H-Rosette formation in T-cell-proliferative diseases

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Summary

In health up to 6% of human lymphocytes will form rosettes with homologous group O rhesus-negative erythrocytes (H rosettes). Increased numbers of H rosettes were found in T-cell-proliferative diseases—namely, infectious mononucleosis, Sézary's syndrome, and T-cell leukaemia. H-rosette formation is thus a marker for a subpopulation of T lymphocytes, and this easily performed test of T-cell activation may have clinical value in characterising changes in blood lymphocyte populations in disease.

Introduction

In several clinical conditions surface markers are used to categorise lymphocytes as derived from either thymus (T) or bone marrow (B). In lymphatic leukaemia they show that the abnormal cells are indeed lymphoid, and in most cases of chronic lymphatic leukaemia these have the characteristics of B cells,¹⁻³ although T-cell leukaemias have been reported.⁴⁻⁵ Acute lymphocytic leukaemia and childhood lymphoblastic lymphoma may also be derived from T cells,⁶⁻⁷ and Sternberg's sarcoma⁸ and Sézary's syndrome⁹⁻¹⁰ have been attributed to a proliferation of T cells. In infectious mononucleosis most of the circulating atypical mononuclear cells are of T-cell origin,¹¹⁻¹² though some, probably infected with Epstein-Barr virus, are B cells¹³⁻¹⁴ and represent the target against which the major T-cell response is directed.¹⁵

Subpopulations of T cells have been found in mice,¹⁶ and they probably also exist in man, though this is less clear. Human peripheral blood T lymphocytes form rosettes with sheep erythrocytes (E rosettes)¹⁷⁻¹⁸ and thymocytes with human erythrocytes (H rosettes)¹⁹ as well as with sheep erythrocytes. A few peripheral blood lymphocytes may form rosettes with human erythrocytes,²⁰ the number being greater when bovine serum albumin is present.²¹

We have shown that when human lymphocytes have been stimulated by mitogens they form far more H rosettes than when unstimulated²² and that this is due to activity of the T cells and not of the B cells. We therefore decided to investigate H-rosette formation in diseases in which the existence of an activated T-cell population might be predicted and to study the conditions under which T cells activated in vitro form H rosettes.

Material and methods

Patients studied included four with infectious mononucleosis (Paul-Bunnell test result positive), two with Sézary's syndrome, one with T-cell leukaemia (peripheral leucocyte count $500 \times 10^9/l$; 95% capable of forming rosettes with sheep erythrocytes), and three with

chronic lymphatic leukaemia. Ten healthy controls (laboratory personnel) were also tested. In addition, synovial fluid lymphocytes from five patients with Still's disease or rheumatoid arthritis and one with acute non-specific synovitis were examined.

Lymphocyte separation and transformation—Lymphocytes were obtained from blood and synovial fluid as described.²³ Mitogen stimulation by phytohaemagglutinin (PHA), measurement of lymphocyte transformation by ¹⁴C-thymidine uptake, and autoradiography were carried out with peripheral blood lymphocytes from healthy controls.²²⁻²³

Preparation of H rosettes²²—A 0.5% suspension (0.2 ml) of thrice-washed human group O rhesus-negative erythrocytes in 10% fetal bovine serum (Flow Laboratories) was added to an equal volume of washed mitogen-treated control cells or cells from the patients and controls and the cell mixtures were incubated at 37°C for 30 minutes, centrifuged at 1000 rpm for five minutes, then reincubated at 4°C for a minimum of one hour. After discarding 0.2 ml of the supernatant the remaining suspension was gently resuspended by tapping the tube and one drop placed on a coverslip. The cells were examined by light microscopy at a magnification of 200. A rosette was defined as a lymphocyte with three or more erythrocytes attached. At least 100 lymphocytes were counted. The H rosette suspension from one patient with Sézary's syndrome was centrifuged at 100 rpm for five minutes. The supernatant was discarded and the pellet fixed in 3% glutaraldehyde in pH 7.3 phosphate buffer for one to two hours, rinsed in several changes of buffer, and resuspended in 30% bovine serum albumin. The suspension was lightly centrifuged to form a pellet, which was refixed in glutaraldehyde, then cut into 1-mm cubes and post-fixed in buffered osmium tetroxide, dehydrated, and embedded in Araldite. Rosettes were selected by light microscopy of toluidine-blue-stained 2-μ sections and ultra-thin sections cut and stained with uranyl acetate and lead citrate.

Preparation of mixed E and H rosettes—This test was performed with PHA-stimulated control lymphocytes and unstimulated T-leukaemia cells. Mixed rosettes were made by adding 0.2 ml of a 0.5% sheep erythrocyte suspension to a suspension of lymphocytes and human erythrocytes already incubated to induce human erythrocyte rosette formation. The rosetting procedure was repeated. Cytocentrifuged preparations of mixed sheep and human erythrocyte rosettes were made and stained with Leishman.

Mixed lymphocyte reaction—A total of 10^6 lymphocytes in RPMI 1640 from three pairs of six unrelated healthy controls were incubated for up to seven days at 37°C. The cells were washed after incubation, the procedure for the formation of H rosettes was carried out, and the rosettes were counted. In one experiment lymphocyte transformation was also measured.

Inhibition experiments (PHA-stimulated control lymphocytes only)—These were carried out to study the effect of functional inhibition of microtubules (using vinblastine and colchicine)²⁴ and microfilaments (using cytochalasin B).²⁵ Rosette formation with sheep erythrocytes is inhibited by cytochalasin B but not by vinblastine or colchicine,²⁶ and the H-rosette-forming cell receptor was therefore similarly studied.

Colchicine (Sigma Chemical Co, St Louis)—Colchicine was dissolved in medium 199 to give concentrations of 1.0 and 0.1 mmol/l. Lymphocytes (10^6) from a control subject were cultured in the presence of PHA for 24 hours, then washed twice in medium 199 and incubated with colchicine at 37°C for two hours. The cells were then washed and H rosettes made as before and counted.

Vinblastine (Sigma Chemical Co)—The procedure was as for colchicine, concentrations of vinblastine ranging from 1.0 to 0.001 mmol/l being used. This test was performed with sheep erythrocytes as well as human erythrocytes.

Cytochalasin B (Aldrich Chemical Co, Ltd, England)—Cytochalasin B (1 and 5 μg) was dissolved in dimethylsulphoxide (DMSO) (BDH Chemicals, England). A total of 10^6 lymphocytes stimulated by PHA were incubated with cytochalasin B at 37°C for one hour, then washed, added to a 0.5% suspension of human erythrocytes, and the rosettes counted immediately. The same suspension was then incubated at 4°C for two hours and H rosettes were again counted, microfilament function having presumably returned.

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Results

The proportion of lymphocytes forming H rosettes in the various diseases are shown in table I. Rosettes are frail and easily dissociated. They often have few adherent erythrocytes, which commonly attach with their biconcave surfaces in the same plane as the plane of viewing; in contrast the flat surfaces of sheep erythrocytes are at right angles to the plane of view in a typical rosette. The H rosettes formed with mitogen-stimulated lymphocytes tended to have more attached erythrocytes and were made stable.

TABLE I—Percentage of lymphocytes forming H rosettes from patients with various diseases and from a group of healthy controls

	No	% of lymphocytes forming H rosettes	
		Range	Mean
Patients with:			
Infectious mononucleosis	4	23-40	31.5
Sézary's syndrome	2	21-52	36.5
T-cell leukaemia	1	28	28
Still's disease or rheumatoid arthritis (synovial fluid cells)	5	0-40	18.3
Non-specific synovitis	1	25	25
Chronic lymphatic leukaemia	3	1-4	2.3
Healthy controls	9	0-6	3.2

The proportion of lymphocytes capable of forming H rosettes was considerably increased in the diseases studied, with the exception of chronic lymphatic leukaemia. There was great variation in the proportion found in rheumatoid synovial fluid, but it appeared that H rosettes are more likely to be found when the duration of the effusion is short. Mixed H and E rosettes were made using mitogen-stimulated lymphocytes and unstimulated lymphocytes from the patient with T-cell leukaemia (see fig 1). E and mixed H and E rosettes were seen but not H rosettes alone, which was taken as evidence that the H receptor is on T lymphocytes. An H rosette from a patient with Sézary's syndrome is shown in fig 2. Autoradiography (fig 3) showed that cells forming H rosettes after incubation with PHA have indeed been stimulated.

The results of mixed lymphocyte reactions are shown in fig 4. H rosettes began appearing in appreciable numbers on or after the fourth day of culture. Three control cultures of lymphocytes from three separate donors gave few or no H rosettes under the same conditions at up to seven days of incubation. In one mixed culture evidence of transformation by ¹⁴C-thymidine uptake was apparent by the third day, while cells forming H rosettes did not in this case appear until the fifth day.

The results of the inhibition experiments are given in table II, which shows that microtubule inhibitors had little effect on the formation of H rosettes, except vinblastine at a concentration of 1 mmol/l. At this concentration H rosettes were more sensitive than E rosettes, but at 0.1 mmol/l the difference was small. On the other

FIG 1—Mixed H and E rosette from patient with T-cell leukaemia. (Cyto-centrifuged preparation stained with Leishman. $\times 1850$.) (NB sheep erythrocytes are smaller than human erythrocytes.)

FIG 2—Electron micrograph of H rosette from patient with Sézary's syndrome. ($\times 8000$.)

TABLE II—Effect on H and E rosette formation by PHA-stimulated lymphocytes of colchicine, vinblastine, and cytochalasin B. Figures are percentages

	Type of rosettes	Concentration in mmol/l				μ g		Diluent alone
		1.0	0.1	0.01	0.001	1.0	5.0	
Colchicine in medium 199	H	58	57					61
	E	49	70					66
Vinblastine in medium 199	H	0	34	40	45			61
	E	4	56					68
Cytochalasin B in DMSO	H					19	0* (26†)	55

*Tube read immediately (room temperature).

†Tube reincubated at 4°C for two hours before reading.

TABLE III—Human T-cell population: discriminatory properties of E and H rosettes

Rosettes	E rosettes	H rosettes in 5% bovine serum albumin	H rosettes in 10% fetal bovine serum
In health	55-80% ²²	Approximately 50% of E rosettes ²¹	6% (table I)
In T-cell-proliferative disorders	Up to 89% ¹²	Not reported to date	21-52% (table I)
Stimulation by PHA	Up to 99% ²¹	Not reported to date	Up to 90% ²¹
Effect of cytochalasin B	Inhibition ¹⁸	More resistant than E rosettes ²¹	Inhibition (table II)
Effect of vinblastine	No inhibition ²⁶ (slight inhibition at 1 mmol/l; table II)	Not reported to date	No appreciable inhibition except at 1 mmol/l (table II)
Effect of colchicine	No inhibition ²⁶	Not reported to date	No inhibition (table II)

FIG 3—Autoradiograph of PHA-stimulated H-rosette-forming lymphocyte. (Counterstained with Giemsa. $\times 2000$.)

FIG 4—Percentage of H-rosette-forming cells appearing after various periods in mixed lymphocyte culture (MLC).

hand, microfilament inhibition produced profound impairment of H-rosette formation even with only $1 \mu\text{g}$ cytochalasin B. On recovery of microfilament function, H rosettes were again demonstrated—that is, after reincubation at 4°C for two hours.

Discussion

H-rosette-forming cells in the blood appear to increase on stimulation by mitogens, on mixed lymphocyte culture, and in certain T-cell-proliferative disorders. Their number in chronic lymphatic leukaemia is no greater than that in health, as might be expected, since this disease is generally regarded as a B-cell-proliferating condition.¹⁻³ The increase in H-receptor-bearing cells in the conditions considered here is likely to reflect an increase in a T-cell subpopulation already bearing this marker, since a few H rosettes are found in health. Interestingly, in infectious mononucleosis and rheumatoid joint effusions, where

the cells showed a high background uptake of ^{14}C -thymidine, unresponsiveness to PHA, and an increase in E rosettes formed,^{11 12 24} H rosettes were found in moderately increased numbers. The H-rosette-forming cells in the patient with T-cell leukaemia may represent a minority T-cell population activated by the presence of a malignant clone of E-receptor-bearing cells. The nature of the receptor for sheep erythrocytes present on the surface of the human T lymphocyte is not known. Its characteristic inhibition by cytochalasin B but not colchicine, however, was also seen with the human erythrocyte receptor, suggesting that integrity of microfilament function is essential for rosette formation of both types.

Hence the ability to bind human erythrocytes is shared by thymocytes, normal peripheral blood T lymphocytes activated by mitogens or by mixed lymphocyte culture, and a subpopulation of peripheral blood lymphocytes in T-cell-proliferative diseases. Only a small proportion (0.6%) of untreated normal peripheral blood lymphocytes form H rosettes except when tested in the presence of bovine serum albumin (table III); the increase this produces may delineate a further subpopulation of T cells. It appears that in man, as in mice, there is heterogeneity of the T-cell population, which may explain the so-called "active rosettes" produced by a short period of incubation of lymphocytes and sheep erythrocytes²⁷ and present in health. The H-rosette test is easily performed and should be useful clinically in other conditions in which changes in lymphocyte populations might otherwise pass unnoticed.

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References

- Johansson, B, and Klein, E, *Clinical and Experimental Immunology*, 1970, 6, 421.
- Wilson, J D, and Nossal, G J V, *Lancet*, 1971, 2, 788.
- Papamichail, M, Brown, J C, and Holborow, E J, *Lancet*, 1971, 2, 850.
- Lille, I, et al, *Lancet*, 1973, 2, 263.
- Stathopoulos, G, et al, *Journal of Clinical Pathology*, 1974, 27, 851.
- Borella, L, and Sen, L, *Journal of Immunology*, 1973, 111, 1257.
- Kaplan, J, Mastrangelo, R, and Peterson, W D, *Cancer Research*, 1974, 34, 521.
- Smith, J L, et al, *Lancet*, 1973, 1, 74.
- Brouet, J C, Flandrin, C, and Seligman, M, *New England Journal of Medicine*, 1973, 289, 341.
- Zucker-Franklin, D, *Mayo Clinic Proceedings*, 1974, 49, 567.
- Sheldon, P J, et al, *Lancet*, 1973, 1, 1153.
- Papamichail, M, Sheldon, P J, and Holborow, E J, *Clinical and Experimental Immunology*, 1974, 18, 1.
- Epstein, M A, in *Oncogenesis and Herpesviruses*, ed P M Briggs et al, p 261. Lyon, WHO International Agency for Research on Cancer, 1972.
- Jondal, M, and Klein, G, *Journal of Experimental Medicine*, 1973, 138, 1365.
- Carter, R L, *Lancet*, 1975, 1, 846.
- Stobo, J D, *Transplantation Reviews*, 1972, 11, 60.
- Lay, W H, et al, *Nature*, 1971, 230, 531.
- Jondal, J M, Holm, G, and Wigzell, H, *Journal of Experimental Medicine*, 1972, 136, 207.
- Baxley, G, et al, *Clinical and Experimental Immunology*, 1973, 15, 385.
- Kaplan, J, *Clinical Immunology and Immunopathology*, 1975, 3, 471.
- Yu, D T Y, *Clinical and Experimental Immunology*, 1975, 20, 311.
- Sheldon, P J, and Holborow, E J, *Journal of Immunological Methods*. In press.
- Sheldon, P J, Papamichail, M, and Holborow, E J, *Annals of the Rheumatic Diseases*, 1974, 33, 509.
- Malawista, S E, Sato, H, and Bensch, K G, *Science*, 1968, 160, 770.
- Schroeder, T E, *Experimental Cell Research*, 1968, 53, 272.
- Kersey, J H, Hom, D J, and Buttrick, P, *Journal of Immunology*, 1974, 112, 862.
- Wybran, J, and Fudenberg, H H, *New England Journal of Medicine*, 1973, 288, 1072.