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Ecotaxis: the principle and its application to the study of Hodgkin's disease

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SUMMARY

A study of the function, characterization and distribution of T and B lymphocytes in five children with Hodgkin's disease is presented. The results, indicating that lymphocyte depletion in the peripheral blood does not necessarily reflect an overall lack of circulating lymphocytes, are presented to demonstrate that failure of ecotaxis (normal lymphocyte migration and distribution) can occur in man. The underlying reasons for such failure and their relevance to the pathogenesis of Hodgkin's disease are discussed.

INTRODUCTION

Since Gowan's critical demonstration that in the rat, lymphocytes are long-lived cells whose rapid turnover in the blood can be explained by their continuous circulation into the lymph through the peripheral lymphoid organs (Gowans, 1959), much has been learned about the circulation of lymphocytes in experimental animals (Ford & Gowans, 1969; Ford, 1975; de Sousa, 1976a).

It has been shown that the continuous circulation of lymphocytes between blood and lymph can be altered non-specifically in a number of ways, i.e. *in vivo* treatment with antigens, *B. pertussis*, dextrans, cortisone, X-irradiation, and treatment of the lymphocytes *in vitro* with a number of substances altering their surface, i.e. enzymes, plant lectins, anti-lymphocyte antiserum, lipopolysacchardide, phospholipases, etc. (Ford, 1975; Freitas & de Sousa, 1976a, 1976b; de Sousa, 1976a). Finally, it is known that in addition to discriminating between lympoid and non-lymphoid organs, T and B lymphocytes also discriminate between distinct microenvironments within the spleen, lymph nodes and Peyer's patches (Parrott & de Sousa, 1971). The ability of lymphocytes to migrate and arrange themselves in specific organs and microenvironments has been called ecotaxis (de Sousa, 1971, 1973, 1976b).

In striking contrast with the considerable knowledge of lymphocyte maldistribution in experimental animals, little is known about lymphocyte maldistribution in man. In this context, it is perhaps worth noting how the mechanism of certain forms of anaemia has been associated with the concept of red-cell maldistribution and seqestration, yet with the exception of mycosis fungoides (Mackie *et al.*, 1976), lymphopenia in man has not been envisaged as the possible reflection of a similar mechanism. Studies of the peripheral blood in Hodgkin's disease have revealed a depletion of T lymphocytes, B lymphocytes, or both types of cells (Andersen, 1974; Case *et al.*, 1976; Cohnen, Douglas & Brittinger, 1974; Gajl-Peczalska *et al.*, 1973; Bobrove *et al.*, 1975), and organ biopsies are available as part of the staging procedure. Hodgkin's disease seemed, therefore, an ideal situation to investigate the possibility of the existence of lymphocyte maldistribution in man.

This paper is a preliminary account of the results of a study of the distribution of T and B cells in the spleen, blood, liver, involved and uninvolved lymph nodes in five children with Hodgkin's disease. The

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study is still under progress and a detailed account of the whole investigation is presently in preparation (Tan *et al.*, 1976, in preparation).

Some of the results presented do support the idea that some forms of lymphopenia, like some forms of anaemia, may reflect a failure of the mechanisms normally involved in the control of cell traffic.

MATERIALS AND METHODS

Patients. Details of the patients' dates of birth, times between apparent onset and diagnosis, histology, presentations and stages are summarized in Table 1.

Controls. Peripheral blood. The results of the immunological analysis of the control young adult pool of the SKI Immunological Parameters Laboratory examined for the period of this study were used as controls. In addition, we examined the peripheral blood lymphocyte content and mitogen responses of a 7-year-old child control to ensure that the values of the young adult controls could be used as controls to the children with Hodgkin's disease.

Spleen. Three adult non-involved spleens obtained from diagnostic splenectomies (one carcinoma of lung, one carcinoma of esophagus and one (?) intracranial malignancy) were the only material available to be used as controls.

Preparation of cell suspensions. Suspensions of lymphoid cells were obtained from areas of the spleen and the liver uninvolved by disease, by teasing the tissue pieces gently into RPMI 1640 under sterile conditions. After preparing a single cell suspension, the lymphoid cells were separated in a Ficoll–Hypaque gradient (d : 1075–1088). It has been pointed out that substantial cell losses occur using similar separation procedures (Habeshaw & Young, 1975). We consider the use of the present method valid because it was applied in the standard way used in the laboratory, to all blood and tissue samples analysed, and any cell losses occurring in one sample must have been identical to those in the other samples, thus making it possible to correlate with some confidence the different results obtained (Table 2).

The peripheral blood lymphocytes were obtained by separation of 50 ml of heparinized blood (diluted in Hanks's balanced salt solution 1 = 1 v/v) in a similar gradient. After two washes, the viability of the cells was checked by trypan blue exclusion, and only suspensions of more than 95% viable cells were used. The suspensions were then divided into two separate samples, one to be used for rosette formation and membrane immunofluorescence, and the other for mitogen stimulation. To the former, 100 λ of 1% latex particles (0·86 μ diameter, Dow Chemical) were added, the cells suspended in RPMI 1640 containing 20% foetal calf serum and incubated for 30–60 min at 37°C. After incubation with the latex particles, the cells were washed twice with RPMI.

Spontaneous rosette formation with sheep erythrocytes. E rosettes (T lymphocytes). For the preparation of E-rosette-forming cells, the concentration of the cell suspensions was adjusted to 3×10^6/ml. A suspension of 0·5% washed sheep red cells (Flow Laboratories, Rockville, Maryland) was used. Duplicate or triplicate samples were prepared in the following way: equal volumes (100 λ/100 λ) of the lymphocyte suspension and of the sheep erythrocyte suspension were incubated in the presence of heat-inactivated and absorbed human AB serum (20 λ) for 5 min at 37°C in a shaking water bath. The mixtures were then centrifuged at 50 *g* for 5 min and left at 4°C for 18 hr. At the end of the incubation, the proportion of E-rosette-

TABLE 1. Summary of clinical data

Patient	Birth	Onset	Diagnosis	Histology	Nodes involved at presentation	Other sites involved	Stage
J.E.	4*/69†	11/73	9/74	LP	Right cervical	—	IA
J.S.	12/59	6/74	8/74	NS	Cervical (right and left) Supraclavicular (right and left)	—	IIA
A.S.	3/67		9/74	MC	Right cervical Right axilla	Spleen	III
C.D.	12/64	7/74	11/74	NS	Left axilla Left supraclavicular	Spleen and para-aortic nodes	IIIB
M.O'K.	7/67	1/75	1/75	NS	Right cervical Left cervical Left supraclavicular	Spleen and para-aortic nodes	IIIB

LP = Lymphocytic predominance; MC = mixed cellularity; NS = nodular sclerosis; R = right; L = left.

* Month.

† Year.

TABLE 2. Proportion of T and B cells in peripheral blood and spleen

Stage	Age	Sex	Compartment	E (%)	EAC (%)	Immunoglobulin-bearing cells						
						pv	μ	γ	α	κ	λ	δ
IA	5	M	Blood 1*	23·5	13·5	14	10	5	0	7	6	—
(J.E.)			Spleen	61	28	37	23	11	5	—	—	24
			Blood 2†	43·5	—	12	23	11	4	26	12	—
IIA	15	F	Blood	45·5	8	—	—	—	—	—	—	—
(J.S.)			Spleen	51	43	—	—	—	—	—	—	—
IIIB	7	M	Blood	83·5	15	18	8	4	1	12	3	—
(A.S.)			Spleen	53	44	33	20	14	9	17	19	21
IIIB	10	F	Blood	62·5	19	9	3	3	0	5	4	—
(C.D.)			Spleen	83	17	16	10	5	2	10	9	9
IIIB	8	F	Blood	84·5	6	13	5	8	0	10	3	—
(M.O'K.)			Spleen	68	—	45	34	5	3	25	20	18
Controls			Blood	83·7±6·3	11·8±4·5	13±6	7±4	6±5	1·5±9	8·7±4	5±2	—
			Spleen	75·8±16	—	18±2	4±3	4·±3	1·3±9	14±5	7±3	8±3

Letters in parentheses are patients' initials.

* Pre-splenectomy.

† Post-splenectomy (3 months).

forming cells was estimated by counting at least 200 cells for each of the samples prepared. Cells with ingested latex particles were not included in the count.

Rosette formation with IgMEAC and IgGEA (B lymphocytes). Sensitized sheep erythrocytes were prepared with IgG and IgM antibodies to whole sheep red blood cells (Cordis Laboratories) in a subagglutination titre, and AKR mouse serum as source of complement (Bianco, Patrick & Nussenzweig, 1970) to prepared IgMEAC complexes.

Equal volumes (100 λ/100 λ) of the white-cell suspensions (3×10^6/ml) and the IgGEA, IgMEAC, IgMEA suspensions (0·5%) were mixed and incubated for 30 min at 37°C in a shaking water bath. At the end of the incubation, the proportion of rosette-forming cells was estimated by counting at least 200 cells for each of the samples prepared. Cells with ingested latex particles or positive trypan blue staining were not included in the count.

Fluorescence microscopy. Aliquots containing approximately 1×10^6 cells were washed once and the pellet resuspended in a volume of 25 λ of 2% bovine serum albumin (Pentex Bovine Albumin, Miles Laboratories) in Hanks's BSS with sodium azide. To this suspension, 25 λ of the FITC-conjugated antisera polyvalent and monospecific anti-IgM, IgG, IgA, K, λ Dako immunoglobulin were added and incubated in ice for 30 min. Cells were then washed three times in Hanks's BSS–2% BSA containing sodium azide and a drop of the cell suspension placed on a slide, covered with coverslip and sealed with nail polish. Specimens were examined under a Leitz Orthostat microscope equipped with Ploem system. At least 200 cells were counted in each sample and the percentage of cells showing positive membrane fluorescence estimated.

Mitogen stimulation. The concentration of the cell suspensions containing no latex was adjusted to 5×10^5/ml in RPMI 1640 containing 20% inactivated, pooled human serum, antibiotics and glutamine (5000 u penicillin, 5000 mcg streptomycin, 2 mM glutamine in 100 ml media). Six mitogen concentrations were used to determine the response of the cells to phytohaemagglutinin and Con A (Difco Laboratories) and 3 concentrations of PWM (Gibco Laboratories). Triplicate samples of the cells (1×10^5/ml) and mitogens were incubated in microtest tissue culture plates No. 3041 (Falcon Plastics) at 37°C in a National Heinicke incubator containing 5% CO_2. At the end of 72 hr incubation, 0·03μ Ci [^{14}C]thymidine (New England Nuclear NEC 156 specific activity 750 m Ci/mM) was added to each sample and incubation carried out for a further 24 hr. The cells were then harvested in a Skatron multiple cell culture harvester and the radioactivity counted in a Packard Tricarb liquid scintillation counter.

RESULTS

Proportion of T and B cells in peripheral blood and spleen

The results of the estimations of the proportions of T and B cells found in the peripheral blood and spleen were summarized in Table 2. Low percentages of T cells in blood were found in patients J.E., J.S. and C.D., in stages IA (23·5%), IIA (45·5%) and IIIB (62·5%) respectively. The other two patients,

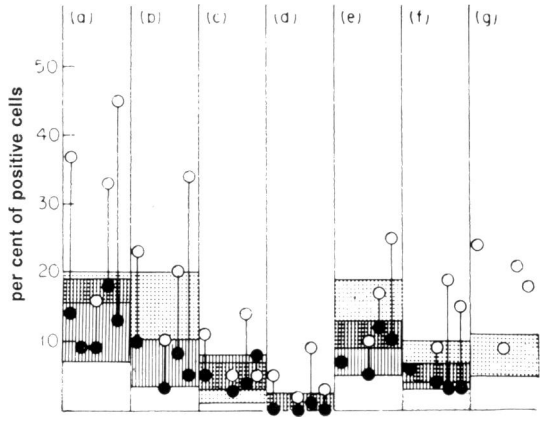

FIG. 1. Distribution of the different classes of B cells in the blood (●) and spleen (○) of 5 children with Hodgkin's disease. The shaded areas represent the control range (mean ± 1 s.d.) for the blood (▥) and spleen (▨). (a) Polyvalent; (b) μ; (c) γ; (d) α; (e) κ; (f) λ; (g) δ.

A.S. and M.O'K., in stage IIIB, had normal percentages of circulating T cells. The proportions of the T cells in the spleens of the three patients with low peripheral blood T cells were higher than in the blood (61%, 51% and 83%). A considerable proportion of the lymphoid cells in the blood of these three patients could not be characterized by any of the surface markers used. HD involvement of the spleen was observed in only one of the three patients (patient C.D., stage IIIB). Macroscopically, the sample analysed was *not* associated with an involved tissue area. The other two patients, J.E. and J.S., staged as IA and IIA respectively, had no involvement of the spleen (confirmed histologically, see Table 1). In all five patients examined, the proportions of 'EAC' rosette and surface immunoglobulin bearing cells (Fig. 1) were consistently high in the spleen. These numbers were not only higher than the corresponding peripheral blood and control blood values, but in three cases (J.E., A.S. and M.O'K.) were also higher than the ones observed in the control spleens. The immunoglobulin classes involved were

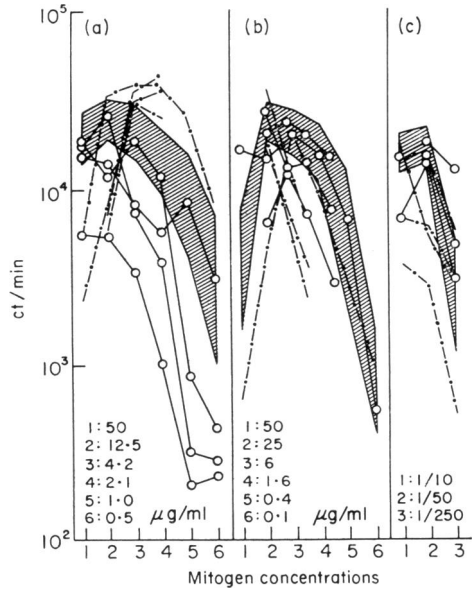

FIG. 2. Comparison between the response of peripheral blood (○——○) and spleen (●——●) lymphocytes to stimulation *in vitro* with decreasing concentrations of mitogens PHA, (a) Con A (b) PWM (c). The shaded area represents the control range (mean ± 1 s.d.) for the peripheral blood.

TABLE 3. Multiple compartment analysis of patient M.O'K.

Stage	Age	Sex	Compartment	E (%)	EAC (%)	Immunoglobulin-bearing cells						
						pv	μ	γ	α	κ	λ	δ
IIIB	8	F	Blood 1*	84·5	6	13	5	8	0	10	3	—
			Spleen	68	—	45	34	5	3	25	20	18
			Liver	53	—	—	—	—	—	—	—	—
			Blood†	65·5	9	19	19	2	2	12	8	—

* Pre-splenectomy.
† Post-splenectomy (6 days).

IgM in two cases, IgG and IgA in another case. In all three cases, the proportions of IgD bearing cells were higher than the control spleen range.

Peripheral blood and spleen cell response to non-specific mitogens in vitro (*Fig.* 2)

The *in vitro* response to stimulation with three non-specific mitogens, PHA, Con A and PWM, was studied. Of the three responses, the PHA was the one that differed most from the control dose–response curve, either by being lower than the control at all six mitogen concentrations tested, or at the two lower concentrations (see Fig. 5). The spleen cell response to PHA, however, was normal or slightly higher than the control blood range, and certainly higher than the range of the control spleen response (shown in Fig. 5). The response of the peripheral blood cells to Con A and PWM did not differ markedly from the one observed in the controls; the spleen-cell response to these two mitogens was lower than the corresponding blood-cell response.

Effect of splenectomy

In view of the differences observed between the number of circulating T cells in the blood and in the spleen of three patients, and of the discrepancy between the peripheral blood and the spleen cell PHA

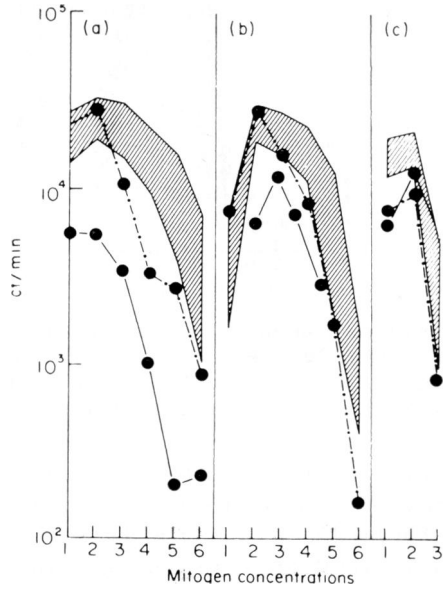

FIG. 3. Comparison between the mitogen response of the peripheral blood lymphocytes of patient J.E., diagnosed as stage IA, before (●——●) and 3 months after splenectomy (●- · - · -●). For details of mitogen concentrations, see Fig. 2. (a) PHA; (b) Con A; (c) PWM.

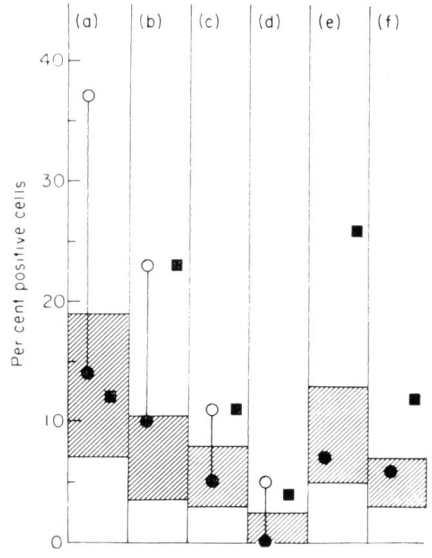

FIG. 4. Comparison between the percentage of B lymphocytes in the spleen (○) and peripheral blood before (●) and 3 months after splenectomy (■). Patient J.E., stage IA. (a) Polyvalent; (b) μ; (c) γ; (d) α; (e) κ; (f) λ.

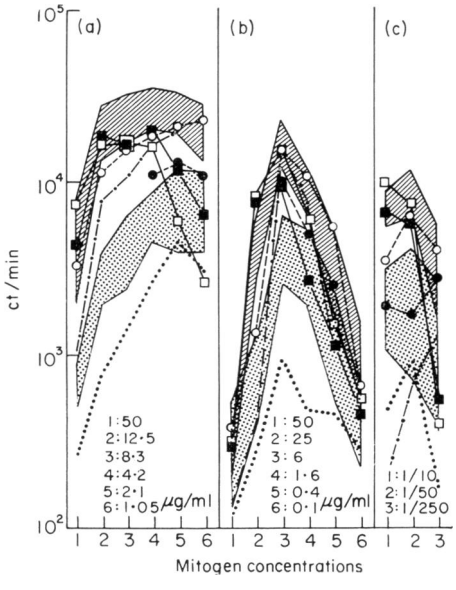

FIG. 5. Comparison between the mitogen responses of lymphocytes obtained from five different organs in patient M.O'K., stage III. Blood before (■——■) and 1 week after splenectomy (□——□). Spleen (○- - -○). Involved (·····) and uninvolved (· - · - · - ·) lymph nodes, and liver (●——●). The shaded areas represent the control range (mean ± 1 s.d.) for the peripheral blood (▨) and spleen (⊡).

response (Fig. 2) observed in all patients, we examined the effect of splenectomy in one patient who was diagnosed as stage IA and received radiotherapy (4000 rad) confined to a small area (irradiated field of 10 × 10 cm over the area involved by the disease, Table 1) of the right cervical region and was, therefore, the best case to analyse the effect of removal of the spleen on numbers of circulating cells. The peripheral blood lymphocyte count at presentation was 1072/mm^3; 3 months after splenectomy, it had increased to 2550/mm^3. The percentage of peripheral blood T cells increased from 23·5–43·5%, and the patterns of the responses to PHA and Con A (Fig. 3) were nearer the control range than the ones observed prior

to splenectomy. Nevertheless, the response at the low concentrations of PHA (Fig. 3) was still abnormal. The other interesting observation in this patient was the considerable increase found in the numbers of immunoglobulin-bearing cells in the perpheral blood after splenectomy (Fig. 4).

Multiple compartment analysis of one single patient at the time of splenectomy

One possible explanation for the results observed so far was that some abnormality of the spleen was inducing it to act as 'trap' of circulating lymphocytes. It was, therefore, necessary to analyse compartments other than the spleen and see if lymphocyte 'trapping' was occurring elsewhere. Accordingly, in patient M.O'K., we examined the blood, involved and uninvolved lymph nodes and liver (subsequently found not to be involved by histological examination, Table 1), in addition to the spleen, at the time of staging laparotomy (Fig. 5, Table 2). This patient had a normal proportion of circulating T (84·5%) and B (6%) cells in the blood. In spite of the normal number of circulating T lymphocytes, however, the dose–response curve to PHA showed the same abnormality observed in the other patients, i.e. a low response at the lower concentrations of the mitogen (Fig. 5). The peripheral blood lymphocyte responses to Con A and PWM were normal. The dose–response curves of the spleen cells were normal in comparison with the control blood curves but higher than the control spleen cell response.

The response of the uninvolved lymph node cells to PHA and Con A was very similar to that of the spleen cells; the PWM response, however, was considerably different from the spleen or peripheral blood cells, increasing with the decreasing concentration of the mitogen.

The response of the involved lymph node cells to all three mitogens was considerably lower than the one observed in all other compartments, including the liver. In the liver, 53% of the lymphoid cells separated in the Ficoll–Hypaque gradient were T cells (Table 2); the response of these cells to PHA and Con A was not significantly different from that of the control blood or spleen cells; the response to PWM was lower than the spleen cell response. No changes were detected in the peripheral blood lymphocyte responses to mitogens at one week after splenectomy.

DISCUSSION

We set out to investigate the possibility that the well documented blood lymphopenia observed in Hodgkin's disease is a reflection not of an absolute decrease in the numbers of circulating lymphocytes, but of lymphocyte sequestration in a solid organ compartment. Accordingly, we studied the proportions of T and B lymphocytes in the blood and spleen of five children diagnosed at different stages of the disease. We consider the finding of low T cell levels in the blood with concomitant high spleen values in three patients preliminary, and we wish to view it with caution. A more extensive study of immunological parameters in blood and tissue lymphocytes in children with Hodgkin's disease is presently in course (Tan *et al.* 1976, in preparation), and we feel entitled to the conclusion that depletion of lymphocytes from one compartment of the lymphoid system, i.e. the peripheral blood or some involved lymph nodes, does not necessarily reflect an absolute decrease of circulating cells.

It was of interest to find that the children with the most marked decrease in the percentage of peripheral blood T lymphocytes were the ones diagnosed at the early stages of the disease (IA and IIA), in whom no HD involvement of the spleen was observed histologically. The finding of high percentages of T cells in the spleens of these two children precludes the doubts and arguments always surrounding the finding of variable numbers of T cells in areas of spleen involved with the disease (Kaur *et al.*, 1974; Payne *et al.*, 1976). Recently, Payne *et al.* (1976), having a much more staisfactory group of control spleens than the ones available for the present study, have decisively demonstrated that T lymphocyte values in the spleens of adult patients are especially high (two standard deviations from mean control values) in uninvolved spleens. Payne *et al.* (1976), suggest from their results that 'elevated T lymphocyte values in uninvolved spleens is an early phenomenon occurring prior to recognized histological involvement'. In their study, however, no simultaneous values of peripheral blood lymphocytes were presented. In fact, though differences between the response of spleen and blood lymphocytes to nonspecific mitogens were first reported by Matchett, Huang & Kremer (1973) in a study of adult HD patients, the present

study is the first in children where surface markers, and response to non-specific mitogens were analysed simultaneously in different compartments of the same patients.

From the observation that in the early stages of Hodgkin's disease low percentages of T cells occur in the blood (Andersen, 1974; Cohnen *et al.*, 1974), simultaneously with high percentages in the spleen, one is tempted to draw an analogy with the recent findings of low levels of peripheral blood T and B lymphocytes in patients analysed at the early stages of mycocis fungoides (Mackie *et al.*, 1976; Nordqvist & Kinney, 1976) and discuss the possible pathogenesis of HD in the light of the same principle applied to the analysis of mycosis fungoides (Mackie, *et al.* 1976, in preparation). Thus, accumulation of T cells in the spleen in the early stages of HD may reflect either an abnormality of the spleen environment or an abnormality of the lymphocyte itself.

The possibility that a splenic abnormality plays a role in the pathogenesis of the disease is somewhat supported by the finding of unusually high amounts of normal ferritin (Eshhar, Order & Katz, 1974) in the spleen of patients with HD. Alternatively, this finding may be reflecting a simultaneous abnormality of red cell traffic. One further piece of evidence supporting 'a primary spleen abnormality' hypothesis is the marked improvement that followed splenectomy in our patient J.E. diagnosed at stage IA. Removal of the spleen resulted in a marked increase ($1072/mm^3$–$2550/mm^3$) in the abolute numbers of circulating T and B cells. This is perhaps not surprising in view of the known changes in the white blood cell count following splenectomy. More relevant to the pathogenesis of the disease was the finding that the *in vitro* response to PHA in this patient improved markedly after splenectomy, and this was related to clinical improvement.

The response to the lower concentrations of PHA, however, was persistently lower than the control response. This confirms a series of recent reports indicating that abnormalities of lymphocyte function are present at the early stages of the disease (Matchett *et al.*, 1973; Levy & Kaplan, 1974; Lang *et al.*, 1974), leading to the alternative hypothesis, which we favour, of 'a primary T lymphocyte defect' only detectable after stimulation with low antigen or mitogen concentrations (Eltringham & Kaplan, 1973; Levy & Kaplan, 1974; Matchett *et al.*, 1973). Whether such a defect is the cause or consequence of production of autoantibodies in later stages of the disease (Grifoni, 1975) remains to be established.

The point we wish to stress is that experimentally induced modifications of lymphocytes or red cells surface lead to the cells' sequestration in the 'wrong' environment (de Sousa, 1976a); when the wrong environment is the spleen, the organ where most important cell interactions occur in the development of immune responses and antibody formation, the consequences may be as varied as the papers written on the pathogenesis of the disease.

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