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LYMPHOCYTE SUBPOPULATIONS IN SARCOIDOSIS

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SUMMARY

Highly purified blood lymphocytes from sarcoidosis patients were characterized by rosette technique for the presence of receptors for complement and by indirect immunofluorescence for the presence of membrane-bound immunoglobulin (B cells). T cells were estimated by spontaneous rosette formation with sheep red blood cells. The proportion of B cells was significantly higher and the proportion of T cells significantly lower than in the control group. Marked lymphocytopenia was found in the patient group. As a consequence the total number of circulating B cells was normal whereas the total number of circulating T cells was significantly depressed. Large atypical mononuclear cells were found in most patients. One-third of these cells had receptors for human complement.

INTRODUCTION

There is now considerable evidence of the presence among small lymphocytes of at least two subpopulations with regard to origin, life-span and function; thymus-dependent (T) lymphocytes responsible for cell-mediated immunity and bone marrow-derived (B) lymphocytes mediating humoral immunity. By extrapolation from animal experiments these two subpopulations can be distinguished in man. Mature B cells carry a high density of immunoglobulin determinants on their surface (Unanue *et al.*, 1971). Most B cells have a membrane receptor for an activated component of complement (C3) and form rosettes with sheep red blood cells (SRBC) which have been sensitized with rabbit IgM antibody and complement (EAC-rosettes) (Bianco, Patrick & Nussenzweig, 1970). This receptor is also found in a small population of non-identified lymphoid cells (Jondal, Wigzell & Aiuti, 1973). T lymphocytes spontaneously form rosettes with SRBC (E-rosettes) (Jondal, Holm & Wigzell, 1972).

In sarcoidosis, which is a non-malignant lymphopathy of unknown aetiology and pathogenesis mainly affecting young people, the cell-mediated immunity is characteristically depressed while the humoral immunity seems to be normal (see Siltzbach, 1971). The

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characteristic epitheloid cell granulomas are found in mediastinal glands and/or lungs in >90% of the cases. Several observers have found that lymphocytopenia occurs in an appreciable number of cases (Hoffbrand, 1968; Böttger, 1971).

As the defect cell-mediated immunity could be reflected in changes in the lymphocyte subpopulations peripheral blood lymphocytes from sarcoidosis patients have been isolated and characterized by morphology and by different cell surface markers. As the different stages of the disease differ in mode of onset, course and prognosis the findings have been correlated to the clinical picture.

MATERIALS AND METHODS

Patients and controls

Thirty-three patients with pulmonary sarcoidosis were investigated. The diagnosis was supported by histopathology of mediastinal lymph nodes and/or by positive Kveim test in all the cases. Patients with concomitant diseases were excluded except for one case with mild psoriatic lesions. None of the patients was on treatment at the time of test. In a few steroid-treated cases the drug had been withdrawn at least 3 months before test. In skin tests with 2 TU PPD tuberculin (Serum Institute, Copenhagen, Denmark) twenty-nine of the patients had a negative reaction and four had a positive reaction.

Stage I (bilateral hilar lymphomas)

Five patients were tested. All had acute onset of the disease with—apart from bilateral hilar lymphomas—erythema nodosum in three, arthralgias in one and parotitis in one. In all the patients symptoms still persisted when tested within 4 months. One was retested after another 3 months after progression of the disease and was included in the stage II group.

Stage II (parenchymal lesions of the lungs)

Twenty-one patients with a duration of disease from 4 months to 8 years were tested. All but one showed signs of radiological activity within the last 2 years.

Stage III (parenchymal lesions with pulmonary fibrosis)

Seven patients with signs of progressive pulmonary fibrosis were tested. The duration of disease was more than 5 years in all the cases.

Eighteen healthy volunteers between 20 and 70 years with normal lymphocyte counts (1200–4000 mm^3) and receiving no drugs served as controls.

White blood cells counts

The total number of white blood cells were counted in Türk's solution. Differential counts were determined on smears of peripheral blood stained with May–Grünwald and Giemsa.

Lymphocyte separation

Peripheral venous blood was defibrinated by shaking with glass beads. A hundred millilitres was mixed with 33 ml of 3% gelatin in Hanks's solution with 0·15 M Tris buffer, pH 7·4 (HT). The blood was allowed to sediment for 1 hr at 37°C. The cells in the supernatant were collected by centrifugation and washed twice in HT. The pellet was suspended in 10 ml HT with 10% pooled heat-inactivated human normal AB serum (HNS) containing

iron powder (0·4 g/10 ml). The tube was incubated in water at 37°C for 30 min and turned every 5th min. Phagocytic cells and remaining iron powder were removed by a magnet (Lundgren, Zukoski & Möller, 1968). The cell suspension was thereafter layered on a Ficoll–Isopaque gradient and centrifuged for 40 min at 400*g* in order to remove remaining red blood cells (Bøyum, 1968). The viability of the cells as revealed by Trypan Blue exclusion was >98% in all the preparations. Aliquots from the final cell suspension were smeared and stained with May–Grünwald–Giemsa.

Phagocytosis

25×10^6 heat-killed *Candida albicans* were suspended with 10^6 cells in conical plastic tubes in a total volume of 2 ml of medium (Parker 199 supplemented with antibiotics—100 iu penicillin and 100 μg streptomycin/ml—2 mM glutamine and 5% heat-inactivated foetal bovine serum (FBS) (Microbiological Associates, Bethesda, Maryland). The tubes were centrifuged at 80 *g* for 5 min and were then incubated at 37°C for 2–18 hr. The number of phagocytosing cells was counted in stained smears. Two hundred cells were counted (Holm, 1972).

E-binding lymphocytes

SRBC were stored at 4°C in Alsever's solution (1/1) and used within the week of bleeding. Before use the cells were washed twice and adjusted to 0·5% suspension in HT. As a test for cells with ability to form spontaneous rosettes with SRBC 0·25 ml (10^6) lymphocytes were mixed with 0·25 ml of SRBC and incubated at 37°C for 15 min. The mixed cell suspension was spun at 90 *g* for 6 min and then incubated in ice overnight. One drop of the cell suspension was mounted on to a glass slide, covered by a coverslip, and sealed with nail-polish. Two hundred lymphocytes were counted, and all lymphocytes binding more than three SRBC were considered positive (E-binding lymphocytes) (Jondal, Holm & Wigzell, 1972).

EAC-binding lymphocytes

Five millilitres of a 5% solution of washed SRBC was incubated for 30 min at 37°C with 5 ml amboceptor (rabbit anti-sheep SRBC) diluted 1/1000 in HT. The cells were washed twice and resuspended in 5 ml of HT. Five millilitres of human complement (fresh serum) diluted 1/20 in HT was then added and the suspension was incubated for 30 min at 37°C. The cells were finally washed twice and adjusted to a 0·5% solution. 0·25 ml of the prepared SRBC (EAC) was mixed with 0·25 ml (10^6) lymphocytes, spun at 90 *g* for 5 min and then incubated at 37°C for 15–30 min. Most of the supernatant was sucked off and the cells were vigorously resuspended on a whirl mixer. Two hundred cells were counted under a sealed coverslip. Lymphocytes binding more than three EAC were regarded as positive (EAC-binding lymphocytes) (Jondal *et al.*, 1972).

In eight patients in stages II and III the EAC lymphocyte preparations were stained with 0·001% (final concentration) Acridine Orange (AO). This facilitates the distinction between different lymphoid cells by staining the nucleus. The cells were examined in a Zeiss fluorescence microscope.

Labelling of immunoglobulin-bearing lymphocytes

0·25 ml (10^6) lymphocytes were pelleted down and the supernatant sucked off. Fifty microlitres of polyvalent rabbit anti-human immunoglobulin serum (Cappel Laboratories,

Downingtown, U.S.A.) diluted 1:16 was added. The cells were incubated for 45 min at 4°C and then washed three times in cold HT. Fifty microlitres of sheep anti-rabbit serum conjugated with fluorescein isothiocyanate (State Bacteriological Laboratories, Stockholm) was added. The cells were incubated for another 45 min at 4°C and thereafter washed three times in cold HT. The cells were mounted on a glass slide in one drop of 50% glycerol in phosphate buffer, pH 7·6 added with NaN_3 1 mg/ml. The membrane fluorescence was looked for by a Zeiss fluorescence microscope. Two hundred cells were counted (Holm *et al.*, in preparation).

Statistical methods

The mean values are given ±s.d. The significance of difference between mean values was tested by Student's *t*-test.

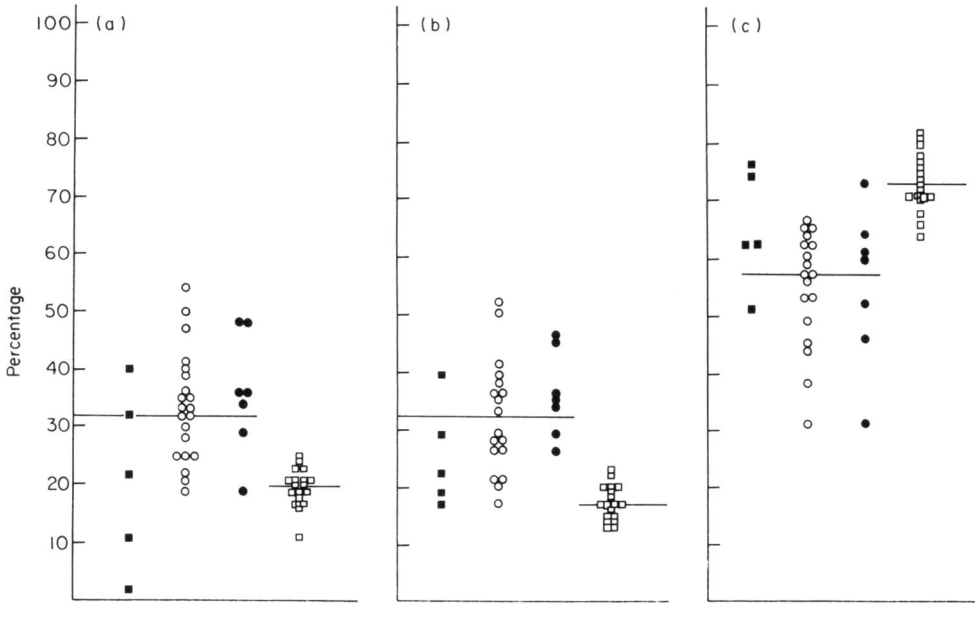

FIG. 1. The proportion of (a) EAC-binding cells, number of patients = 33, (b) immunoglobulin-staining cells, number of patients = 30, and (c) E-binding cells, number of patients = 30, in sarcoidosis patients, (■) stage I, (○) stage II, (●) stage III, and (□) healthy controls.

RESULTS

The total lymphocyte count was 1240 ± 111 in the patient group and 2227 ± 160 in the controls ($P < 0{\cdot}001$). Atypical mononuclear cells were found in the differential counts of the blood of the patients except in some cases in stage I. The cells were found in 10–20% of the total white cell count. Atypical mononuclear cells were not found in the control group.

After separation the cell suspensions were almost completely free of red blood cells and contained in the control group >99% small lymphocytes. In all the lymphocyte preparations in the patient group admixture of large atypical mononuclear cells were seen in varying amounts.

Parallel determinations of lymphocyte subpopulations in unfractionated blood and puri-

fied lymphocyte suspensions in patients and normals have not shown selection of lymphocyte subpopulations during the purification (Holm *et al.*, in preparation).

Cell suspensions from fourteen patients were tested for phagocytosis of *Candida albicans*. Ten were negative. In four 0·5–2% phagocytic cells were seen. The atypical cells were not phagocytic.

The proportion of EAC-binding cells was 32±2% in the patient group and 20±1% in the controls ($P<0·001$) (Fig. 1). The corresponding figures for immunoglobulin-positive cells were 32±2% and 17±1% respectively ($P<0·001$) (Fig. 1). In one of the patients with acute sarcoidosis with erythema nodosum only 2% of EAC-binding cells were detected and in another one with the same clinical picture the value was below normal (11%). In both cases the numbers of immunoglobulin-positive cells were normal, 19% and 17% respectively. In all the others normal to highly increased proportions of EAC-binding and immunoglobulin-positive cells were found.

TABLE 1. EAC-lymphocyte preparations stained with Acridine Orange

Case	Rosette-forming cells (%)		Non-rosette-forming cells (%)	
	Small lymphocytes	Atypical cells	Small lymphocytes	Atypical cells
1	29	10	34	27
2	27	6	49	18
3	36	14	36	14
4	45	3	45	7
5	15	22	31	32
6	32	7	53	8
7	27	14	39	20
8	15	7	68	10

The proportion of E-binding cells varied inversely with the proportion of EAC-binding and immunoglobulin-bearing cells. The mean value was 57±2% in the patient group and 73±1% in the control group ($P<0·001$) (Fig. 1). The sums of EAC-binding or Ig-positive cells and E-binding cells were about 90% in both groups.

The morphological evaluation of the AO-stained EAC preparations from eight patients is shown in Table 1. Atypical cells were found in 27±5%. About one-third of these (10±2% of total) formed rosettes with EAC. The total proportions of EAC-positive cells were in these patients 39±3% in the AO-stained preparations and 41±3% in the non-stained ones.

The total numbers of cells in the lymphocyte subpopulations are shown in Fig. 2. The difference between total number of E-binding cells in the two groups is highly significant 714±75 and 1646±129 respectively ($P<0·001$). The values of EAC-binding and immunoglobulin-positive cells in the patient group did not differ from the control group.

No significant differences between total numbers of cells in the subpopulations were found between the different clinical stages.

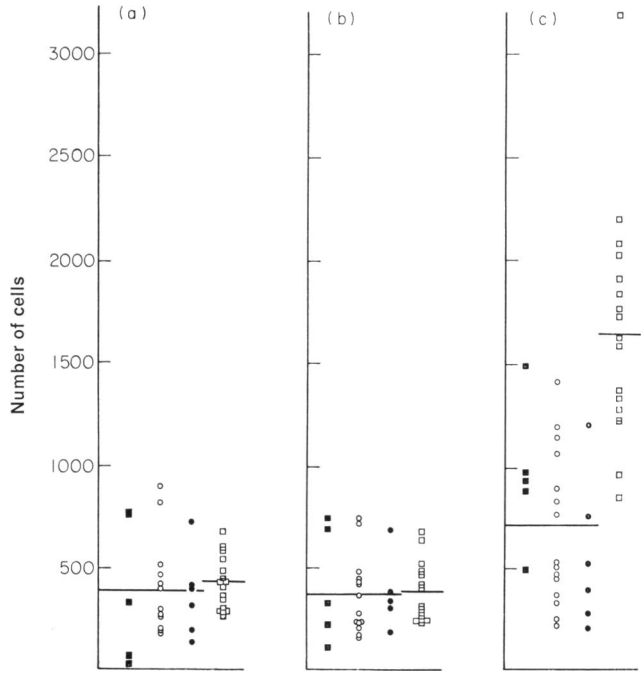

FIG. 2. Total numbers of (a) EAC-binding cells per mm^3, number of patients = 28, (b) immunoglobulin-staining cells per mm^3, number of patients = 26, and (c) E-binding cells per mm^3, number of patients = 26, in sarcoidosis patients (■) stage I, (○) stage II, (●) stage II, and (□) healthy controls.

DISCUSSION

The total lymphocyte counts in patients with pulmonary sarcoidosis were lower than in healthy controls. This finding confirms earlier investigations (Hoffbrand, 1968; Böttger, 1971). The difference was most pronounced in long-standing severe cases. This has also been found earlier (Hoffbrand, 1968).

In the patient group the proportions of the EAC-binding and immunoglobulin-positive cells were increased whereas the proportion of E-binding cells was decreased. This is in agreement with a preliminary study of five sarcoidosis patients (Papamichail *et al.*, 1972).

Since selective losses of lymphocytes do not occur during the purification (Holm *et al.*, in preparation) the total number of cells in the subpopulations could be calculated. High proportions of EAC-binding and immunoglobulin-positive cells were not in any case equivalent with an increased number of circulating B cells. On the other hand low proportion of E-binding cells was reflected by an absolute T-cell deficiency in the peripheral blood.

The results obtained are in agreement with the clinical findings of impaired delayed hypersensitivity with preserved normal humoral immunity in most patients (see Siltzbach, 1971). The cause of the T-cell deficiency which is present from the onset of the disease remains unknown. Long-standing severe cases tended to be more T-cell deficient, but the difference between patients in stage I and III was not statistically significant.

Atypical mononuclear cells were found in most sarcoidosis patients. The number of

patients with these cells as well as the total number tended to be higher in late stages of the disease. Morphologically indistinguishable from these cells are the atypical cells found in the peripheral blood in Hodgkin's disease (Crowther, Fairley & Sewell, 1967; Holm *et al.*, in preparation) during the acute phase of several viral illnesses as well as in several other states (Wood & Frenkel, 1967; Crowther, Fairley and Sewell, 1969). Although the atypical cells are morphologically heterogenous most observers consider them as *in vivo* activated lymphocytes (Wood & Frenkel, 1967). However, their relationship to the humoral and/or cellular immune responses has not been fully established. Recent studies indicate that in infectious mononucleosis and Sézary's syndrome the large atypical cells seem to be T cells (Virolainen *et al.*, 1973; Brouet, Flandrin & Seligmann, 1973). In the sarcoidosis patients AO-staining of EAC-rosetted cells indicated that some atypical cells had receptors for complement.

In healthy persons the complement receptor is found not only on most B cells (Bianco *et al.*, 1970) but also on cells belonging to the monocytic series (Huber *et al.*, 1968) as well as in a small population of non-identified lymphoid cells (Jondal *et al.*, 1973). Further studies are necessary to evaluate the nature and significance of atypical cells found in a disease in which widespread formation of epithelioid cell granulomas occurs in the presence of acquired T-cell deficiency.

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