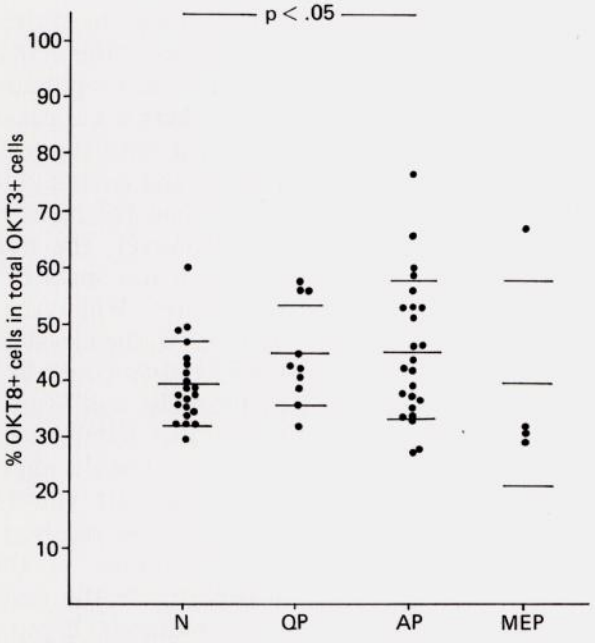

Fig. 5 The percentages of OKT8+ cells in total OKT3+ cells in normal subjects (N), quiescent pulmonary (QP), active pulmonary (AP) and miliary and extra-pulmonary (MEP) tuberculosis patients. The statistical significance of the difference between each group to each of the rest was analysed, but p values are shown only for those comparisons with a significant difference.

the two methods employed, while significant differences for the other groups could be shown only by one or the other methods. As far as the degree of depression was concerned, the group with miliary and extra-pulmonary tuberculosis appeared to be the most depressed; the patients in that group could be shown to be significantly more depressed than those in the quiescent pulmonary group when the OKT3+ cells were compared. However, owing to a relatively smaller number of cases in this group, the results were not conclusive, being at best suggestive. Although the results obtained with the two methods for detecting T cells were not always consistent with one another with regard to the statistically significant level of changes among the groups, the direction of the changes were in general quite consistent except for the case of miliary and extra-pulmonary tuberculosis when compared to either the active or quiescent pulmonary patients. This relatively good agreement of results from the two methods is substantiated by a rather good correlation coefficient. Others who have compared the two methods found essentially the same degree of correlation.[30,31] Moreover, new MoAb OKT11 and 9.6 have been found to correlate even better with E-RFCs; they have also been shown to bind to the site of or near the receptors for SRBCs on the surface of lymphocytes.[32-36] This finding of a depressed number of T cells is consistent with an earlier report by Katz *et al,*[8] who employed the SRBC rosetting technique alone with a resulting reduction in the number of T cells among patients with active pulmonary tuberculosis. The present studies further added to this observation, particularly with regard to the quiescent pulmonary, miliary and extra-pulmonary groups. Furthermore, the present studies also supported the notion of depressed CMI by showing an elevation of TG in all groups of tuberculosis patients, while Katz[8] demonstrated this only

Table 1 Effect of RMP treatment of E-RFCs, TG lymphocytes, OKT3+, OKT4+ and OKT8+ cells in patients with active and quiescent tuberculosis

Time of RMP treatment		No. of cases	Percentage of ($\bar{x} \pm SD$)				
			E-RFC / lymphocytes	TG / E-RFC	OKT3+ / lymphocytes	OKT4+ / OKT3+	OKT8+ / OKT3+
Normal		21	79.8 ± 7.8	4.4 ± 2.3	68.7 ± 7.7	68.9 ± 8.2	39.6 ± 7.3
Active tuberculosis							
Week 0	RMP	11	68.8 ± 13.1	12.9 ± 4.4	59.1 ± 12.8	65.9 ± 19.6	45.1 ± 14.4
Week 2		11	60.0 ± 20.7	12.9 ± 6.0	59.6 ± 13.2	56.1 ± 13.5*	46.3 ± 16.5
Week 4		10	73.8 ± 15.7	15.8 ± 8.9	70.7 ± 12.0*	52.7 ± 11.4*	37.5 ± 12.3†
Week 6		8	61.3 ± 13.2†	14.9 ± 8.3	54.4 ± 15.0	60.3 ± 17.1*	40.6 ± 15.9
Week 8		7	63.3 ± 9.8*	15.5 ± 11.0	49.1 ± 12.7	63.8 ± 17.1	44.4 ± 16.0
Quiescent tuberculosis							
Week 0	RMP	9	61.9 ± 20.4	11.3 ± 4.9	63.5 ± 9.7	60.0 ± 18.0	45.0 ± 9.4
Week 2		9	61.2 ± 20.9	15.5 ± 8.0*	58.9 ± 11.9*	53.4 ± 19.2	52.0 ± 15.2
Week 4		8	69.8 ± 11.1	16.6 ± 8.6*	57.8 ± 13.5	57.4 ± 17.4	48.5 ± 14.6
Week 6		8 (7)	68.5 ± 12.5	15.3 ± 5.9	56.1 ± 13.9*	64.4 ± 18.3	48.2 ± 10.8
Week 8		6 (5)	74.8 ± 9.6	14.3 ± 5.3	59.4 ± 16.0	55.3 ± 10.7	47.6 ± 15.3

( ) = number of cases for OKT8+ cells
* = significant difference ($p < 0.05$) from pretreatment (Week 0)
† = significant difference ($p < 0.001$) from pretreatment (Week 0)

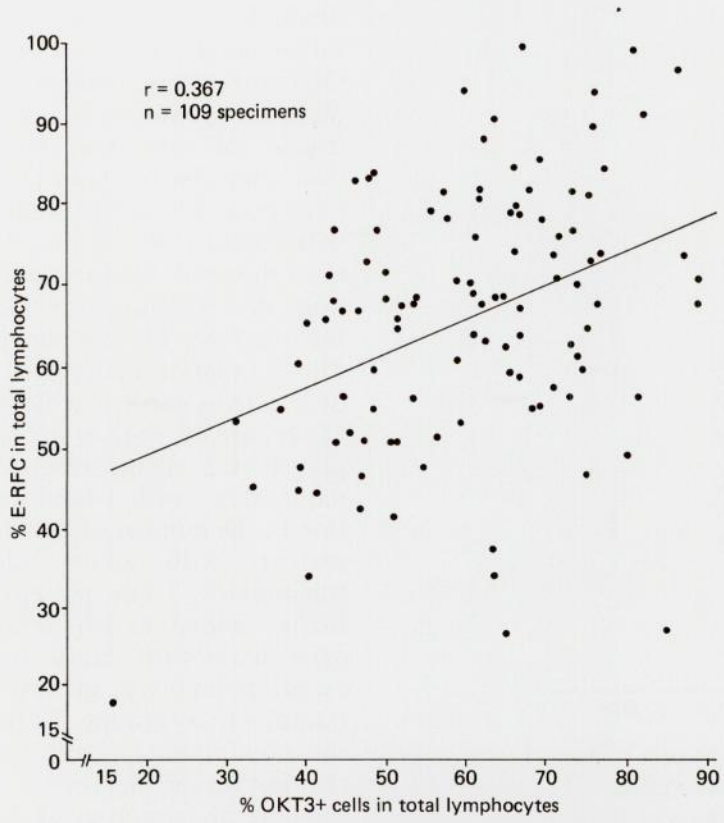

Fig. 6 Correlation between the percentage of E-RFC and OKT3+ cells in total lymphocytes in tuberculosis patients.

among active pulmonary tuberculosis patients. In the present studies, the degree of TG elevation was the least among the quiescent cases as compared to the active, the miliary and the extra-pulmonary groups. Again there is a suggestion from the data that the group of patients with miliary and extra-pulmonary tuberculosis had the highest elevation of TG. However, the number of subjects was too small to allow such a conclusion. When the OKT moAb were used, the elevation of OKT8+/OKT3+ cells could be shown to be statistically significant only among the active pulmonary tuberculosis patients. The discrepancy between the results of the two methods could not be resolved easily in the present studies as the correlation was poor. In the past few years, as both methods began to be more and more widely used, others have also reported conflicting results when the two methods were used separately in other diseases, e.g. SLE.[37,38] When the two tests were

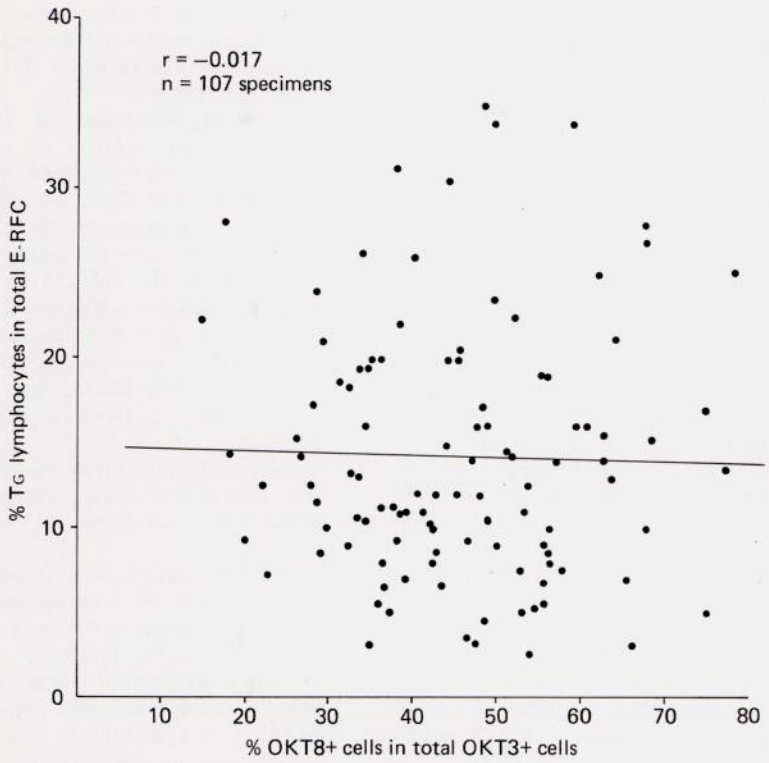

Fig. 7 Correlation between the percentage of TG in total E-RFC and OKT8+ cells in total OKT3+ cells in tuberculosis patients.

compared directly, only some 20 per cent of TG were OKT8+ cells[39] and around 70 per cent were OKT3+ cells. This suggested that either some TG cells were non T cells or that the OKT3 MoAb does not stain all T cells. At any rate, the correlation among these marker systems and between each system's function is far from settled. If one must decide whether TG or OKT8+ cells provide a better marker for suppressor cells, one probably would rely more on TG than OKT8+ cells at this time, particularly for diseases in which the correlation between OKT8+ cells and functional tests have been quite poor.[38] In the meantime, it is wise to use more than one marker system at least for confirmation. Thus, based on the present studies, one may quite confidently say that there is an elevation in suppressor cells among patients with active pulmonary tuberculosis because a statistically significant elevation could be shown with both marker systems in this group. The elevation in the other two groups is suggested on the basis of elevated TG alone.

The reduction in the proportion of helper T cells (OKT4+/OKT3+, Fig. 3) among both active and quiescent pulmonary tuberculosis patients is also consistent with the depressed CMI demontrated earlier. Since the rosetting technique for the detection of helper T cells (TM) was not included in the present studies, no correlation was possible. However, other reports in the literature concerning the correlation between OKT4+ cells and TM and between either of them and a functional test have been less problematic than is the case with suppressor cells.[19] In fact, correlation was quite good.

Together these results indicate that in tuberculosis there is a reduction in the number of total T cells and in some groups a reduction in the number of helper cells as well as an elevation in the number of suppressor cells in all groups of patients. These findings are all consistent with earlier ones regarding depressed CMI and they help to explain the chronicity of tuberculous infections. In immunoregulatory terms, this picture reflects a natural course of immune response in which the location of the invading mycobacteria, their macromolecular compositions and products, their resistance to rapid elimination or their sheer antigenic load gradually accumulated, thus leading to a preferential suppressor response. Once this is established, such a suppressor response can in turn play a role in enabling the organism to persist, thus leading to the chronicity of the infection. If unchecked by chemotherapy, the active disease inevitably progresses and/or the quiescent disease is reactivated.

The effect of RMP treatment on the T cells and their subpopulations is of interest because it has been reported that RMP itself may be immunosuppressive and because of the possible effect of antigen release owing to its bactericidal effects. The results of the present studies show that a recovery based on the changes in total T and T suppressor cells is consistent with the findings of Ktaz *et al*[8] who studied only active tuberculosis cases. However, the recovery in the relative number of TM as a result of treatment could not be found among our patients. In fact, there was an indication of a further reduction in the number of OKT4+ cells among the patients with active disease. Upon termination of RMP and continuation of conventional treatment, these effects of RMP were gradually lost and the T cells and T-cell subpopulations slowly returned to the pretreatment levels.

The lack of similar effects when RMP is used for treating quiescent cases is of interest for several reasons. Firstly, it suggests that the effects found in the active cases could be related to the massive killing of mycobacteria which are consequently removed from the system, whereas this massive killing

does not occur among quiescent patients in whom the number of mycobacteria may be substantially lower. Secondly, the suppressive response in quiescent patients and those with longer term disease may be so entrenched that chemotherapy cannot alter it much. It is in this quiescent group that a further elevation of suppressor cells (as detected by TG) was statistically significant during the course of RMP administration. It is possible that RMP itself could be immunosuppressive as suggested by others who reported a reduction in *in vitro* lymphocyte response and a reduction in the number of E-RFC.[5,9,10,11,13] However, this should again be taken as only suggestive; further investigation is needed.

One further precautionary note must also be added because the results of the cellular changes during and after RMP treatment varied greatly from patient to patient even among those in the same group. Thus, all that may be said is that the results suggest only a tendency and that the *in vitro* situation during infection, and particularly during chemotherapy, is extremely complicated and that there is an interphay of several chains of dynamic events. Some of these, e.g. changes in the mycobacterial antigenic load and a possible direct effect of RMP on certain cells, could be of immunoregulatory importance.

Despite these reservations, these results represent our first glimpse into this complicated situation and suggest a number of lines for further investigation.

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