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Studies on the High- and Low-Affinity Rosette-Forming T-Lymphocytes in Patients with Hodgkin's and Non-Hodgkin's Lymphoma*

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Abstract—*The total number of lymphocytes and the number and proportion of high-* (29°C) *and low-* (4°C) *affinity E (erythrocyte) rosette-forming cells (RFC) from the peripheral blood of* 33 *patients with Hodgkin's and* 41 *patients with non-Hodgkin's lymphoma was assessed, and compared with results obtained from normal controls. Analysis of results was performed on groups of patients subdivided according to age, histological type and stage of disease. Patients with Hodgkin's disease showed a decrease in RFC which was attributed to a reduction in low-affinity RFC; this decrease could not be related to any particular histological type or stage of disease. Patients with non-Hodgkin's lymphoma showed, in addition to a decrease in low-affinity RFC, a significant decrease in high-affinity RFC. The observed decrease in peripheral blood T cell numbers of non-Hodgkin's lymphoma patients did not correlate with the type of disease, but patients with clinical stage* IV *showed a more pronounced reduction in the number of high- and low-affinity RFC when compared with other groups.*

INTRODUCTION

THE PROPERTY of human thymus-dependent lymphocytes (T cells) to form rosettes with sheep red blood cells *in vitro* provides a well established method for determining the number of these cells in blood specimens [1, 2].

More recently, the test has been refined to distinguish between cells which spontaneously rosette with sheep erythrocytes only at 4°C following overnight incubation (termed low-affinity rosettes) and those which rosette at 4°C following overnight incubation and at 29°C following a 1 hr incubation (high affinity rosettes) [3, 4]. The terms "high-" and "low-"affinity rosettes may not truly reflect the mechanism of erythrocyte–lymphocyte binding, but have been used in previous studies to denote spontaneous rosette formation under different experimental conditions. These terms are used here in this context.

An alternative method for distinguishing between these two populations of T cells is the identification of Fc-receptor sites on high-affinity cells using a fluorescent-antibody technique [4]; however, this test is probably less easily interpreted than the rosetting assay. Application of the temperature-dependent rosetting test to the lymphocytes of patients with malignant disease has indicated a deficiency of high-affinity T lymphocytes in patients with cancer [4–9]; however, it is not known whether this deficiency is limited to certain malignant diseases or is a feature of all types of cancer. In the present study we have investigated the number of lymphocytes and the relative proportion of high- and low-affinity E-rosette-forming lymphocytes in mononuclear cell suspensions isolated from patients with Hodgkin's and non-Hodgkin's lymphoma. The results have been analysed with respect to age, histological type and stage of disease.

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MATERIALS AND METHODS

Patients

Peripheral blood samples were collected in heparin (100 units/ml final concentration) from all patients with Hodgkin's or non-Hodgkin's lymphoma admitted to Weston Park Hospital, Sheffield, during an 8-month period and prior to any treatment; control samples were collected from staff and patients at the Royal Hallamshire Hospital during the same period, and were age, sex and time matched as closely as possible. In total, blood specimens were obtained from 33 patients with Hodgkin's disease, 41 patients with non-Hodgkin's lymphoma and 79 control subjects. All blood samples were prepared within 3 hr of their being collected. Histological examination of tumours from patients with Hodgkin's disease allowed a division of the cases into four groups: lymphocyte predominant, mixed cell, lymphocyte depleted and nodular sclerosing, according to the Rye classification [10] and clinical investigation allowed a division of the patients into four stages by the Ann Arbor criteria [11]. The results obtained from lymphocyte evaluation were separately compared with both the type of tumour and the stage of disease. A classification of patients with non-Hodgkin's lymphoma was also made, and the results of lymphocyte counts compared with the staging of disease and the histological type according to Rappaport *et al.* [12] or its equivalent in the Lukes and Collins classification [13]. For analysis, the non-Hodgkin's lymphomas were grouped histologically as favourable in type. Patients with recurrent disease of Hodgkin's or non-Hodgkin's type were also studied.

Preparation of peripheral blood leukocytes

A 20 ml sample of heparinized (100 units/ml final concentration) blood from lymphoma patients or controls was separated on Ficoll–Triosil in 10 ml plastic centrifuge tubes (Sterilin R.T.D.). Each sample was centrifuged at 400 ***g*** for 30 min and mononuclear cells (MNC) which concentrated at the sample–gradient interface were collected and washed three times in Hanks Balanced Salt Solution (HBSS). The cells were re-suspended in HBSS, counted and adjusted to a concentration of 2×10^6 cells/ml. This preparative technique yielded approximately 40% of the total mononuclear cells present in 10 ml of blood, but this did not lead to loss of lymphocytes which increased overall by 10–15%. Monocyte contamination remained at between 5 and 7% of the total population following separation in Ficoll–Triosil.

Assay of lymphocyte populations

Total lymphocytes. The total number of MNC in the isolated cell suspension was calculated by direct counting in a modified Neubauer counting chamber, and the total number of MNC recovered from each 10 ml blood sample was determined.

High- and low-affinity T lymphocytes. The technique used to determine the numbers of high- and low-affinity T lymphocytes was the same as described by other workers [14, 15]. We have used the term "low affinity" to denote lymphocytes which form spontaneous rosettes only at 4°C and the term "high affinity" in the case of cells forming rosettes at 29°C. MNC suspensions prepared on Ficoll–Triosil were re-suspended in HBSS at a concentration of approximately 2×10^6/ml. To an aliquot of lymphocyte suspension was added equal volumes of 0.5% (v/v) sheep red blood cells (SRBC; Oxoid Ltd.) and foetal calf serum; the mixture was incubated at 37°C for 5 min. After incubation the cells were divided into two portions and sedimented by centrifugation at 200 ***g*** for 5 min, after which one aliquot was incubated for 1 hr at 29°C, to determine the number of high-affinity T lymphocytes, and the remainder incubated at 4°C for 18 hr to determine the number of combined high- and low-affinity T lymphocytes. Tests were performed in duplicate on each blood sample. Following the incubation, the deposited cells were gently re-suspended in 10 μl of a 1% (w/v) methylene blue solution in PBS and approximately 100 cells in each test were examined, and the number of high- and low-affinity T cells calculated. Thus, a total of 200 cells were counted from each blood sample. Statistical analysis was performed on the group results using Student's t test; differences were considered significant if $P = < 0.01$ in two tailed tables.

RESULTS

Lymphocyte populations in patient and control groups

The number and type of lymphocytes present in peripheral blood specimens from control and patient groups are shown in Table 1. For normal subjects the total number of lymphocytes recovered from 10 ml of peripheral blood was approximately the same for volunteers aged < 40 and > 40 years. This distribution allowed significant numbers of patients to be ascribed to each group. For patients

Table 1. *Lymphocyte number and high- and low-affinity rosette-forming cells in patients and control groups*

Clinical group	Age (mean ±S.D.)	No. of patients	Total lymphocytes* ($\times 10^6$)±S.D.	Number and percentage RFC 29°C Nos.	29°C %	4°C Nos.	4°C %
Normal controls	18–39 (29±5.6)	51	6.0±3.9	1.8±1.1	34.8±15.8	3.0±1.8	52.0±12.7
	40–72 (54±10.2)	28	6.35±2.8	1.7±1.1	28.8±13.8	2.6±1.4	41.0±11.2
Hodgkin's disease	18–39 (29±7.3)	16	4.7±1.8	1.5±1.5	30.7±19.1	2.0±1.4	43.3±14.9
	40–72 (60±13.3)	14	4.7±1.5	1.4±1.0	29.8±17.7	1.7±0.6	37.5±11.0
Non-Hodgkin's disease	18–39 (26±9.4)	15	5.3±1.8	1.1±0.9	19.5±11.2	1.8±1.0	33.4±19.3
	40–72 (61±12.1)	33	4.8±1.8†	1.2±0.6†	24.0±12.4	1.9±1.0	40.0±15.6

*Recovered from 10 ml blood samples.
† $P = <0.01$.

with Hodgkin's disease, the total number of recoverable lymphocytes was less than found in controls, but the difference was not significant ($P = >0.01$). The reduction observed was probably due to smaller numbers of low-affinity T cells; for example, in Hodgkin's disease patients of <40 years the number of these cells was $2.0 \pm 1.4 \times 10^6$ and for a comparable group of normal subjects the number was $3.0 \pm 1.8 \times 10^6$. Similar results were obtained for the >40 years age group, where patients with Hodgkin's disease had a marginally lower number of low-affinity T lymphocytes, as compared to normal subjects (Table 1). For patients with non-Hodgkin's lymphoma, the total number of lymphocytes present in the peripheral blood was less than in controls for both age groups, and this difference was statistically significant for the >40 years age group ($P = <0.01$). These results suggest that deficiency of RF lymphocytes was probably due to lower numbers of high-affinity T lymphocytes; thus, T lymphocytes numbered $1.2 \pm 0.6 \times 10^6$ in the <40 year age group of non-Hodgkin's lymphoma patients, compared to $1.7 \pm 1.1 \times 10^6$ in control patients ($P = <0.01$), and $1.1 \pm 0.9 \times 10^6$ and $1.8 \pm 1.1 \times 10^6$, respectively, for the >40 year age group, although the difference between these groups was not statistically significant ($0.01 < P < 0.05$).

Lymphocyte population related to stage and type of disease

Hodgkin's disease. The number of high- and low-affinity T lymphocytes in 10 ml blood specimens from patients with Hodgkin's disease was compared with that of control subjects and grouped according to the histological type of tumour. The results are shown in Table 2. The total number of lymphocytes in the blood of patients with Hodgkin's disease was lower than found in control subjects; however, this difference was not found to be significant ($P = >0.01$). Such differences that were observed related specifically to the number of low-affinity T lymphocytes when patients were grouped together. Thus, these numbered $1.8 \pm 1.1 \times 10^6$ in the patient group, and $2.8 \pm 1.7 \times 10^6$ in the control group; this difference was found to be statistically significant ($P = <0.01$) and correlated with a significant reduction in the proportion of low-affinity E-rosetting cells. This effect could not be related to any particular histological type of tumour.

The number of T lymphocytes in 10 ml blood specimens from patients with Hodgkin's disease was also analysed with reference to stage of disease. When these results were compared to control subjects, patients with Hodgkin's lymphoma had marginally lower numbers of lymphocytes, which was chiefly due to a significant decrease in the number of low-affinity T lymphocytes ($P = <0.01$); however, the deficiency was equally spread over the four stages and there was no evidence to suggest that a T lymphocyte deficiency was associated with any stage of Hodgkin's disease (Table 3). In patients with recurrent disease a lower number of high-affinity rosetting cells was observed, but due to the small sample

Table 2. High- and low-affinity rosette-forming cells in patients with Hodgkin's lymphoma: correlation with histological type

Disease type	No. of patients	Age (mean ±S.D.)	Total lymphocytes* ($\times 10^6$)±S.D.	Number and percentage RFC 29°C No.	29°C %	4°C No.	4°C %
Lymphocytes predominant	3	48±18	4.0±2.7	2.1±1.5	49.0±20.9	1.5±0.9	41.3±8.1
Mixed cell	17	46±18	4.5±1.6	1.6±1.4	30.4±18.7	2.0±1.4	42.5±13.1
Nodular sclerosing	2	72±1	3.8±1.7	1.0±0.1	39.0±2.8	1.2±0.1	38.5±4.9
Lymphocyte depleted	3	39±1	7.7±0.4	3.0±0.3	32.0±20.8	3.0±0.2	38.6±23.4
Recurrent	8	33±9	4.7±1.1	1.0±0.8	22.3±16.3	1.7±0.7	36.4±14.3
Total patients	33	44±18	4.7±1.7	1.5±1.2	30.9±18.5	1.8±1.1†	40.3±13.3
Normal controls	79	38±14	6.1±3.6	1.8±1.1	32.8±15.4	2.8±1.7	48.0±13.2

*Recovered from 10 ml blood samples.
†$P = <0.01$.

Table 3. High- and low-affinity rosette-forming cells in patients with Hodgkin's lymphoma: correlation with clinical stage

Disease stage	No. of patients	Age (mean ±S.D.)	Total lymphocytes* ($\times 10^6$)±S.D.	Number and percentage RFC 29°C No.	29°C %	4°C No.	4°C %
I	4	3.8±25	4.4±2.1	1.9±0.9	34.3±17.6	1.9±0.87	44.0±13.7
II	5	35±3	5.6±1.9	2.2±1.2	39.0±20.8	2.2±0.7	39.4±4.6
III	11	57±17	4.7±1.8	1.7±1.8	31.8±21.8	2.1±1.7	43.3±16.5
IV	5	41±20	4.2±1.5	1.15±0.2	31.8±15.8	1.5±0.2	39.6±15.7
Recurrent	8	33±9	4.7±1.1	1.0±0.8	22.3±16.3	1.7±0.7	36.4±14.3
Total patients	33	44±18	4.7±1.7	1.5±1.2	30.9±18.5	1.8±1.1†	40.3±13.3
Normal Controls	79	38±14	6.1±3.6	1.8±1.1	32.8±15.4	2.8±1.7	48.0±13.2

*Recovered from 10 ml blood samples.
†$P = <0.01$.

size the results were not statistically significant; a similar trend was found with stage IV patients.

Non-Hodgkin's disease. The total lymphocyte population and the number of high- and low-affinity T lymphocytes was estimated for 48 patients with non-Hodgkin's lymphoma and the results compared with those of control subjects. Patients were grouped according to their prognosis based on the histology of the tumour. The results are shown in Table 4. Patients with non-Hodgkin's disease had lower numbers of lymphocytes than control subjects, although the difference was not significant ($P = >0.01$). In contrast, the number of high- and low-affinity lymphocytes in blood specimens from all the patients, $1.2 \pm 0.74 \times 10^6$ and $1.9 \pm 1.1 \times 10^6$, respectively, were both shown to be significantly lower than those found in control subjects ($P = <0.001$ and $P = <0.01$). Grouped results also showed a marked decrease in the percentage of high- and low-affinity T lymphocytes. This reduction was not related to any specific type of non-Hodgkin's disease, since all four types showed lower numbers of low- and high-affinity T lymphocytes (Table 4).

The lymphocyte populations in patients with non-Hodgkin's disease were also analysed with reference to disease stage. These results

are shown in Table 5. No statistically significant reduction or increase in the total number of lymphocytes was found for patients with stage I, II or III, compared to control; neither did patients in these groups show a significant decrease in high- or low-affinity T lymphocytes. However, for patients with stage IV disease the mean low-affinity T lymphocyte population in 10 ml of blood was $1.4 \pm 0.8 \times 10^6$ and the mean number of high-affinity T lymphocytes was $0.7 \pm 0.4 \times 10^6$; these values were shown to be significantly lower than controls ($P = < 0.01$). In addition, a decrease in the percentage of high- and low-affinity T lymphocytes was observed in stage IV patients compared with controls.

DISCUSSION

Membrane markers have been used to discriminate between the various sub-populations of lymphoid cells [16]; this has afforded an opportunity to study the status of peripheral blood lymphocytes in patients with malignant disease. Using the ability of T lymphocytes to form spontaneous rosettes with sheep red blood cells, distinction can be made between those lymphocytes forming low-affinity E-rosettes (at 4°C) and those which form high-affinity rosettes (at 29°C) [3, 4]. Previous workers have found that a decrease in the number of high-affinity rosette-forming lymphocytes occurs in malignant disease [5–9, 17]

Table 4. High- and low-affinity rosette forming cells in patients with non-Hodgkin's lymphoma: correlation with disease type

Disease type	No. of patients	Age (mean ±S.D.)	Total lymphocytes* ($\times 10^6$)±S.D.	Number and percentage RFC 29°C No.	29°C %	4°C No.	4°C %
Good (favourable)	12	45±23	4.8±1.9	1.26±0.9	26.7±14.3	2.0±1.0	43.0±15.7
Intermediate	14	48±20	4.9±1.7	1.1±0.8	22.3±13.2	2.0±1.4	39.2±21.4
Aggressive (unfavourable)	11	48±21	4.9±2.3	1.1±0.6	23.8±10.8	1.9±1.0	39.3±16.7
Recurrent	4	57±8	4.5±0.6	1.1±0.6	23.0±15.9	1.8±0.7	41.0±13.3
Total patients	41	48±20	4.8±1.8	1.2±0.74†	24.1±12.7	1.9±1.1	40.5±17.4
Normal controls	79	38±14	6.1±3.6	1.8±1.1	32.8±15.4	2.8±1.7	48.0±13.2

*Recovered from 10 ml blood samples.
†$P = < 0.001$.
‡$P = < 0.01$.

Table 5. High- and low-affinity rosette forming cells in patients with non-Hodgkin.s lymphoma: correlation with clinical stage

Disease stage	No. of patients	Age (mean ±S.D.)	Total lymphocytes* ($\times 10^6$)±S.D.	Number and percentage RFC 29°C No.	29°C %	4°C No.	4°C %
I	12	54±18	4.5±1.75	1.3±0.8	31.7±13.1	1.9±0.8	45.3±13.1
II	7	42±24	6.2±2.0	1.4±1.0	23.4±14.8	2.5±1.6	39.5±19.8
III	7	33±14	4.6±2.1	0.8±0.4	23.7±12.6	2.1±1.2	40.8±26.4
IV	11	55±21	4.3±1.9	0.7±0.4†	17.8±8.0	1.4±0.8†	30.0±12.2
Recurrent	4	57±8	4.5±0.6	1.1±0.6	23.0±15.9	1.8±0.7	41.0±13.3
Total patients	41	48±20	4.8±1.8	1.2±0.74†	24.1±12.7	1.9±1.1†	40.5±17.9
Normal controls	79	38±14	6.1±3.6	1.8±1.1	32.8±15.4	2.8±1.7	48.0±13.2

*Recovered from 10 ml blood samples.
†$P = < 0.01$.

and in patients suffering from viral infections or alcoholic liver disease [3, 18, 19].

However, most of the studies undertaken on cancer patients have not related the findings to the clinical stage of the disease.

A decrease in low-affinity RFC in Hodgkin's patients prior to treatment has been reported. Holm *et al.* [20] found a positive correlation between decreased RFC and advancement of the disease, whilst Bobrove *et al.* [21] demonstrated that Hodgkin's patients with low numbers of RFC had a reduced responsiveness to T cell mitogen stimulation, indicating that alteration in lymphocyte surface properties may be closely associated with impaired immunological funtion. Low peripheral blood lymphocyte counts have been reported in Hodgkin's and non-Hodgkin's disease patients [22] and other workers have reported the presence of low levels of RFC in Hodgkin's patients out of remission, inferring in this instance that the recovery of normal T lymphocyte populations following cancer therapy does not readily occur [7]. Evidence suggesting that depressed *in vitro* immune activity may relate to *in vivo* defective immune function has come from a recent study by Thatcher *et al.* [5]. Non-Hodgkin's lymphoma patients showed a significant decrease in the number of low-affinity rosette-forming T lymphocytes, and the capacity of the patients' peripheral blood lymphocytes to function as effector cells in T, non-T and K-killing was impaired. This decreased lymphocyte reactivity observed *in vitro* correlated with a low level of responsiveness to antigens *in vivo*, and the effect was closely associated with patients having unfavourable prognosis. Collectively these observations suggest that changes in lymphocyte membrane properties and function is a reflection of decreased *in vivo* immunocompetence. The results shown here reflect the difference in T cell populations between patients with Hodgkin's and non-Hodgkin's lymphoma, which appear to show decreased numbers of low- and high-affinity E-RFC, respectively. The observed decrease in T lymphocyte numbers could not be ascribed to any particular tumour histology; however, the results suggest that patients with widespread disease are more likely to have decreased T lymphocyte levels.

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