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Spleen lymphocyte populations in patients with Hodgkin's disease—properties of cells with different densities

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SUMMARY

Spleen lymphocytes from five patients with Hodgkin's disease (HD) type mixed cellularity and five normal controls were studied. An increased percentage of 'null cells' was observed in three out of the five patients studied. The two other patients had increased percentage of T lymphocytes. Lymphocytes from spleen with HD had a decreased ability to respond to suboptimal concentrations of T cell mitogens. When these cells were fractionated in a discontinuous density gradient a subpopulation of cells, unable to respond to Con A, was recovered from the dense cell fractions. The implications of the present findings to the understanding of the physiopathological changes in HD are discussed.

INTRODUCTION

Hodgkin's disease (HD) is a progressive disorder of the lymphoid system often accompanied by lymphopenia and deficits of cell-mediated immunity (CMI), including inability to reject skin grafts (Kelly, Laneb & Good, 1960), anergy to *in vivo* challenge with antigens (Aisenberg, 1962) and changes in the *in vitro* response to T cell mitogens (Levy & Kaplan, 1974). Changes in CMI in Hodgkin's disease were attributed to a defect of the lymphocyte either intrinsic to the cell (de Sousa *et al.*, 1977) or consequent to the presence of serum blocking factors (Fuks, Strober & Kaplan, 1976). Alternatively, changes in lymphocyte distribution, due to the sequestration of immunocompetent cells in the lymphoid organ involved by the disease (de Sousa *et al.*, 1977) could justify the immunological defect observed in the peripheral blood lymphocytes of these patients. Therefore, the study of lymphocyte function in major lymphoid organs may contribute to understanding the pathogenesis of HD.

In the present work, spleen lymphocytes from five patients with HD type mixed cellularity and from five patients with splenic rupture due to accident, were studied. In HD a decreased percentage of B lymphocytes was observed, when compared with normal cells. Three of the five patients studied also had increased numbers of 'null cells'. In the other two patients, an increased percentage of ERFC was observed. HD spleen lymphocytes had lower ^{3}H-thymidine incorporation in the presence of suboptimal doses of T cell mitogens (PHA and Con A) and when these cells were fractionated in a BSA discontinous density gradient, a subpopulation of cells unresponsive to Con A was recovered from the dense cell fractions. The implications of the present findings to the understanding of the physiopathological changes in HD are discussed.

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MATERIALS AND METHODS

Patients. All patients studied had Hodgkin's disease type mixed cellularity, according to the Rye modification of the Lukes and Butler classification (Lukes *et al.*, 1966) and had not received any therapy before splenectomy for staging. The spleens used as controls were obtained from five normal individuals with splenic rupture due to traffic accidents. In any case, the spleens were kept at 4°C in sterile saline until used and spleen cell suspensions were prepared from macroscopically normal splenic tissue within 4 hr after splenectomy.

Cell suspensions and cell surface markers. Spleen cell suspensions were prepared by teasing the organ with two needles in ice cold Minimum essential medium (MEM; GIBCO, USA). Tissue clumps were removed by decantation and cells were washed twice in MEM, before further use.

Density fractionation of spleen cell suspensions was done in a 2% step discontinuous BSA (Bovine Serum Albumin Fraction V, Sigma Chemical Co, St Louis, Missouri) density gradient, ranging from 31% to 17% w/v BSA (Dicke, Tridente & Van Bekkun, 1969). The cells, resuspended in 17% BSA, were layered into the top of the gradient and centrifuged at 1000 ***g*** for 45 min at 10°C. For each experiment the pH, osmolarity and refractive index of the various density fractions were carefully adjusted. Spleen cells sedimenting at the various gradient interphases were collected with a Pasteur pipette and washed three times in large volumes of cold MEM.

Lymphocytes able to form rosettes with sheep erythrocytes (SRBC) were determined as described (Jondal, Holm & Wigzell, 1972). The number of cells with membrane Igs was determined by direct immunofluorescence using a rabbit anti-human Ig antiserum (Behring). Macrophages were determined as cells able to incorporate latex beads (Bianco, 1976). The percentage of cells with T and B cell markers was determined in the non-phagocytic cell population.

Cell cultures. Spleen cells, at the final concentration of $2{\cdot}5 \times 10^5$/ml were cultured in triplicate in RPMI 1640 (GIBCO, USA) containing 4×10^{-3} M glutamine, 100 units penicillin–streptomycin, 2×10^{-2} M HEPES buffer (GIBCO, USA) and 10% pooled AB serum in microculture plates (0·2 ml/culture) at 37°C for 72 hr in a 5% CO_2 humidified atmosphere. The following mitogen concentrations were tested: Concanavalin A (Con A, Sigma Chemical Co., St Louis, Missouri) 1, 2·5, 5, 10, 25, 50 μg/ml. Phytohaemagglutinin (PHA-P, Wellcome) 0·1, 1, 2·5, 5, 10, 25 μg/ml. For determination of DNA synthesis a 4 hr pulse of 0·1 μCi/culture of ^{3}H-thymidine (5 Ci/nmol, Radiochemical Centre, Amersham, UK) was given. Results are expressed as mean c.p.m./culture.

RESULTS

Spleen cells from patients with Hodgkin's disease (HD) have a decreased frequency of cells bearing surface immunoglobulin (Table 1). In patients No. 6, 9, 10 the percentage of T cells was within normal values, and a clear increase in cells without T or B cell markers ('null cells') was observed (Table 1). A slight increase in E rosette forming cells (E-RFC) was observed in patient No. 7. In contrast, patient No. 8, which spleen was macroscopically involved by the tumour had a marked increase in E-RFC (Table 1).

Fig. 1 shows the dose-response curve to Con A and PHA of spleen lymphocytes from five normal donors (shaded area) and five patients with HD. No noticeable differences in optimal dose of mitogen, or maximum intensity of response are observed between HD lymphocytes and controls. Patients with HD, however, have reduced responses in the presence of suboptimal doses of T cell mitogens, more marked in the presence of Con A. When the intensity of ^{3}H-thymidine incorporation, in the presence of PHA or Con A is compared in each individual spleen, normal lymphocytes respond similarly to both mitogens, while HD lymphocytes respond preferentially to PHA (Table 2).

Density distribution of T cells, B cells (Table 3), total number of cells and phagocytic cells (not shown) is similar in HD patients and control spleens. T cells accumulate in 21/23 and 23/25 interphases while B cells are enriched in lighter and denser fractions (Table 3) and phagocytic cells distribute homogeneously throughout the gradient (not shown). This density distribution is similar

Table 1. Percentage of T and B lymphocytes in normal spleens and in the spleens of patients with Hodgkin's disease

Patient No.	Sex/age	Diagnosis*	% T	% B
1	M/18	SR	32	45
2	F/28	SR	47	52·5
3	M/25	SR	45	50
4	M/42	SR	43	50
5	F/8	SR	30	50
6	M/6	HD	26	3
7	M/11	HD	56	37
8	M/35	HD	75†	2·5†
9	M/42	HD	32	18
10	M/37	HD	27	24

* SR = Splenic rupture; HD = Hodgkin's disease mixed cellularity. T cells were determined as the percentage of cells able to form rosettes with SRBC and B cells by direct immunoflourescence using a FITC-rabbit anti-human Igs antisera.

† Spleen macroscopically involved by the tumour.

to that of human peripheral blood lymphocytes (Geha, Rosen & Merler, 1973) and human tonsil lymphocytes (Geha & Merler, 1974).

In contrast, response to T mitogens of cells recovered from the various density fractions differs markedly in contols and in patients with HD (Figs 2, 3, Table 2).

In normal spleens, dose-response curves of every density fraction are similar throughout the

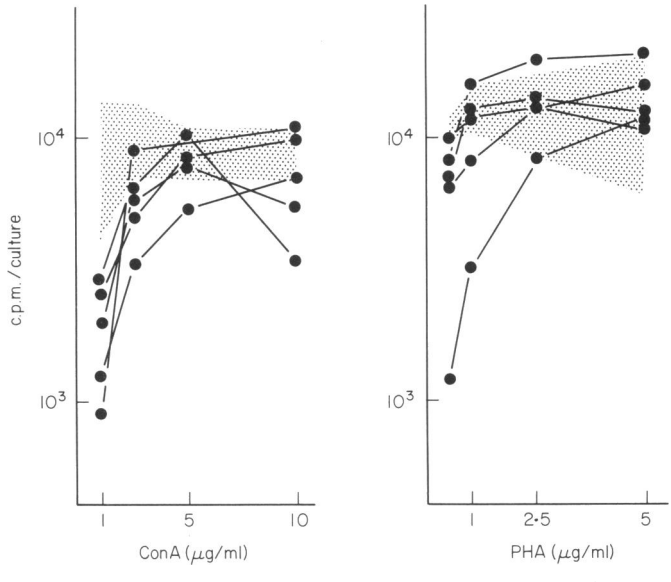


Fig. 1. Dose-response curve to Con A (a) and PHA (b) of spleen lymphocytes from five normal donors (shaded area) and five patients with Hodgkin's disease (●——●). Results are expressed as mean c.p.m./culture of triplicate cultures.

Table 2. Response to PHA and Con A of spleen lymphocytes with different densities

Patient No.	UNF		17/19		19/21		21/23		23/25		25/27		27/29	
	Con A	PHA	Con A	PHA	Con A	PHA	Con A	PHA	Con A	PHA	Con A	PHA	Con A	PHA
1	13·8	13·2	17·3	16·6	7·1	12·9	12·7	10	7	12	n.d.	n.d.	10	11·8
2	10	11·5	20·6	17·5	12·6	14·3	6	8	5·7	8·4	2·1	4·5	4·3	4·5
7	11·8	21	1	2·2	7·8	12·2	7·7	16·7	1·6	17·6	n.d.	n.d.	0·5	7
8	7·6	16·8	2·6	5·5	7·3	11·8	12·2	17·1	5·3	20·4	0·8	9·6	0·2	3·3
9	11·3	13	10	14	11·6	14·8	14·2	19·4	11·3	16·4	0·8	10·9	0·6	8·08
10	5·3	11·8	6·4	7·4	4·7	7·8	8·5	13·5	5·3	17·6	1·1	11·9	0·4	10

Spleen lymphocytes from patients with splenic rupture (1, 2) or Hodgkin's disease (7–10) were fractionated in a discontinuous BSA density gradient, and cells collected from the interphases were cultured from 72 hr with various concentrations of PHA and Con A (see Materials and Methods). Results represent different fractions mitogen response to the optimal concentration of PHA and Con A, expressed as c.p.m. $\times 10^{-3}$.

gradient (Figs 1, 2). Maximum response to Con A is observed in the 17/19 cell fraction, while response to PHA is more intense in the 17/19 and 19/21 cell fractions (Figs 1, 2; Table 2). Dense cells are less responsive to both mitogens than the unfractionated spleen cell population.

Although light cells respond preferentially to Con A while dense cells have higher responses in the presence of PHA, there are no major differences in the ability of cells with same density to respond in the presence of either mitogen (Table 2).

Spleen lymphocytes from patients with HD, obtained from the 17/19 interphase, are not enriched in cells highly reactive to PHA and Con A (Figs 1, 2, Table 2). Maximum response to Con A is observed in the 19/21 and 21/23 cell fractions, while the response to PHA is more intense with cells obtained from the 21/23 and 23/25 interphases.

The response to PHA of dense spleen cells from patients with HD is slightly lower than that of unfractionated spleen cells (Table 2). In contrast to normal lymphocytes, however, the response of HD to Con A is very low (Fig. 2, Table 2). Also, the dose-response curve of dense cell fractions differs from that of other fractions in that suboptimal responses are obtained with Con A concentrations ranging from 1–5 μg/ml (Fig. 2).

The differences in response to T cell mitogens of cells with different densities cannot be

Table 3. Distribution of T and B lymphocytes in different BSA density fractions

Density fraction	% T					% B				
	C	7	8	9	10	C	7	8	9	10
17/19	17	10	31	17	10	n.d.	27	12	18	27
19/21	33	18	30	15	7	34	47	4	39	41
21/23	48	56	59	49	29	19	18	7	11	18
23/25	48	58	68	53	28	17	19	1	15	7
25/27	28	21	21	32	19	27	45	1	41	20
27/29	n.d.	18	17	11	17	n.d.	58	5	43	25

Spleen cell suspensions were fractionated in discontinuous BSA density gradients. T cells were determined as the percentage of cells able to form rosettes with sheep erythrocytes, and B cells staining with a FITC-rabbit anti-human Igs antisera. (C = normal spleen (representative case). 7, 8, 9, 10 refer to Table 1.)

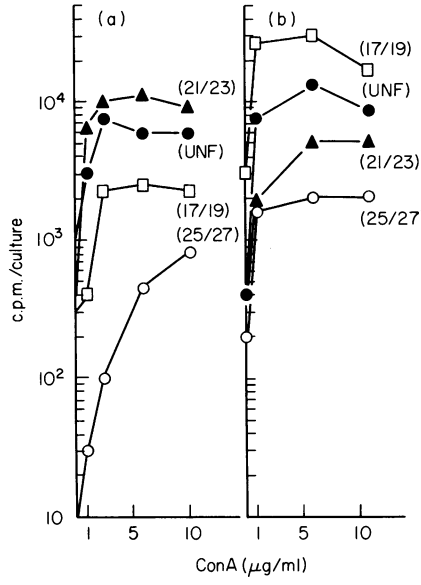
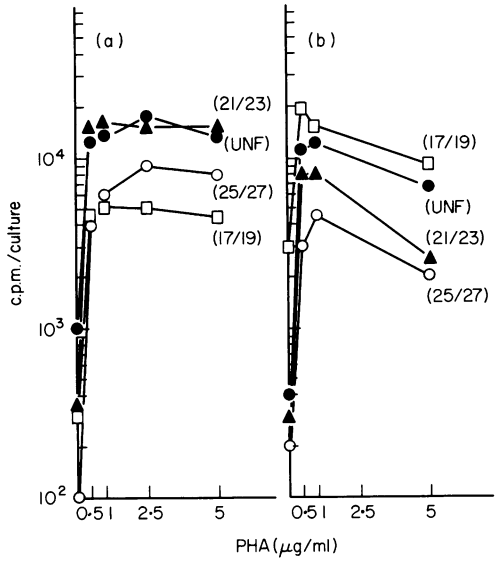


Fig. 2. Dose-response curve to Con A of cells with different densities of patients No. 8 (a) and control No. 2 (b). Similar results were obtained with the other four patients studied. 19/21 and 23/25 dose response curves are similar to respectively 21/23 and 25/27 response. Results are expressed as mean c.p.m./culture of triplicate cultures.



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attributed to a selective enrichment of macrophages, since phagocytic cells were found to distribute homogeneously throughout the gradient (not shown). Also, there is no correlation between T cell numbers and the intensity of mitogenic response (Tables 2, 3). In effect light cell fractions from normal spleen cells, that are depleted in E-RFC (Table 2) have higher mitogenic responses than medium density cell fractions (Table 3) enriched in E-RFC (Table 2). On the other hand cells recovered from light or dense cell fractions, with similar percentage of E-RFC, respond differently to T cell mitogens (Tables 2, 3).

DISCUSSION

Immunological dysfunction of patients with HD has been attributed to a functional defect of the lymphocyte (Moroz *et al.*, 1977; Fuks *et al.*, 1976; de Sousa *et al.*, 1977). Alternatively, sequestration of immunocompetent cells in other lymphoid compartments (de Sousa *et al.*, 1977) could result in depletion of these cells from the peripheral blood of patients with HD. In effect several authors have referred to an increase in the percentage of T lymphocytes present in lymphoid organs involved by the tumour (Aisenberg & Long, 1975; Grifoni *et al.*, 1975; Hunter *et al.*, 1977; Joseph & Belpomme, 1975) that we have also observed (Table 1, Patient No. 8).

The study of indium 111-labelled lymphocytes migration in patients with HD has shown lymphocyte accumulation into lymph nodes (Wagstaff *et al.*, 1981). As it has been thoroughly demonstrated that changes in the membrane induce modifications of migration of lymphocytes (reviewed in Freitas, 1981) it is possible that both mechanisms coexist in the disease. It must be noted that an increased percentage of non-phagocytic mononuclear cells, morphologically identical to lymphocytes, without T or B cell markers ('null cells') were observed in the spleen of three out of five patients with HD (Table 1). The other two patients had increased percentage of E rosette forming cells (Table 1).

Spleen lymphocytes from patients with HD have reduced ^{3}H-thymidine incorporation in the presence of suboptimal concentrations of T cell mitogens (Fig. 1). When the response in the presence of PHA or Con A is compared in each individual spleen, HD lymphocytes have reduced ability to respond to Con A (Table 3). When spleen lymphocytes from patients with HD are fractionated in a discontinuous BSA density gradient, the dense cell fractions are unable to respond to Con A (Figs 2, 3). It is therefore possible that changes in mitogen reactivity of HD spleen lymphocytes can be attributed to depletion of a Con A-reactive dense cell population. A cell population able to respond exclusively to Con A was described in the mouse, and shown to be sessile, sensitive to adult thymectomy, resistant to *in vivo* administration of ALS (Stobo & Paul, 1973), functionally behaving like cytotoxic and suppressor T cell precursors (Hayry *et al.*, 1976) and having the Ly $1^{+}2^{+},3^{+}$ phenotype (Cantor & Boyse, 1976). On the other hand dense spleen cells were shown to mediate suppressor effects in both mouse (Whisler & Stobo, 1976) and rat (Rocha, Freitas & de Sousa, 1979) and preliminary evidence suggests that it is also the case in normal human spleen (Rocha, unpublished observation).

It must be noted, however, that results obtained by several investigators on the properties of cells reacting to PHA or Con A are contradictory. This fact may be due to the complex cell interactions involved in cell activation and proliferation in the presence of T mitogens. Dense suppressor T cells were shown to be involved in the regulation of the response to T cell mitogens, in the rat (Rocha *et al.*, 1979). Also T cell proliferation in the presence of mitogenic lectins was dissociated into two separate events—production of Interleukin II (IL-2), which is macrophage dependent and expression of receptors for IL-2 (Larsson, Iscove & Coutinho, 1980) and different mitogens were shown to induce either step preferentially (Larsson & Coutinho, 1980). Therefore, lack of response to Con A of dense cells from patients with Hodgkin's disease can either result from depletion of a cell population able to express receptors for IL-2 in the presence of this mitogen, or to limiting conditions on IL-2 production that ultimately can result from lymphocyte or macrophage dysfunction. It must be noted that Reed–Sternberg cells were shown to contain fibronectin (Resnick & Nachman, 1981) a characteristic of normal macrophages.

These alternatives are presently being investigated.

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