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T and B lymphocytes and Reed–Sternberg cells in Hodgkin's disease lymph nodes and spleens

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SUMMARY

Lymphoid cells from twenty-four untreated Hodgkin's disease biopsies were examined for spontaneous sheep erythrocyte and sensitized ox erythrocyte rosette formation for the identification of T cell and cells with Fc and C3 receptors and surface immunoglobulin. Compared with normal tissues mean T-lymphocytes values were elevated in both involved lymph nodes and uninvolved spleens from Hodgkin's patients. Lymphocytes bearing C3 receptors were correspondingly reduced in these tissues. Involved spleen T-cell values fell within the normal range. In normal tissues the sum of lymphocytes with surface immunoglobulin and sheep erythrocyte receptors fell in the range 89–108%. In six biopsies of Hodgkin's tissue the sum was outside the normal range (121–142%). This observation is compatible with surface immunoglobulin-coated T cells. Surface marker characteristics and intracellular immunoglobulin studies of small lymphocytes, lymphoblasts and Hodgkin's cells suggested that the neoplastic cells were of B lymphocyte origin.

INTRODUCTION

It has been established that a defect in cellular immunity exists in Hodgkin's disease (HD) measured as cutaneous anergy, either to natural antigens (Lamb *et al.*, 1962), or to chemical allergens (Aisenberg, 1962). The further demonstration by *in vivo* and *in vitro* techniques that this defect may exist in early localized disease (Levy & Kaplan, 1974) is thought to support the concept that a deranged immunological reaction is implicated in the aetiology of the disease, as postulated in the concept of 'lymphocyte war' (Kaplan & Smithers, 1959; Order & Hellman, 1972).

More recently it has become recognized that lymphocyte populations may be sub-divided according to their origin, either as thymus-derived lymphocytes (T lymphocytes), identified by their capacity to bind unsensitized sheep red blood cells (RBC) (Jondal, Holm & Wigzell, 1972), or as bone marrow-derived lymphocytes (B lymphocytes) which possess surface immunoglobulin (Ig) determinants (Pernis *et al.*, 1971; Hallberg *et al.*, 1973) and receptors for bound complement, C3 (Bianco, Patrick & Nussenweig, 1970). These surface marker techniques have been used to identify lymphocyte subpopulations in HD, though, to date, the majority of workers have concentrated on peripheral blood lymphocytes in this disease (Andersen, 1974; Frøland & Natvig, 1973; Cohnen, Douglas & Brittinger, 1974; Grifoni *et al.*, 1975; Gergely *et al.*, 1973).

We wish to report our investigations using techniques to detect surface markers and intracellular immunoglobulin, of lymph node and spleen cell populations from untreated HD patients and from controls.

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MATERIALS AND METHODS

Lymph node and spleen. Tissue was obtained from six female and eighteen male patients with untreated HD either at the time of diagnostic lymph node biopsy or at staging laparotomy. The patients ranged in age from 17 to 88 years. Uninvolved lymph nodes from patients with carcinoma or nodes showing non-specific reactive hyperplasia were used as controls. Control spleens were obtained from patients undergoing splenectomy following traumatic rupture or incidental to abdominal surgery.

Cell suspensions. Biopsy tissue was finely minced and teased in cold HEPES buffered Eagles' minimal essential medium (HEPES–MEM, Biocult Laboratories, Paisley, Scotland), filtered through gauze and layered over Ficoll–Triosil (Thorsby & Bratlie, 1970). Cells collected at the interface were washed three times by centrifugation (150 *g*, 10 min) and the final pellet was resuspended at a density of 2×10^6 cells/ml in HEPES–MEM with 0·2% bovine serum albumin (BSA), for analysis. The cell suspensions contained greater than 90% lymphoid cells.

Rosette tests. (1) *Indicator cells.* Full details of cell preparations for the sheep rosette test, Fc rosette test, C3 rosette test and mixed antiglobulin (MAG) reaction are described in previous publications (Hallberg *et al.*, 1973; Smith & Haegert, 1974). In the C3 rosette test both human R_3 reagent (zymosan-treated whole serum) and (BALB/c) mouse serum were used as a source of complement.

(2) *Rosette formation.* Except for minor modifications this has been described fully elsewhere (Smith & Haegert, 1974). The indicator red cells and test lymphocyte suspensions in HEPES–MEM with BSA were mixed in plastic tubes (50 × 4 mm). Two per cent heat-inactivated calf serum was added to this medium in the sheep red cell rosetting test and the tube was incubated for 5 min at 37°C, centrifuged at room temperature, and the pellet was kept on ice for 1 hr before scoring. In the other rosetting reactions the lymphocyte–indicator red cell mixtures were centrifuged immediately at 200 *g* for 3 min at room temperature and kept on ice for at least 30 min before scoring.

The tests were scored after cytocentrifuge preparation as previously described (Collins *et al.*, 1974; Smith & Haegert, 1974). The slide preparations were stained with Leishman's stain and scored. In these preparations cells forming rosettes with multiple erythrocytes were easily identified. Cells with nearby individual erythrocytes were not scored as positive unless four or more of these were clearly attached to the cytoplasmic membrane. Aggregates of more than four cells were not scored. In each test the number of lymphocytes rosetting was expressed as a percentage of the total lymphoid cells present. In the MAG test it was not always possible to exclude monocytes from the lymphoid populations; polymorphs could easily be excluded.

Immunofluorescence staining. (1) *Cell suspensions* (*membrane immunofluorescence*). Polyvalent rabbit antisera to human immunoglobulins and rabbit or sheep antisera to human G, A, M, D heavy chains and to κ and λ light chains were given by Professor G. T. Stevenson of the Tenovus Research Laboratory, Southampton. Test cells were stained directly with these antisera conjugated to fluorescein. In some tests the cells were stained by the indirect method, using unconjugated rabbit polyvalent antisera and fluorescein-conjugated goat anti-rabbit immunoglobulin. Controls of normal rabbit and/or normal sheep sera, conjugated with fluorescein, were included in all experiments.

(2) *Slides* (*cytoplasmic immunofluorescence*). Cell smears were fixed overnight in dry acetone at −20°C and stained by the direct method with fluorescein-conjugated antisera, described above. Controls of fluorescein-conjugated normal rabbit and/or sheep sera were included in all experiments.

Both fluorescein-labelled cell suspensions and fluorescein-labelled smear preparations were examined using a Leitz Orthoplan microscope fitted with a HB 200 mercury vapour Ploem illuminator.

RESULTS

T- and B-lymphocyte populations in control lymph nodes and spleens

T- and B-lymphocyte ratios were examined in fresh preparations from six normal spleens and twenty-two control lymph nodes. Mean values and standard deviations are given in Table 1. In control tests cells did not rosette with unsensitized ox cells. T- and B-lymphocyte percentages in normal peripheral blood, assessed after Ficoll–Triosil separation and cotton wool filtration (Collins *et al.*, 1974), have been included for comparison. In normal reactive spleens and lymph nodes from patients with no carcinoma, the total percentage of lymphocytes rosetting with sheep cells and reacting in the MAG test fell within the range 89–108%. The total cells detected by these techniques in uninvolved spleens and lymph nodes from carcinoma patients also fell within this range except on two occasions. In two tests with suspensions from uninvolved nodes from patients with breast cancer the total percentage of lymphocytes detected was 112% and 116%.

T- and B-lymphocyte populations in biopsy specimens from Hodgkin's patients

In histologically involved Hodgkin's splenic tissue the percentage of T lymphocytes identified by sheep RBC rosetting did not differ significantly from normal values (Table 2).

TABLE 1. T and B markers on lymphocytes from normal peripheral blood, lymph nodes and spleens

Tissue	No. studied	T lymphocytes*	B lymphocytes*				
		Sheep RBC rosettes	Fc rosettes	C3 rosettes		MAG rosettes	FITC† surface Ig
				Mouse C	Human R3		
Peripheral blood	18	58±10	26±10	15±5	15±5	32±10	24±10‡
Lymph nodes	22	53±12	9±7	43±12	32±14	47±11	25±13
Spleens	6	51±9	38±14	38±22	38±12	49±11	34±15

* Mean values and standard deviations.
† Direct fluorescence unless marked ‡ which denotes indirect.

In involved lymph nodes and uninvolved spleens, however, the proportion of T lymphocytes present was elevated by comparison with those observed in the corresponding control tissues. Further, in seven out of sixteen HD lymph nodes and two out of four histologically uninvolved spleens taken at laparotomy, the T-lymphocyte value exceeded two standard deviations of the mean control value. No significant correlation was observed between increased T-cell values and either clinical stage or histological type of the disease.

In involved lymph nodes, the mean proportions of C3 receptor-bearing lymphocytes and immunoglobulin-bearing lymphocytes measured by immunofluorescence and the MAG test, were reduced. In involved lymph nodes the proportion of C3 receptor-bearing lymphocytes was significantly reduced in situations corresponding to T-cell elevation (Table 2: cases DEA, QUI, MOU, CHI, BAR, COR). In six biopsies the total percentage of cells detected by sheep RBC rosetting and the MAG test exceeded the range for normal tissues (89–108%). In four of these biopsies (LEM LN and Spl; DEA LN and RUS Spl) this overlap exceeded 30% (Table 2). In these overlap cases the percentage of non-lymphoid cells in the preparations was less than 8%.

Surface marker analysis of normal and neoplastic cells in HD biopsies

The cell populations including small lymphocytes, blast cells, mononuclear HD cells and Reed–Sternberg (RS) cells in five biopsies of HD tissue were critically evaluated for sheep RBC and Ox Fc rosetting (Table 3). In all biopsy specimens RS cells were accompanied by a proliferation of large mononuclear cells broadly divisible into either normal lymphoblast cells or abnormal cells interpretable as mononuclear Hodgkin's cells (Dorfman *et al.*, 1973).

All Reed–Sternberg cells observed and the large majority of mononuclear HD cells did not rosette with sheep RBC (Table 3). Some of the mononuclear cells did rosette in the Fc test. A small minority of Reed–Sternberg cells formed weak attachment with a small number of sensitized ox cells in the Fc test. The majority of normal blast cells (79%) observed in cytocentrifuge preparations were T cells, as identified by spontaneous sheep RBC rosetting. A smaller percentage (15%) were identified by rosetting with Ox Fc indicator erythrocytes.

Intracellular immunoglobulin

Direct immunofluorescence was performed on acetone-fixed smears of cell preparations from lymph node biopsies of three patients with HD (LEM, PLU and MOU). In two biopsies (LEM and MOU) mononuclear HD cells and RS cells, identified by phase contrast, exhibited cytoplasmic fluorescence with κ light chain and γ heavy chain antisera. In LEM the large majority of cells examined (30/35) exhibited intracellular Ig. In MOU a minority of cells (9/20) exhibited fluorescence. No staining was observed with other antisera. In a third case (PLU) mononuclear HD cells (5/11) stained with κ light chain antisera, insufficient material was available for determinations of heavy chain class. In this case RS cells did not stain with light chain antisera. The intensity of fluorescence in HD cells was less than that in the accompanying plasma cells which were polyclonal with respect to their immunoglobulin content though with a predominance of IgG κ.

TABLE 2. T and B markers on lymphocytes from Hodgkin's disease lymph nodes and spleens

			T lymphocytes	B lymphocytes				Sheep RBC rosettes and
Patient	Tissue	Histology	Sheep RBC rosettes	Fc rosettes	C3* rosettes*	MAG rosettes	FITC S. Ig†	MAG rosettes
SHE	Lymph node	L.P.	62					
ANG		L.P.	40					
DEA		L.P.	84	8	4**	55	32	139
QUI		L.P.	86	2	9**	18	3	104
MOU		N.S.	80					
WIL		N.S.	65				17	
PLU		N.S.	52				25‡	
LEM		N.S.	85	2	13**	57	12	142
MOU		N.S.	97	2	3	10	6	107
CHI		N.S.	70	8	4	19	2	89
BAR		N.S.	67	11	8	29	21	96
COR		N.S.	73	6	6	22	10	95
MDO		N.S.	82	23	24	40	32	122
EAR		M.C.	70				16‡	
PRI		M.C.	88				12‡	
HAW		M.C.	68	37	34		18	
Mean ± s.d.			73 ± 15	11 ± 12	12 ± 11	31 ± 18	16 ± 10	
MOU	Involved spleen	N.S.	45					
LOV		N.S.	49	55				
PLU		N.S.	60	19				
THO		N.S.	42	31	35	79	45	121
JON		M.C.	47					
BER		M.C.	31	18				
EAR		M.C.	60				30‡	
HAW		M.C.	42	37	58		23	
BEA		M.C.	57	35	45	27	18	84
RUS		M.C.	57	54	56	77	50	134
Mean ± s.d.			49 ± 9	36 ± 15	49 ± 11	61 ± 29	33 ± 14	
HUS	Uninvolved spleen	L.P.	66					
BEW		N.S.	53	43	53**			
FIS		N.S.	74	35	33	34	11	108
LEM		N.S.	73	39	31**	67	37	140
Mean ± s.d.			67 ± 10	39 ± 4	39 ± 12	52	28 ± 14	

L.P., lymphocyte predominant; N.S., nodular sclerosing; M.C. mixed cellularity.

* Mouse complement used unless marked ** indicating a human R_3 reagent was used.

† Direct fluorescence unless marked ‡ indicating indirect.

DISCUSSION

Normal lymph nodes, spleens and peripheral blood lymphocyte suspensions have similar proportions of T cells as measured by sheep RBC rosetting (Table 1). In peripheral blood more Fc than C3 receptor cells were observed in contrast to lymph node populations where the situations were reversed. In spleens the Fc and C3 receptor cell proportions were similar. The finding that the proportions of C3 and Fc receptor cells vary between the tissues investigated probably reflects the variation in B-cell maturation to be found in these lymphoid tissues. There were no significant differences between BALB/c mouse serum and human R3 reagent as a complement source for the C3 test system in investigations of normal

lymphoid cells. The proportion of lymphocytes with surface Ig was consistently greater when detected by the MAG test than by direct fluorescein-labelled antibody staining confirming the increased sensitivity claimed for this test (Hallberg *et al.*, 1973).

Involved lymph nodes and uninvolved spleens from HD patients contained a significantly higher percentage of T lymphocytes than control tissues. This finding confirms the earlier reports of Braylan, Jaffe & Berard (1974) and Kaur *et al.* (1974). Unlike Kaur *et al.* (1974), we have not observed an increase in the percentage of spontaneous sheep RBC rosetting lymphocytes in histologically involved HD spleens. Kaur's values for sheep RBC rosetting lymphocytes in HD spleens fall within our normal range. While we cannot eliminate the possibility of sample error in our studies, we suggest that elevated T-lymphocyte values in uninvolved spleens is an early phenomenon occurring prior to recognizable histological involvement. This elevated T-cell population does not appear to persist in involved spleens. No correlation was observed between T-lymphocyte elevation and either clinical stage or histological type of HD.

TABLE 3. T and B markers on lymphocytes and Hodgkin's cells in five cases

No. of cases	Rosetting cells	Lymphocytes				Hodgkin's cells			
		Small lymphocytes		Lymphoblasts		Mononuclear Hodgkin's cells		Reed–Sternberg cells	
		Sheep RBC rosettes	Fc rosettes	Sheep RBC rosettes	Fc rosettes	Sheep RBC rosettes	Fc rosettes	Sheep RBC rosettes	Fc rosettes
4	No. positive	504	94	166	22	3	10	0	3
Lymph nodes	Total	800	600	211	146	72	47	24	10
	Percentage positive	63	16	79	15				
1	No. positive	120	32	67	20	0	1	0	0
Spleen	Total	200	200	100	104	13	15	5	4

The central involvement of immunocytes in the aetiology of HD (Smithers, 1967; Order & Hellman, 1972) is suggested by qualitative changes in the *in vitro* mitogen transformation studies of peripheral blood lymphocytes in early stage HD patients and the relative or absolute reductions in T lymphocytes in the peripheral blood of HD patients (Levy & Kaplan, 1974; Lang *et al.*, 1974; Gergely *et al.*, 1973; Andersen, 1974; Cohnen *et al.*, 1974). Our results suggesting the commitment of large number of T lymphocytes within early HD lesions are in full agreement with such concepts of 'lymphocyte war'.

In normal lymphocyte suspensions the total lymphocyte population detected by the MAG test and by spontaneous sheep RBC fell within the range 89–108%. Small error (3–4%) can occur in scoring cytocentrifuge rosette tests (Collins *et al.*, 1974) and totals exceeding 100%, but within the normal range, probably reflect this error. The MAG test is best applied to pure lymphoid suspensions to avoid problems with cell identification; polymorphs can be easily excluded but small monocytes may often be included. In this study contaminating non-lymphoid cells were less than 10% and in those cases where there was significant overlap the non-lymphoid cells did not exceed 8%. It seemed unlikely therefore that contaminating cells contributed to this significant overlap. From all the tests with control and non-HD lymphoid preparations a significant overlap was seen in only two lymph node preparations, both biopsies from patients with breast cancer; whether these represent 'normal' controls is questionable and warrants further study. Significant overlap occurred in six biopsies of HD tissue (LEM LN and Spl; DEA LN; RUS Spl; MDO LN and THO Spl; Table 2). In one case (DEA LN) where there was significant

overlap, the overlap was also observed after fluorescent antibody staining of the lymphocyte surface Ig. This overlap is consistent with immunoglobulin coating of lymphocytes in HD tissue. Lymphocytotoxin activity has previously been postulated in HD following the demonstration of IgG coating of peripheral blood lymphocytes (Grifoni *et al.*, 1975; Chin *et al.*, 1973).

In recent years there has been considerable interest in the nature of the pathognomic RS cell in HD. Although disputed by Dorfman (1964) it has been suggested to have a histocytic origin. A derivation from immunoblasts has been suggested by Tindle, Parker & Lukes (1972) and more specifically from T cells (Thomson, 1955; Order & Hellman, 1972). If this is the case, RS cells might be expected to rosette with sheep RBC as do other neoplastic cells of the T-lymphocyte series (Seligmann, Preud'Homme & Brouet, 1973; Catovsky *et al.*, 1973; Smith *et al.*, 1973).

In five biopsies (Table 3), however, where mononuclear HD cells were identified, minimal rosetting with sheep RBC was observed (3/185 cells) and negligible sheep RBC rosetting was seen with classical RS cells in cytocentrifuge preparations. A small number of neoplastic cells were observed to rosette weakly with Ox Fc indicator erythrocytes. By contrast, the large majority of lymphoblasts recognized in both spleen and lymph node preparations in HD did form spontaneous sheep RBC rosettes suggesting that these cells represent the reactive population. Direct immunofluorescence performed on acetone fixed smears of washed cell preparations from three biopsy specimens revealed monotypic intracellular immunoglobulin (IgG, κ) within some but not all RS cells and their mononuclear counterparts. This observation confirms previous reports (Denton, 1973; Taylor & Burns, 1974; Garvin *et al.*, 1974) and together with the surface receptor pattern of HD cells reported in this study and the surface immunofluorescence studies of Leech (1973) and Kadin *et al.* (1974) is evidence for the origin of neoplastic Hodgkin's cells from the B-lymphocyte series.

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