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INCREASED PROLIFERATION OF T LYMPHOCYTES IN THE BLOOD OF PATIENTS WITH HODGKIN'S DISEASE

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SUMMARY

We investigated the number of DNA-synthesizing T lymphocytes in the blood of patients with Hodgkin's disease, with infectious mononucleosis and in normal controls. T cells were characterized by their ability to form rosettes with unsensitized neuramidase-treated sheep red blood cells. Cells in DNA synthesis were evaluated autoradiographically after *in vitro* incubation with [^{3}H]thymidine. Our results indicated a preferential proliferation of T lymphocytes in the blood of patients with Hodgkin's disease and infectious mononucleosis and suggested an increased turnover of these cells.

INTRODUCTION

Recent studies suggest an increased renewal rate of blood lymphocytes in patients with Hodgkin's disease (Crowther, Fairley & Sewell, 1969a; Huber *et al.*, 1970; Schick *et al.*, 1973). This conclusion was based on the evaluation of DNA-synthesizing lymphocytes in the blood and was further substantiated by the finding of an increased lymphocyte turnover after [^{3}H]thymidine labelling *in vivo*.

The number of proliferating blood lymphocytes in Hodgkin's disease was proportional to the production rate of these cells (Schick *et al.*, 1973), but further characterization of these cells has not yet been achieved. For a classification of lymphoproliferative states, surface markers specific for T or B lymphocytes have been widely applied (Bianco, Patrick & Nussenzweig, 1970; Grey, Rabellino & Pirofsky, 1971; Michlmayr & Huber, 1970; Papamichail *et al.*, 1971; Wilson & Nossal, 1971; Jondal, Holm & Wigzell, 1972; Dickler *et al.*, 1973; Seligmann, Preud'homme & Brouet, 1973; Huber *et al.*, 1974a, b). The combined use of these markers and *in vitro* labelling of DNA-synthesizing blood lymphocytes may give further information about the proliferative state of the various lymphocyte subpopulations.

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Lymphocytes incorporating [^{3}H]thymidine were evaluated in terms of their reactivity with unsensitized sheep red blood cells (SRBC). Results were compared with those obtained in normal and in a virus-induced lymphocyte response. Our experiments indicated a preferential proliferation of T lymphocytes in Hodgkin's disease and suggested an increased turnover of these cells.

MATERIALS AND METHODS

Patients

Hodgkin's disease. A total of twenty-two patients was studied. Eleven of these patients had been treated previously. For clinical details see Table 1.

Infectious mononucleosis. Five patients with the typical clinical, haematological and serological features of the disease were studied during the early stages of the illness.

TABLE 1. Clinical data of patients with Hodgkin's disease

Number	Age (years)	Stage	Staging laparatomy and splenectomy*	Duration of illness (years)	Lymphocytes (per ml × 10^3)	Treatment†	
						Previous	At time of investigation
1	69	IA	n.d.	< 1	1·8	None	None
2	23	IIIA	Yes	< 1	1·3	None	None
3	51	IIIB	Yes	< 1	0·4	None	None
4	27	IIIB	Yes	< 1	2·4	None	None
5	61	IVB	Yes	< 1	1·5	None	None
6	31	IIIB	Yes	< 1	1·1	None	None
7	49	IIA	n.d.	< 1	1·7	None	None
8	37	IIIB	Yes	< 1	1·9	None	None
9	24	IIE	Yes	< 1	2·1	None	None
10	21	IIB	Yes	< 1	1·7	None	None
11	24	IIA	Yes	1	1·7	None	None
12	21	IVB	n.d.	2	0·4	MOPP	MOPP
13	64	IVB	n.d.	3	0·9	COPP	CCNU-Vinblastine
14	34	IVB	Yes	2	0·7	COPP	Vinblastine weekly
15	47	IIIB	n.d.	4	0·9	Radiotherapy, COPP	COPP
16	41	IIIB	n.d.	4	0·5	Radiotherapy, COPP	Vinblastine weekly
17	71	IVB	Yes	< 1	1·2	None	COPP
18	63	IIIB	Yes	1	0·3	None	COPP
19	31	IVB	Yes	4	1·2	Radiotherapy, procarbacin	COPP
20	34	IIIB	Yes	2	0·5	COPP	Vinblastine weekly
21	38	IIIB	Yes	< 1	0·8	COPP	None
22	27	IIIB	n.d.	2	1·4	COPP	Vinblastine weekly

* n.d. = Not determined.

† COPP = cyclophosphamide, oncovin, procarbazin, prednisolone; MOPP = mustagen, oncovin, procarbazin, prednisolone.

Controls. Ten healthy adults without haematological or immunological abnormalities were studied.

Methods

Isolation of blood lymphocyte. Blood lymphocytes were isolated by centrifugation on a Ficol (Pharmacia Uppsala, final concentration 5%) Ronpacon (Ronpacon 440, Cilug-Chemie, final concentration 8%) gradient. Suspensions containing more than 95% viable lymphocytes were obtained. They were washed three times with TC 199 culture medium (Burroughs Wellcome, London).

Neuraminidase treatment of SRBC (E_N). 0·1 ml of neuraminidase (obtained from *Vibrio comma*, Behringwerke AG, Marburg, Germany) (500 units/ml) was added to 4 ml of 5% SRBC suspension in HBSS. After incubation for 15 min at 37°C the mixture was washed three times in TC 199 at room temperature. E_N instead of E were used since this method yields the highest percentage of rosetting cells (Weiner, Bianco & Nussenzweig, 1973; Michlmayr *et al.*, 1974; Fink *et al.*, in preparation) and since E_N rosettes were more stable when smeared on slides.

Evaluation of E_N-binding lymphocytes (E_N-*L*). 2×10^6 Purified lymphocytes in 0·5 ml of TC 199 were mixed with 0·5 ml of 5% E_N. The mixture was incubated for 15 min at 37°C, centrifuged at 200 *g* for 10 min at room temperature and then placed in an ice bath for 15 min. Subsequently the pellet was gently resuspended in 1 drop of 35% bovine serum albumin (BSA, Pentex Inc., Kankakee, Illinois) and smeared on gelatinized slides. After Giemsa staining a minimum of 200 lymphocytes were counted at a magnification of × 800. Rosette formation was considered if a minimum of four red cells was bound to a lymphocyte. In fifteen experiments the percentage of rosetting cells was also counted in a haemocytometer. Closely comparable values were obtained with both methods.

[^{3}*H*]*Thymidine labelling in vitro.* 2×10^6 Leucocytes were incubated with 1 μCi of [^{3}H]thymidine (Radiochemical Centre, Amersham, Bucks; specific activity 5000 mCi/mmol) in a total volume of 0·5 ml of TC 199 for 45 min at 37°C. For simultaneous detection of both E_N-L and DNA synthesis 0·5 ml of E_N was subsequently added and the suspension further treated as described above. After centrifugation cells were resuspended to 1 drop of BSA and smeared on gelatinized slides which were subsequently prepared for autoradiography. For the evaluation of the [^{3}H]thymidine labelling index a minimum of 5000 lymphocytes was counted. Labelling indices for E_N-L and for lymphocytes without rosettes (non E_N-L) were calculated separately.

In the fifteen experiments the [^{3}H]thymidine labelling indices were evaluated on purified and unseparated blood lymphocytes and similar results were obtained. Most experiments were then performed with purified lymphocyte suspensions.

Detection of surface immunoglobulin-bearing lymphocytes (*SIg-L*) *and aggregated gamma-globulin-binding lymphocytes* (*AGG-L*). For the evaluation of B lymphocytes two markers, a ^{125}I-labelled rabbit anti-human heavy-chain serum (^{125}I-GAM) (Fa. Dakopats, Denmark) and a heat-aggregated human gamma-globulin (^{125}I-AGG) were used in parallel. Details of these methods have been published elsewhere (Huber *et al.*, 1974a, b).

Autoradiography. Slides were fixed in absolute methanol for 12 hr. Autoradiograms were performed using the dipping technique with Ilford G5 emulsion (Ilford, Essex). The slides were exposed at 4°C for 7 days, developed and stained with Giemsa at pH 5·85. For details of the method and evaluation of the slides see (Huber *et al.*, 1970).

RESULTS

The percentage of B and T lymphocytes in the blood of patients with Hodgkin's disease was investigated. Results were compared with those obtained in patients with infectious mononucleosis and in normal controls.

The mean percentage of lymphocytes binding unsensitized SRBC (E_N-L), of lymphocytes binding aggregated IgG (AGG-L) and of lymphocytes with surface immunoglobulin (SIg-L) were in the same range as that of the normal controls (Table 2). Untreated and treated

TABLE 2. Frequencies of T and B lymphocytes in the blood of patients with Hodgkin's disease and with infectious mononucleosis

	Hodgkin (all cases)	Hodgkin (untreated)	Hodgkin (treated)	Infectious mononucleosis	Controls
Number of patients	22	11	11	5	10
E_N-binding lymphocytes					
Percentage	52±4	52±5	52±5	74±7	66±2
Per microlitre ($\times 10^2$)	7±1	10±2	6±2	45±11	10±1
^{125}I-AGG binding lymphocytes					
Percentage	29±5	29±8	29±5	n.d.	29±4
Per microlitre ($\times 10^2$)	5±1	5±1	3±0·1	n.d.	5±1
^{125}I-GAM binding lymphocytes					
Percentage	27±4	22±5	32±6	18±6	26±5
Per microlitre ($\times 10^2$)	4±0·1	5±1	3±0·1	11±4	5±1

n.d. = Not determined.

patients differed only in their absolute lymphocyte counts. In patients with infectious mononucleosis decreased percentages of AGG-L and SIg-L and concomitantly increased numbers of E_N-L were observed (Table 2). In absolute counts the increase in the numbers of T lymphocytes markedly exceeded that of B cells, in which the counts were only slightly elevated.

After [^{3}H]thymidine labelling *in vitro* a severalfold increase in the number of DNA-synthesizing lymphocytes were found in patients with Hodgkin's disease, when compared with the normal controls (Table 3). In patients under treatment the absolute number of DNA-synthesizing blood lymphocytes was lower than in patients without treatment. The highest values of thymidine-incorporating lymphocytes were observed in the patients with infectious mononucleosis.

The majority of [^{3}H]thymidine-incorporating cells were larger than 15 μm in diameter, with abundant pale-staining cytoplasm, and showed monocytoid- or lymphoblast-like nuclei. These cells in the blood of patients with Hodgkin's disease were morphologically indistinguishable from the atypical lymphocytes observed in patients with infectious mononucleosis. Approximately 10% of the labelled cells resemble medium-sized lymphocytes, most of them with hyperbasophilic cytoplasm.

When DNA-synthesizing lymphocytes were evaluated in terms of rosette-forming cells, a striking difference between Hodgkin's patients and the normal controls was observed. In the controls the majority of the labelled cells were negative, whereas in patients with Hodgkin's

TABLE 3. DNA-synthesizing lymphocytes in the blood of patients with Hodgkin's disease and with infectious mononucleosis

	Hodgkin (all cases)	Hodgkin (untreated)	Hodgkin (treated)	Infectious mononucleosis	Controls
Number of patients	22	11	11	5	10
I DNA-synthesizing blood lymphocytes					
Per thousand	7·4±1·0*	5·4±0·9*	10·0±1·7*	43·8±15·6*	1·7±0·4
Per microlitre	8·3±1·2*	9·3±1·7*	7·5±1·6*	394·4±250·0*	2·2±0·2
II E_N-binding lymphocytes in DNA synthesis					
Per thousand	9·6±1·7*	7·6±2·4*	11·6±3·7*	65·3±27·7*	1·4±0·4
III Non E_N-binding lymphocytes in DNA-synthesis					
Per thousand	6·5±1·7	3·5±1·0	8·0±2·4	17·7±6·8*	1·9±0·3
Ratio of II:III	1·5	2·2*	1·4	3·7*	0·7

* P values versus normal controls $< 0{\cdot}05$.

disease the majority showed rosette formation (Table 3). Of five [^{3}H]thymidine-incorporating lymphocytes, an average of about three exhibited binding of the T-cell marker in Hodgkin's patients, whereas only two out of five showed this property in the controls. In absolute terms, the number of labelled non-rosette-forming cells was also slightly increased. The differences compared with the normal controls, however, were not significant. Whereas DNA-synthesizing E_N-L were almost always large atypical lymphoid cells, the rare medium-sized lymphocytes with hyperbasophilic cytoplasm were relatively frequent in the non-E_N fraction (mean value 21%). The highest relative and absolute numbers of DNA-synthesizing E_N-L were observed in patients with infectious mononucleosis (Table 3). Of five [^{3}H]thymidine-incorporating lymphocytes four exhibited rosette formation and these mostly showed the morphological features of atypical lymphocytes.

DISCUSSION

The appearance of increased numbers of large DNA-synthesizing blood lymphocytes in patients with Hodgkin's disease is well documented and further substantiated by the present study (Crowther *et al.*, 1969a; Huber *et al.*, 1970; Schick *et al.*, 1973). At least three possible explanations for these findings have been proposed: (1) the large atypical lymphoid cells are malignant transformed lymphocytes; (2) they represent normal lymphocyte precursors released from lymph nodes involved in the disease process; (3) they indicate a proliferative response caused by tumour surface antigens, tumour products, anti-lymphocytic antibodies or unidentified (virus) infections.

At the moment experimental evidence to support the first hypothesis is lacking. In contrast to Hodgkin cells the large atypical blood lymphocytes show an euploid DNA content (Huber *et al.*, 1970; Peckham, 1973). The second mechanism seems also unlikely since no correlation has been found between the degree of lymphoid blood proliferation and the

extent of organ involvement (Crowther, Fairley & Sewell, 1969a; Huber *et al.*, 1970; Fairley *et al.*, 1973). Moreover, only slightly increased numbers of DNA-synthesizing blood lymphocytes were observed in carcinoma patients with multiple lymph node metastases (Huber *et al.*, 1971). The last hypothesis is supported by several findings. Increased numbers of proliferating blood lymphocytes have been found under conditions of known antigenic stimulation (Crowther *et al.*, 1969b) as well as in virus infections (Huber *et al.*, 1971b). These atypical lymphocytes resemble transformed lymphocytes observed after antigenic or mitogenic stimulation *in vitro* (Crowther, 1969a, b; Huber *et al.*, 1970, 1971b; Peckham, 1973).

The majority of DNA-synthesizing blood lymphocytes in Hodgkin's disease belong to the T-cell series. In the normal controls, by contrast, many of the cells in DNA synthesis were non-rosette-forming lymphocytes, which include the B cells. In infectious mononucleosis, the proliferative response of T lymphocytes was accompanied by elevated counts of T cells, whereas in Hodgkin's disease their absolute numbers were normal or slightly reduced.

Order & Hellman (1972) presented a hypothesis which relates viral and host immunity to the pathogenesis of Hodgkin's disease. During viral infection T cells are thought to undergo an antigenic alteration while normal T lymphocytes react against these altered cells. A chronic immune reaction similar to graft-versus-host disease would then lead to the appearance of neoplastic reticulum cells and to a depletion of T cells. Our study indicates a preferential involvement of T lymphocytes in the proliferative response which, together with the normal or reduced number of T lymphocytes, supports the concept of a T cell-directed destructive agent.

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