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SURFACE IMMUNOGLOBULINS OF LEUKAEMIC CELLS

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SUMMARY

We have examined leukaemia cells from twenty-eight patients for the presence of surface immunoglobulins by a migration-inhibition technique. Immunoglobulins were identified on the cells of fourteen out of nineteen patients with chronic lymphatic leukaemia but were not present on cells from patients with chronic myeloid leukaemia or acute leukaemia. The results obtained with the migration technique were confirmed by identification of immunoglobulins in eluates and lysates of the leukaemia cells. Serial elution and lysis studies indicated that in some cases the immunoglobulins were produced by the leukaemia cells at a low but steady rate, possibly indicating a relationship between those cells and 'B' cells. In other cases the immunoglobulins were readily removed from the cells and were present only in the initial eluates from the cells, suggesting that they had been adsorbed and might possibly be specific antibody. The immunoglobulin negative chronic lymphatic leukaemia cells may be related to 'T' cells, a possibility supported by a high level of phytohaemagglutinin induced transformation in one case examined by this technique.

INTRODUCTION

Normal human peripheral lymphocytes, relatively homogeneous by light microscopy, can be subdivided by the density and nature of their membrane immunoglobulins (Grey *et al.*, 1971; Wilson & Nossal, 1971; Pernis *et al.*, 1971; Papamichail *et al.*, 1971), complement receptors (Bianco *et al.*, 1970), rosette-forming capacity (Brain, Gordon & Willetts, 1970) and transformability by mitogens such as phytohaemagglutinin (PHA) (Greaves & Bauminger, 1972). Accumulating evidence therefore supports the view that human lymphocytes are divisable into thymus-dependent (T) lymphocytes and bone marrow (or bursa-dependent) (B) lymphocytes, as is the case with chickens and rodents. There is some evidence that leukaemic (Wilson & Nossal, 1971; Grey *et al.*, 1971; Papamichail *et al.*, 1971; Shevach *et al.*, 1972)

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and lymphomatous (Klein *et al.*, 1968; van Furth *et al.*, 1972) lymphoid cells show some of the characteristics of T or B lymphocytes, presumably depending on their histogenesis. We have classified chronic lymphatic laukaemia cells on the basis of their reactivity with anti-immunoglobulin sera in a leukaemia cell migration inhibition technique (MIT) and by their responsiveness to PHA. The nature of the surface immunoglobulins of leukaemia cells has been examined by serial elution studies and by cell lysis.

MATERIALS AND METHODS

Leucocytes from thirty-one patients were studied. Twenty-two had chronic lymphatic leukaemia (CLL); seven, chronic myeloid leukaemia (CML); one, acute myeloid leukaemia; and one, acute lymphoblastic leukaemia. The diagnosis was confirmed by bone marrow biopsy in all cases. All patients had a markedly leukaemic blood picture, more than 90% of the peripheral blood leucocytes being neoplastic.

Leukaemia cell migration inhibition test (MIT)

This has been described in detail perviously (Cochran, 1971). Briefly, leukaemic cells migrate actively and this activity is inhibited by antibodies to molecules present at the cell surface (Cochran *et al.*, 1972). Leukaemia cells from the peripheral blood are washed three times and placed in capillary tubes which are then sealed at one end with inert clay (Clay Adams Inc.). The capillaries are centrifuged to displace the cells to the sealed end and then cut at the cell-fluid interface. The capillary stumps are mounted on the base of disposable culture plates (Univers. Mek. Verkstad AB, Sweden), medium with or without additional serum is added, and the plates are closed with a coverslip. After incubation for 18–24 hr at 37°C, migration areas are measured by planimetry.

In this study, migration plates contained: (1) Eagle's minimum essential medium + 10% foetal calf serum (EMF)—*EMF control;* or (2) EMF + 1/100 swine normal serum—*serum control*; or (3) EMF + 1/100 swine antihuman immunoglobulin (IgG, IgM or IgA)—*test.*

The antisera (Fraburg Ltd) used were swine antihuman IgA (anti-α), swine antihuman IgM (anti-μ) and swine antihuman γFc. The sera were tested against IgG coated erythrocytes and only the anti-γFc serum caused agglutination. In immunodiffusion and immunoelectrophoresis the anti-Fc and anti-IgM sera gave single lines against IgG and IgM respectively. Unfortunately the anti-IgA serum was not tested in either of these systems.

A minimum of four capillaries was prepared for assessing cell migration in each antiserum and in the serum and EMF controls. Areas of migration in test antisera were compared to those in serum controls and a migration index (MI) calculated from the ratio:

$$\frac{\text{Mean of migration areas in test}}{\text{Mean of migration areas in serum control}}.$$

Migration areas in tests and controls were compared by the Mann–Whitney U-test of Ranking and significance assessed at the 5% level.

Studies on the nature of cell surface immunoglobulins

In an attempt to confirm the findings with the MIT, and to examine the kinetics of

immunoglobulin release from the leukaemic cells, eluates and lysates were prepared and examined for their immunoglobulin content.

1. *Eluates.* Leukaemic cells were washed three times in phosphate buffered saline (PBS) and incubated in EMF at 37°C for up to 4 hr with frequent intermittent shaking. The suspension was then centrifuged at 800 *g* for 8 min and the supernatant concentrated five-fold by lyphogel (Hawksley, Sussex). For serial elution studies the cells were washed in PBS, and re-incubated in fresh EMF as often as appropriate. The eluates were stored at −20°C until tested.

2. *Lysates.* Leukaemia cells were washed three times in PBS and then resuspended in PBS at a concentration of 3×10^7 cells/ml. The cells in 1 ml of PBS were lysed by alternately freezing and thawing them four times. The lysate was then centifuged and the supernatant stored at −20°C until tested.

Detection of immunoglobulins in eluates and lysates

Antiglobulin consumption tests were used for this purpose.

To detect IgG, serial doubling dilutions of swine antihuman IgG were distributed in rows of tubes. An equal volume of EMF was added to each tube in one row, and equal volumes of the eluates or lysates to each tube in the other rows. After 10 min at room temperature, slide agglutination tests for anti-IgG were performed on the contents of the tubes, using D-positive human red cells sensitized with 'incomplete' anti-D (Ortho Diagnostics). A reduction of titre of 2 or more tubes was accepted as evidence of the presence of IgG in a lysate or eluate.

To detect IgM in the eluates and lysates, use was made of the observation that anti-IgM inhibited the migration of leukaemia cells with IgM on their surface (as evidenced by membrane immunoflourescence as well as migration inhibition) and that this inhibition was neutralized by pre-incubating the anti-IgM with purified human IgM. Eluates and lysates were examined for their capacity to neutralize anti-IgM and rough quantification of the amount of IgM present was possible by reference to the effect of known amounts of pure IgM.

Varying amounts of a 1 in 6 dilution of eluate or lysate (200–5 μl vol.) were added to tubes containing 0·5 cc of a 1/200 dilution of anti-IgM. Controls were anti-IgM plus various amounts of IgM, anti-IgM alone, anti-IgM plus various volumes of the diluent (PBS or EMF) and IgM alone in various amounts. The tubes were mixed, incubated for 1 hr at 37°C and the contents were then tested for their ability to inhibit the migration of IgM positive indicator cells. A statistically significant reduction in migration inhibition (U-test) relative to the effect of anti-IgM alone was regarded as indicative of the presence of IgM in the test material.

RESULTS

1. *Migration in the presence of antisera to immunoglobulins* (Table 1)

(a) *Chronic lymphatic leukaemia.* In fourteen out of nineteen cases, migration was inhibited significantly by one or more of the antiglobulin sera used, indicating the presence of surface immunoglobulins on the leukaemic cells of these cases. IgG only was detected in seven cases, and IgM only in one case. IgG and IgM were detected in three cases, IgG and IgA in one case, while all three immunoglobulins were detected in two cases.

Two or more samples of blood drawn on different dates were investigated in five cases. In three cases the results of repeated examinations were identical. The results for the other two (W.L. and A.D.) are described more fully below.

TABLE 1. Immunoglobulins on leukaemia cells as assessed by inhibition of migration in antisera to human immunoglobulin

	Number of cases	Immunoglobulin present		
		IgG	IgM	IgA
Chronic lymphatic leukaemia (nineteen cases)	2	+	+	+
	3	+	+	−
	1	+	−	+
	7	+	−	−
	1	−	+	−
	5	−	−	−
Chronic myeloid leukaemia (seven cases)	7	−	−	−
Acute leukaemia (two cases)	2	−	−	--

In three additional cases the absolute migration of cells in the EMF and normal serum controls was too small to allow the demonstration of any inhibitory effect of antisera.

(b) *Chronic myeloid leukaemia.* Immunoglobulins were not detected by the MIT in seven cases.

(c) *Acute leukaemias.* Immunoglobulins were not detected in two cases studied.

2. *Absolute migration (AM)* (Table 2)

In the EMF and normal-sera controls, the leukaemic cells from patients with chronic myeloid leukaemia were the most actively migratory leukaemic cells. In addition they covered a larger area in 18 hr than did a similar number of normal polymorphs. The cells of chronic lymphatic leukaemic patients were the least actively migratory of the leukaemias but even they were more active than a similar number of normal peripheral blood lymphocytes.

TABLE 2. Absolute migration of leukaemic and normal leucocytes in medium

Type of cells	No. of cases	Mean area of migration (mm^2)	SD
Chronic lymphatic leukaemia (MIT −ve)	5	11·6	±2·4
Chronic lymphatic leukaemia (MIT +ve)	14	5·2	±2·6
Chronic myeloid leukaemia	3	33·4	±7·2
Acute myeloid leukaemia	1	21·2	—
Acute lymphoblastic leukaemia	1	17·1	—
Normal polymorphonuclear leucocytes (peripheral blood)	1	27·0	—
Normal lymphocytes (peripheral blood)	1	3·1	—

An interesting observation was that cells with no demonstrable immunoglobulins on their surface were more motile in EMF than those showing surface immunoglobulins.

Cells from the acute leukaemias were intermediate in activity between those of CML and CLL.

3. *Elution and lysis studies*

Eluates of leukaemic cells from four patients were prepared at the same time as the migration studies and were examined for immunoglobulins. In two patients studied (J.M. and E.B.) poor absolute migration precluded the use of the MIT to detect surface Ig and therefore only elution and lysis results are available. In the other two cases (A.L. and W.L.), the presence of immunoglobulins in eluates and lysates exactly paralleled the inhibition of migration by anti-Ig sera (Table 3).

TABLE 3. A comparison of the MIT results and the occurrence of Ig in lysates and eluates of CLL cells

Patient	Migration inhibition by		Eluate contains		Lysate contains	
	Anti-IgM	Anti-IgG	IgM	IgG	IgM	IgG
A.D.	+	−	+	−	ND	ND
W.L. small cells (second experiment)	−	+	−	+	ND	ND
W.L. large cells (second experiment)	+	+	+	+	ND	ND
W.L. large cells (third experiment)	+	+	+	+	+	+

ND, test not performed.

4. *Serial studies* (Tables 4, 5 and 6)

In order to detect whether the surface Ig on leukaemic cells represented a secretory product of the cell, or alternatively Ig adsorbed from the plasma serial elution studies were

TABLE 4. Migration of chronic lymphatic leukaemic cells before and after prolonged incubation in medium (migration areas in mm^2)

Case	Preincubation migration	Migration after first incubation	Migration after second incubation
Cells with surface Ig			
W.L.	6·85	6·58 (24 hr at 4°C)	6·85 (48 hr at 4°C)
A.D.	10·0	13·7 (12 hr at 4°C)	
J.McM.	2·06	3·29 (4 hr at 37°C)	2·37 (24 hr at 4°C)
E.B.	1·37	3·00 (4 hr at 37°C)	3·52 (24 hr at 4°C)
Cells with no surface Ig			
(a)	11·4	10·6 (24 hr at 4°C)	
(b)	9·15	7·15 (24 hr at 4°C)	8·00 (48 hr at 4°C)
(c)	10·28	6·87 (24 hr at 4°C)	

TABLE 5. A comparison of inhibition of migration by antisera to immunoglobulins and the presence of immunoglobulins in eluates and lysates of the cells of two patients with chronic lymphatic leukaemia

Patient	Pre-elution migration index in antiserum to		First eluate		First post-elution migration index in antiserum to		Second eluate		Second post-elution migration index in antiserum to	
	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
A.D.	0·69	0·93	+++	−	0·90	ND	ND	ND	ND	ND
W.L. (small cells—F1) (second experiment)	0·81	0·56	−	+	ND	ND	−	−	ND	ND
W.L. (large cells—F2) (second experiment)	0·36	0·80	++	+	ND	ND	+++	−	ND	ND
W.L. (large cells) (third experiment)	0·68	0·71	+++	+	0·44	0·87	++	−	0·68	0·90

ND, test not done.

TABLE 6. Immunoglobulins in the eluates and lysates of cells from two chronic lymphatic leukaemia patients with non-migratory cells

Patient	Pre-elution lysate		First eluate		Lysate after first elution		Second eluate		Lysate after second elution	
	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
J.M.	++	−	+++	−	+	−	−	−	−	−
E.B.	+++	++	+++	+	++	++	+	+	ND	ND

ND, test not done.

performed on cells incubated at 37°C. The opportunity was also taken to determine whether removal of immunoglobulins by elution affected cell motility.

Patient A.D. This patient's cells were tested on two occasions. On the first assay no Ig was demonstrated by the MIT. On the second occasion, when the patient had septicaemia, the migration of patient's cells was inhibited by anti-IgM serum but not by anti-IgG. Following incubation in EMF, the eluate was found to contain a considerable quantity of IgM but no IgG. Migration studies after elution showed an increase in absolute migration, and anti-IgM serum no longer caused migration inhibition. These results suggest that the surface Ig in this case was adsorbed from the plasma.

Patient W.L. This patient was initially diagnosed as having CLL in 1965, when the majority of white cells in his peripheral blood were small lymphocytes. He relapsed in August 1971 when his white count was 800,000/mm^3 and a differential count showed 57% small lymphocytes and 41% lymphoblastoid cells. We first examined W.L.'s cells in November 1971, following treatment with vincristine, chlorambucil and prednisolone. The white count was then 76,000/mm^3 of which 50% were lymphoblastoid. Migration was inhibited by antisera to IgM, IgG and IgA. After incubation at 4°C for 24 hr, however, the anti-IgG no longer caused inhibition, suggesting that any IgG present was not structural.

In January 1972 W.L.'s white count was 150,000/mm^3 and most of the leukaemic cells were lymphoblastoid. The large and small leukaemic cells were partially separated by repeated sedimentation in autologous plasma, providing a first fraction (F1) in which 75% of cells were small lymphocytes and a second fraction (F2) in which 80% of cells were lymphoblastoid.

F1. The migration of these cells was strongly inhibited by anti-IgG and minimally by anti-IgM. Elution studies showed IgG in the first eluate but not in the second. IgM was not demonstrated in either eluate. The eluates were not tested for the presence of IgA.

F2. The migration of the predominantly lymphoblastoid cells was strongly inhibited by anti-IgM and weakly by anti-IgG. IgG was found in the first eluate of these cells but not in the second. IgM was readily detectable in both eluates. These findings suggest that the IgG associated with the cells of both fractions was adsorbed from the plasma. The IgM on the larger cells, however, seems likely to be structural.

Two months later the white count was 200,000/mm^3 and virtually all leucocytes were of lymphoblastoid type. These cells were inhibited by antisera to IgM and IgG. IgG was detectable only in the first eluate while IgM appeared in both the first and second eluates. Anti-IgG had no inhibitory effect on the cells after the first elution while anti-IgM inhibited cell

migration after both elutions. The findings are thus essentially the same as with F2 (above).

The leukaemia cells of three patients did not migrate sufficiently to allow the assessment of migration inhibition by antisera to Ig. We have attempted to identify and analyse the nature of any Ig associated with cells from two of the cases by lysis and elution techniques (Table 6).

Patient J.M. IgG was not demonstrated in any of the lysates or eluates from this patient. IgM, however, was present in considerable amounts in the pre-elution lysate and in the first eluate. Lesser amounts of IgM were present in the lysate prepared after the first elution and in the second eluate. A lysate prepared after the second elution was free of Ig. These results suggest that the IgM had been adsorbed from the plasma, a view supported by a small increase in absolute migration of the cells after the first elution.

Patient E.B. Lysates of this patient's cells contained a constant amount of IgG whether prepared before or after the elution incubation, and the same amount of IgG in the second eluate as in the first, suggesting that the IgG is in this case structural. However, there was much less IgM in the second eluate than in the first and this in conjunction with an increase in absolute migration after the first and second elution (Table 4) suggests that adsorbed IgM may have been eluted.

The increase in migration of immunoglobulin positive cells after incubation is the more striking when compared with the reduced migration of all immunoglobulin negative cells after incubation for a similar period (Table 4).

Analysis of sera for paraproteins

Electrophoresis analysis of the sera of all nineteen CLL patients showed no evidence of significant paraproteinaemia. The sera were examined after storage for 1 year at −20°C during which time they were thawed and refrozen several times. This is recorded since the effect of such treatment on paraproteins is not certainly known.

DISCUSSION

From these results it is concluded that immunoglobulins are demonstrable on the leukaemia cells from most CLL patients by inhibition of migration in anti-immunoglobulin sera and by elution and lysis techniques. Similar treatment of the cells of chronic myeloid leukaemia and acute leukaemia has not demonstrated any cell-associated Ig. The Ig-negative CLLs are of interest as they may represent T cell-derived leukaemias. This possibility is supported by the high level of PHA-induced transformation of the leucocytes of one such case tested. PHA is said to transform T cells preferentially (Greaves & Bauminger, 1971) and previous reports have indicated that CLL cells transform poorly with PHA (Rubin *et al.*, 1969; Bouroncle *et al.*, 1969; Fröland & Stavem, 1972; Smith *et al.*, 1972; König *et al.*, 1972).

Identification of the nature of the Ig on the cells of most cases of CLL is not complete and the situation is probably complex. Wilson & Nossal (1971) observed stability of the density of surface labelling of the cells of three CLL patients by anti-Ig sera during incubation in medium for 1 hr at 37°C and interpreted this as indicating that the Ig was a structural surface component of the cells. Johansson & Klein (1970) reported a patient with CLL whose cells initially had surface IgM and IgG, but which after maintenance in culture retained only IgM, and suggested that while the IgM was probably structural, the IgG was more likely to be adsorbed to cell surface antigen(s).

Our elution studies were based on the observations of Cone *et al.* (1971) who reported that when mouse B lymphocytes are incubated in culture medium at 37°C, surface Ig, like other surface proteins, is continuously shed into the medium, and yet the amount of surface Ig remains constant as a result of continuous synthesis by the cell. By contrast, Ig adsorbed from the plasma was found to decline rapidly in amount in successive eluates. On this basis, our findings suggest the presence of non-structural immunoglobulins on the cells of all four patients tested. However, it should be noted that in the case of the large-cell population from W.L., stable surface IgM was maintained even after elution of IgG. Conversely, E.B.'s cells were found to have a stable surface IgG but the IgM was readily eluted.

The increase in absolute migration of the cells after elution of non-structural Ig is of interest. It has been shown that mouse leukaemia cells treated with antibody to surface antigens *in vitro*, or exposed to such antibodies *in vivo*, are poorly motile by comparison with uncoated cells (Cochran *et al.*, 1972).

The migration studies on the cells of W.L. provide strong evidence of structural surface IgM on his large cells and indirect evidence of structural IgA on his small cells (migration of mixed small and large cells was inhibited by anti-IgA, but a population of predominantly large cells was not inhibited by anti-IgA). From the evidence available, we cannot say whether the two cell populations represent two malignant cell clones, or whether the large IgM positive cells are the precursors of the smaller cells and the change in population due to an arrest in maturation.

It would be of considerable interest to know whether the Ig regarded from elution studies as non-structural consists of antibody reacting specifically with surface components of the leukaemic cells. Leukaemia cell-specific antigens have been described (Fridman & Kourilsky, 1969; Viza *et al.*, 1969; Bach *et al.*, 1969), as have autoantibodies to leukaemia cells, including CLL cells (Yoshida & Imai, 1970).

The detection of immunoglobulins on the surface of CLL cells by means of anti-immunoglobulin sera labelled with fluorescent or radioactive markers, has been reported by several workers (Grey *et al.*, 1971; Pernis *et al.*, 1971; Papamichail *et al.*, 1971; Wilson & Nossal, 1971). This has been regarded as evidence of non-thymic (B cell) origin of such cells, but our elution studies support the view that surface Ig in this situation may be either a structural component, probably indicative of B cell origin, or Ig, possibly specific antibody, adsorbed from the plasma.

Subdivision of cytologically homogeneous chronic lymphatic leukaemias by analysis of the Ig status of their cell membranes may have important prognostic and therapeutic implications. Neither the numbers of patients studied nor the duration of study are sufficient to allow critical clinical correlation with our findings, but it is of interest that the three patients with non-structural IgM on the surface of their leukaemia cells had remained clinically well without treatment, in two cases for more than 5 years.

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