Br. J. Cancer (1982) **45**, 491

DISTRIBUTION OF T-LYMPHOCYTE SUBSETS IN HODGKIN'S DISEASE CHARACTERIZED BY MONOCLONAL ANTIBODIES

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Received 18 September 1981 Accepted 15 December 1981

Summary.—Mononuclear-cell suspensions of lymph nodes, spleen and blood from 24 patients with active Hodgkin's disease (HD) were studied for possible imbalance of T and B lymphocytes, and T-lymphocyte subsets, using monospecific anti-T antibodies and other reagents. A profile showing T-cell predominance was demonstrated in lymph nodes and blood, with total T-cells ranging from 50–70% of the cell count. As defined by monoclonal antibodies, 70–85% of the latter comprised the "inducer" subclass, the remainder being "suppressor" cells. There were no essential differences between histologically involved and uninvolved lymph nodes from HD patients, though total T-cell proportions were lower in "normal lymph node" controls. The profiles of spleens electively removed, as part of pre-treatment staging procedures, showed reduced total T-cell numbers, whether these were involved with HD or not. These differences are accounted for principally by fewer T "inducer" cells (24%, in spleen, v. 54% in involved lymph nodes and 47% in "normal" control nodes). Possible explanations for these findings are discussed. Our results demonstrate similar profiles in histologically diseased and normal tissue, rather than any clear imbalance of T-cell proportions which might explain the profound disturbances of T-cell function frequently demonstrated *in vivo* and *in vitro*.

THE NATURAL HISTORY of Hodgkin's disease (HD) is that of a chronic progressive illness which, untreated, is almost invariably fatal. It is manifested clinically and pathologically by features common to both a true malignancy and a chronic inflammatory process.

The association of HD with often profound disturbances of cell-mediated immunity, *in vivo* and *in vitro*, is well recognized (Reed, 1902; Parker *et al.*, 1932; Steiner, 1934; Schier *et al.*, 1956; Aisenberg, 1966; Levy & Kaplan, 1974) and has prompted considerable research to determine whether this is intrinsic to the pathogenesis of HD (Levy & Kaplan, 1974; Eltringham & Kaplan, 1973; Bobrove *et al.*, 1975; Jackson & Parker, 1947; Young *et al.*, 1973), or a secondary phenomenon related to stage of disease, and reversible after successful therapy (Jackson & Parker, 1947; Sokal & Primikirios, 1961; Sokal, 1973).

The recognition of 2 functionally distinct major T-cell subclasses, "inducer"/"helper" and "suppressor"/"cytotoxic" (Rowley *et al.*, 1973; Dutton, 1975; Webb & Jamieson, 1976; Moretta *et al.*, 1977; Evans *et al.*, 1978) has led to the recent development of monoclonal antibodies against T-cell antigens (Kung *et al.*, 1979; Hoffman *et al.*, 1980; Reinherz *et al.*, 1979*a*, *b*, 1980) which has thus provided a consistently reproducible means of rapidly and accurately quantifying these cells. We report below on our experiments using these reagents, in single-cell suspensions of tissues obtained

from HD patients, in order to identify possible T-cell imbalances.

MATERIALS AND METHODS

Patients

Material from 24 patients with active, untreated Hodgkin's disease forms the basis of this report. This comprised lymphocyte suspensions prepared from lymph nodes, spleen and peripheral blood. Histological classification was according to the Rye nomenclature (Lukes & Butler, 1966). Clinical and pathological staging was according to the Ann Arbor method (Carbone *et al.*, 1971). Details of the pre-treatment patients are given in Table I.

TABLE I.—*Pre-treatment patient details*

Males, 15; Females, 9; Total, 24
(mean age 30·5 yr)

Histological types		Clinical/pathological stages
Lymphocyte predominance	6	IA 6
Nodular sclerosis	12	IIA 12
Mixed cellularity	5	IIIA 1
		IIIB 1
Lymphocyte depletion	1	IVB 4

An additional 3 patients were submitted for elective laparotomy and splenectomy, after successful completion of combination chemotherapy, to exclude residual, active intra-abdominal HD, and to act as a guide for future management decisions. For comparison the spleens from these patients were studied using the reagents detailed below.

Controls

A group of 15 normal blood donors (mean age 31 years) provided controls for the patient group. A "normal" lymphoid-tissue control group (mean age 24·5 yr) was provided by 8 tonsils from patients undergoing elective tonsillectomy, and histologically reactive lymph nodes removed to exclude possible relapse, in 2 patients treated several years previously for HD.

In addition, 2 histologically normal spleens obtained at diagnostic laparotomy were studied; one was removed from a patient with Wegener's granuloma and renal failure, the other from a patient with suspected intra-abdominal lymphoma. This diagnosis was unequivocally refuted after histological examination.

Tissue preparation

Lymph node.—Surgical biopsy specimens were bisected for histological examination and phenotyping. A single-cell suspension of the latter portion was prepared by teasing out in RPMI 1640. The cells were washed twice in the same medium.

Tonsil.—Single-cell suspensions were prepared as for lymph node.

Peripheral blood.—Lymphocytes were obtained from 50–60 ml of blood separated by centrifugation on a Ficoll-Triosil (Lymphoprep^R) gradient. The cell layer at the interface was harvested and washed twice in RMPI 1640.

Spleen.—About 8 ml of tissue obtained from spleens removed at staging laparotomy were teased into single-cell suspensions. Mononuclear cells were separated on Lymphoprep^R and washed twice in PRMI 1640.

Reagents

Monoclonal antibodies reactive with T lymphocytes included the OKT series (Ortho Pharmaceuticals, Raritan, NJ, U.S.A.) and the Leu series (Becton Dickinson Antibody Center, Sunnyvale, CAL, U.S.A.). The T-cell-subset specificity of these reagents has been extensively documented (Kung *et al.*, 1979; Hoffman *et al.*, 1980; Reinherz *et al.*, 1979*a*, *b*, 1980. Becton Dickinson, 1981) (Table II).

TABLE II.—*T-cell specificity of monoclonal antibodies*

OKT1/Leu1	Pan T cell (mature peripheral T cells, some thymocytes and some B cells)
OKT3	Mature, peripheral T cells
OKT4/Leu-3A	T "inducer"/"helper" subset
OKT6	Cortical thymocyte
OKT8/Leu2A	T "suppressor"/"cytotoxic" subset

B lymphocytes were defined as cells bearing surface immunoglobulin, detected by staining with polyvalent rabbit anti-human Ig (G+M+A+D). Cells expressing HLA-DR were also counted, using antibody DA-2 (antimonomorphic-HLA-DR: Ia-like antigen) (Brodsky *et al.*, 1979). Light-chain restriction of B cells was assessed using fluorescein isothiocyanate (FITC)-labelled rabbit anti-human antibody against κ and λ light chains (Behringwerke).

Other monoclonal reagents included monomorphic anti-HLA-A,B,C:W6/32 (Sera Lab, Crawley Down, West Sussex) expressed on all leucocytes, which provided a positive control. Anti-platelet (Glycoprotein I) monoclonal antibody AN51 (McMichael *et al.*, 1981) provided a negative control.

Techniques

Viability of leucocyte suspensions.—This was assessed by Trypan-blue dye exclusion.

Immunofluorescence.—Quantities of medium (25 μl) containing 10^6 cells each were incubated with 5 μl aliquots of monoclonal antibody of known titre and antibody concentration for 30 min at 4°C. After washing twice in phosphate-buffered saline (PBS) the cell suspension was incubated with 5 μl of FITC-labelled goat $F(ab')_2$ anti-mouse reagents for 30 min at 4°C. For detection of cell surface Ig, 10^6 cells were incubated directly with 5–15 μl of FITC-labelled (deaggregated) rabbit anti-human heavy- or light-chain sera, for 30 min at 4°C. Cells were washed twice in PBS and examined under UV, using a Zeiss Photomicroscope III equipped for epifluorescence microscopy. Positive scores were assessed as live cells showing bright surface-membrane staining as clear rings, caps or distinct speckling. To exclude background or non-specific fluorescence, cells were also incubated with the FITC-labelled second-layer antibody alone.

E rosettes.—These were performed using washed sheep erythrocytes according to methods described by Habeshaw *et al.* (1979). Positive E rosettes were scored as mononuclear leucocytes with 3 or more adherent sheep erythrocytes.

RESULTS

T lymphocytes

The T-cell proportions found in blood and lymph nodes from patients and controls are detailed in Table III and IV. As defined by our results these tissues show a T-cell predominance in which E-rosetting (Er^+) and OKT/Leu$^+$ cells comprise 50–70% of all the mononuclear cells. The majority (70–85%) of T lymphocytes, thus defined, are comprised of cells expressing OKT4/Leu-3A (T "inducer") antigens. In the main, the proportions of cells expressing OKT1/Leu-1-reactive antigen are lower than either OKT3$^+$ cells or the sum of (OKT4/Leu-3A + OKT8/Leu-2A) but the differences are not significant. The percentages of OKT3$^+$ cells do, however, correlate well with the sum (OKT4/Leu-3A + OKT8/Leu-2A)$^+$ cells, *i.e.* T ("inducer" + "suppressor"). Differences in the relative proportions of Er^+ and pan-T antigen-expressing cells vary but are not significant. OKT6 antibody to thymic cortical cells was negative in all tissues.

The differences between the percentages of Er^+ cells in involved lymph nodes and "normal" controls are significant (Table III). There is also an apparently significant difference in the mean proportions of OKT3$^+$ cells between "normal" controls and diseased nodes but not in total T-cell proportions, defined by the sums of $(OKT4 + 8)^+$ and $(Leu\text{-}3A + 2A)^+$ cells.

The surface-antigen profiles of mononuclear cells from "disease control" lymph nodes, do not differ significantly from those in involved nodes. However, there is a significant difference between mean OKT8/Leu-2A (T "suppressor") values in the "disease control" and "normal control" groups (18% and 11% respectively; $P = 0{\cdot}01$).

No significant difference in results from patient and control blood has been demonstrated. The profiles are comparable to those from lymph nodes.

The data from spleen-cell suspensions however showed several differences (Table V): the mean totals of mononuclear cells expressing T-reactive antigens in spleens from patients with untreated HD, were lower than in either blood or lymph node ($P < 0{\cdot}001$). They are accounted for by smaller relative proportions of OKT4/Leu-3A$^+$ cells.

The proportions of Er^+ cells in pre-treatment spleens equate with those in lymph node and blood, and it follows that the difference between the Er^+ and OKT/Lev$^+$ population, in spleen, is also significant ($P < 0{\cdot}01$). In addition the

TABLE III.—*Cell surface phenotypes in HD (% of total viable mononuclear-cell count)*

Lymph nodes		E rosettes	OKT1/ Leu-1	OKT3	Mean* Pan T	OKT4/ Leu-3A	OKT8/ Leu-2A	T "helper"+ "suppressor"	Ia	SmIg	κ	λ
Involved	No. obs.	11	6	5	11	15	16	15	7	8	5	5
	Mean	60	65	73	69	54	14	68	21	21	13	7
	s.e.	3	7	3	4	5	2	5	4	4	2	2
	Range	42–76	29–85	68–87		23–78	2–39		5–44		8–20	2–12
Uninvolved, from patients with active HD	No. obs.	15	8	1	9	16	11	10	5	11	6	5
	Mean	63	55	78	60	52	18	69	17	20	15	11
	s.e.	1·5	3		3·5	4	2	5	3	2	5	3
	Range	40–79	13–74			24–79	8–28		9–24	4–40	4–36	5–21
		$P=0·001$	$0·01>P>0·001$	$0·02>P>0·01$		$0·02>P>0·01$	$0·01>P>0·001$					
"Normal" controls (2 reactive nodes, 8 tonsils)	No. obs.	8	8	9	17	16	13	13	10	9	9	9
	Mean	39	55	51	53	47	11	58	23	16	11	4
	s.e.	6	6	4·5	4·5	3	1	2	2	3	2	1
	Range	20–65	26–78	27–71		15–63	5–20		9–35	8–28	2–20	1–9

* Mean value total OKT1, Leu-1, OKT3.
Statistical analysis by *t* test.

TABLE IV.—*Cell surface phenotypes in HD (% of total viable mononuclear-cell count)*

Blood		E rosettes	OKT1/ Leu-1	OKT3	Mean Pan T	OKT4/ Leu-3A	OKT8/ Leu-2A	T "helper"+ "suppressor"	Ia	SmIg	κ	λ
Patients with HD	No. obs.	9	8	5	13	15	12	12	6	5	—	—
	Mean	57	51	58	54	41	16	59	12	18		
	s.e.	4	2	1·5	2	3	1·5	3·5	3	5		
	Range	43–72	36–61	53–63		26–74	7–33		4–21	4–35		
Normal controls	No. obs.	14	12	13	25	21	18	18	11	10	—	—
	Mean	53	51	62	57	43	21	63	17	14		
	s.e.	3	4	4	3	2	2	3	1	2		
	Range	33–82	26–76	41–77		28–58	9–33		13–22	6–33		

No significant differences between patient and control blood. The dashes indicate 'not measured'.

TABLE V.—*Cell surface phenotypes in HD (% of total viable mononuclear-cell count)*

Spleens		E Rosettes	OKT1/ Leu-1	OKT3	Mean Pan T	OKT4/ Leu-3A	OKT8/ Leu-2A	T "helper" + "suppressor"	Ia	SmIg	κ	λ
All pre-treatment	No. obs.	9	11	5	16	19	14	14	5	12	7	7
	Mean	54	29	43	33	24	18	43	26	18	15	6
	s.e.	4	4	6	4	2	2	3	4	2	3	1·5
	Range	37–78	8–49	23–65		11–40	8–29		19–42	7–37	8–28	2–14
All pre-treatment uninvolved	No. obs.	7	8	4	12	15	11	11	4	10	6	6
	Mean	49	26	48	33	25	18	42	28	18	13	5
	s.e.	3·5	5	5	5	2	2	3	4	2	2	2
	Range	37–64	8–49	36–65		16–40	8–29		22–42	7–32	8–22	2–14
Pre-treatment involved	No. obs.	2	2	1	3	3	2	2	1	1	—	—
	Mean	61	31	23	28	16	22	38	16	20	—	—
Post-treatment	No. obs.	3	3	3	6	6	6	6	3	1	—	—
	Mean	39	31	25	28	13	18	31	36	19	—	—
	s.e.				2	1	1	1				
	Range					11–18	17–21					
"Normal" control	No. obs.	2	1	2	3	3	3	3	2	—	—	—
	Mean	61	37	49	45	35	27	62	13	—	—	—

Comparing Table V with III–VI, the differences in total T cell proportions, defined by monoclonal reagents, between pre-treatment spleens and other tissues, judged by t test, are all significant at $P < 0{\cdot}001$, except in "normal" control lymph nodes, where the differences are less marked. Mean proportions of E-rosetting (Er^+) cells are the same as those found in other tissues. The difference between Er^+ cells and total OKT/Leu^+ cells defined in spleen, is significant at $P < 0{\cdot}01$. The smaller number of OKT/Leu^+ cells is accounted for by lower proportions of T inducer cells and compared to other tissues, the differences are all highly significant ($P \ll 0{\cdot}001$). In addition, the T inducer: suppressor ratio which is 3–4:1 in other tissues, approaches 1:1 in spleen.

TABLE VI.—*Cell surface phenotypes in HD (% of total viable mononuclear-cell count)*

Involved lymph nodes, by histological type		E Rosettes	Pan T	OKT4/ Leu-3A	OKT8/ Leu-2A	T "helper"+ "suppressor"	Ia	SmIg
Lymphocyte predominance	No. obs.	4	3	5	5	5	2	3
	Mean	59	67	55	10	65	15	21
	s.e.	4		8	2	8	—	—
Nodular sclerosis	No. obs.	5	5	5	6	5	2	4
	Mean	61	69	55	21	76	16	23
	s.e.	4	2	8	3·5	7	—	5
Mixed cellularity	No. obs.	1	2	3	3	3	2	1
	Mean	62	78	59	8	67	20	12
Lymphocyte depletion	1 node	—	29	15	7	22	44	—

T "inducer":"suppressor" ratio defined by OKT4/Leu-3A$^+$:OKT8/Leu-2A$^+$ cells approximates to 1:1, compared with 3–4:1, in blood and lymph node. Our data reveal no phenotypic differences between diseased and normal spleen. Phenotype profiles of 3 spleens obtained at laparotomy, after completion of chemotherapy, show decreased proportions of T cells, as defined by Er$^+$, OKT1, OKT3, Leu-1 and OKT4/Leu-3A$^+$ cells.

Data from lymph nodes were analysed according to the histological type of HD (Table VI). In lymph nodes involved with HD of lymphocyte-predominant type, there is an apparently greater predominance of T "inducer" (OKT4/Leu-3A) to T "suppressor" (OKT8/Leu-2A) lymphocytes (ratio 5:1). One node involved with HD of lymphocyte-depleted type shows an overall reduction in T lymphocytes, but with retention of T "helper" predominance. The numbers in each group are too small to allow statistical comparison.

B lymphocytes

The estimated proportions of cells defined either as surface-immunoglobulin-bearing cells (SIg$^+$) or HLA-DR (Ia$^+$) antigen-expressing cells, ranged from 12 to nearly 30%. Although there were some differences in the numbers counted by the two methods, these were not significant. When assessed by light-chain antisera, the B cells show a polyclonal profile.

Controls

All cells were strongly positive with W6/32 antibody. No positive reactions were seen with AN51, except with occasional platelet fragments which appeared as tiny, brilliantly fluorescing particles. Background fluorescence was almost always absent, apart from occasional faint, diffuse intracytoplasmic fluorescence.

DISCUSSION

HD is characterized by the presence, in affected tissue, of high levels of "reactive" T lymphocytes. This could indicate either a marked antigenic stimulation by the neoplastic cell component of the lesion, or a persistent non-specific immune reaction. The frequently observed rise in serum immunoglobulins and circulating immune complexes (Madalinski *et al.*, 1970; Wagener *et al.*, 1976; Amlot *et al.*, 1978; Brown *et al.*, 1978) lends support to theories of general over-reactivity to chronic antigenic stimulation. On the other hand, a T inducer:suppressor imbalance in which the population of T cells consisted entirely or largely of inducer/helper cells, might lead to unbalanced and persistent inappropriate stimulation of B lymphocytes. In view of the abnormal cellular immunity so frequently observed, such lymphocytes might well be defective, either in stimulating or responding to antigenic challenge. Whilst our data demonstrate a T-cell-

predominant (Er^+, $OKT1^+$, $OKT3^+$, $LEU\text{-}1^+$) cell population in diseased lymph nodes and peripheral blood from the patient population, the results are essentially identical to those obtained with the respective control tissues: uninvolved lymph nodes from the patients and normal donor blood.

Our results would appear to be at variance with an earlier study (Romagnani *et al.*, 1978) which showed an imbalance of subsets of peripheral-blood T-cells subsets in HD patients. Such cells identified as Er^+ cells expressing receptors for either IgM($T\mu$) or IgG ($T\gamma$), have been described by others (Moretta *et al.*, 1977) as subserving "helper"/"inducer" and "suppressor"/"cytotoxic" functions respectively. Romagnani was able to demonstrate a decrease in $T\mu$ and an increase in $T\gamma$ subsets though $T\mu$ lymphocytes remained overall predominant. Gupta & Tan (1980) demonstrated a small but non-significant increase in $T\gamma$ lymphocytes in the blood of children with untreated HD. These reports would thus suggest a relative increase in the "suppressor"/"cytotoxic" T-cell population in the blood of HD patients. However, the methods for assaying these subpopulations involve reactions with quite different antigenic determinants from those recognized by the monoclonal antibodies, and it cannot be assumed that identical subsets are identified by the two methods.

Neither tonsils nor reactive lymph nodes from patients "cured" of HD might be regarded as appropriate control material for normal lymph nodes, and this reflects the difficulty in obtaining non-diseased lymph nodes, particularly from an age-matched group. Nonetheless, our data do show similar phenotype profiles, particularly in respect of the T "helper":"suppressor" ratios, though the overall degree of T-cell predominance is marginally less than that in lymph-node material from the patient population. The findings accord with previous reports of cell suspensions and frozen sections of normal tissues (Reinherz & Schlossman, 1980; Janossy *et al.*, 1980; Poppema *et al.*, 1981).

Because of the limited selection of histological types available for study, no conclusions can be drawn about possible T-cell differences in the 4 major histological types, though there is a suggestion of a higher proportion of T "inducer" lymphocytes in nodes involved with lymphocyte-predominant HD than in other subclasses. One lymph node from a patient aged 14, with mixed-cellularity HD, showed a T-helper population > 90% of all cells, a marked predominance of this T subset. Since most of our patients presented with limited disease, it has not been possible to relate our findings to stage, or subsequent clinical progress.

Splenic T-cell populations may differ from those of lymph node or peripheral blood. A substantial proportion of Er^+ cells fails to express either pan-T antigens (OKT1, OKT3, Leu-1) or "helper"/"inducer" antigens (OKT4, Leu-3A). This would imply the presence of an additional subset of T-cells, being either an immature population at an intermediate stage of differentiation, or a subset evolved in response to, or intrinsic to the disease process. Alternatively, it is conceivable that such a population could be resident in normal adult spleen. Gupta & Tan (1980) have demonstrated increased proportions of T-cells bearing IgM receptors in the spleens of their patients, who were, however, all children, mainly with advanced disease or splenic involvement.

The theory of "ecotaxopathy" proposed by De Sousa *et al.* (1976) implies a failure in HD of normal T-cell migration and recirculation (ecotaxis) with a preferential accumulation of these cells within the spleen. De Sousa's study of peripheral blood and spleen from 5 patients suggests that this abnormality is intrinsic to HD, and independent of stage or splenic involvement. Hunter *et al.* (1977) also demonstrated an overall increase of Er^+ cells in the spleens of patients, but most had advanced disease and heavy splenic infiltration, the highest

Er values being obtained from the involved spleens. In contrast, Payne *et al.* (1976) found the highest Er levels in uninvolved spleens, those in diseased spleens being within normal limits. Kaur *et al.* (1974) found normal levels of Er^+ cells in the spleens of HD patients, though responsiveness to PHA was increased.

We have not demonstrated an overall increase in Er^+ cells in the spleens of our patients although as discussed above these may well be distinct from those in peripheral nodes and blood. Only 1 patient, a 16-year-old female with nodular sclerosing HD, had heavy splenic involvement. Intriguingly, in this case the proportion of Er^+ cells was 78%, whilst 37% of the cells reacted with OKT1.

We conclude that our results, obtained from single-cell suspensions of lymph node, tonsil and peripheral blood, show phenotypic similarities in these tissues rather than any clear abnormalities. Compared with involved and uninvolved lymph nodes, there is a slightly lower T-cell proportion in the "normal" lymphoid control group. The proportional distribution of cells expressing "helper" and "suppressor" antigens is, however, very similar. The proportions of B cells defined by our methods do not vary significantly between tissues, and account for $\sim 20\%$ of leucocytes. A normal polyclonal profile is observed, findings similar to those of Bobrove *et al.* (1975).

The significance of the findings in the spleens of patients must remain speculative until appropriate numbers of normal controls for comparison are available.

Despite *in vivo* and *in vitro* demonstration of defective T-cell function in HD, this does not appear to result from deficiencies in the proportions of the T or B cells or T-subset ratios in diseased tissues. This suggests that some intrinsic functional immune deficiency is a significant feature in the pathogenesis of HD. Further work will attempt to clarify the micro-anatomical relationships of T and B subsets within the HD lesion, and to study some of the functional attributes of T cells from diseased tissues, in the hope of elucidating the nature of Hodgkin's disease.

We are grateful to Dr M. F. Greaves for his invaluable help, both in providing reagents and for advising on the design of this study. We also thank Professor J. S. Malpas for allowing us to study his patients and Dr A. G. Stansfeld of the Department of Histopathology who reviewed all the histological material. Our thanks also go to Jill Davey for typing the manuscript.

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