Clin. exp. Immunol. (1973) **13**, 487–496

QUANTITATION OF SURFACE-MEMBRANE AND INTRACELLULAR GAMMA, MU AND KAPPA CHAINS OF NORMAL AND NEOPLASTIC HUMAN LYMPHOCYTES

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(*Received* 22 *August* 1972)

SUMMARY

Using automated haemagglutination and haemagglutination-inhibition methods which are capable of measuring gamma, mu or kappa chains in the nanogram range, we have examined a variety of normal and neoplastic human lymphocyte populations for the amounts of their surface-membrane and internal immunoglobulins. We find IgG to be the predominant immunoglobulin on the surface-membrane and within various normal human lymphocytes, including thymocytes. IgM, in addition to IgG, is found on the surface of tonsil cells. Chronic lymphatic leukaemic lymphocytes consistently had small amounts of surface-membrane immunoglobulin and larger amounts internally (usually IgM), whereas large amounts of immunoglobulin (usually IgM) were present on the surface-membranes and internally in leukaemic lymphosarcoma cells. Both types of cells had the B-specific surface-antigens. Different types of lymphatic leukaemias may represent neoplastic counterparts of different stages of B cell differentiation.

INTRODUCTION

There is currently a great interest in the surface membrane-bound immunoglobulins of lymphocytes. These immunoglobulin molecules are believed to function as specific receptors for antigen and provide a potential mechanism for understanding the processes of antigen recognition, lymphocyte stimulation and clonal expansion, immunologic memory, lymphocyte cooperation and tolerance (Mitchison, 1967; Ada, 1970; Raff, 1971). These membrane-bound immunoglobulins of human lymphocytes have been detected by mixed agglutination (Coombs, Feinstein & Wilson, 1969), and by fluorescent (Pernis *et al.*, 1971; Cooper, Lawton & Bockman, 1971; Papamichail, Brown & Holborow, 1971) or radioautographic (Wilson & Nossal, 1971) methods. Surface membrane and internal IgG content has been quantitated in tissue culture lines of human lymphocytes (Lerner, McConahey & Dixon, 1971) and Fab antigens have been determined on peripheral blood lymphocytes (Smith *et al.*, 1970). In this study we present quantitative data of surface-bound and internal immunoglobulins of various populations of human lymphocytes.

MATERIALS AND METHOD

Lymphocyte preparations

Defibrinated or EDTA samples of venous blood were obtained from normal laboratory worker donors and from adult patients with chronic lymphatic leukaemia (CLL) or lymphosarcoma with peripheral leukaemia (LS). Only patients with high lymphocyte counts ($>20,000/mm^3$) were used to minimize contamination by non-leukaemic cells. The normal and leukaemic lymphocytes were purified by the Ficoll-Hypaque gradient centrifuge method (Boyum, 1968). The lymphocyte preparations from the leukaemic patients were >95% pure by morphologic criteria following Giesma staining, as well as the absence of a non-specific esterase reaction, which identifies monocytes (Yam, Li & Crosby, 1971) on cyto-centrifuge preparations. However, the lymphocyte preparations from normal blood usually required further purification to remove the 15–30% contaminating monocytes. This was accomplished by one of three methods: (a) the partially purified lymphocytes could be layered on a second gradient composed of a lower layer of the usual 9% Ficoll-Hypaque and an upper layer of 4% Ficoll-Hypaque. After centrifugation, the monocytes remained on the surface and the lymphocytes sedimented to the interphase of the two layers; (b) the monocytes could be removed by addition of autologous serum and passage over washed-nylon fibres (Leuko-pak, Fenwal) at 37°C (Walford, Gallagher & Troup, 1965); or, (c) the monocytes were allowed to phagocytize gum acacia-coated iron particles in the presence of autologous serum at 37°C and were then removed with a magnet (Levine, 1956). Each of these methods yielded normal lymphocyte preparations of at least 93% purity.

Thoracic duct lymphocytes (TDL) were obtained from three patients who were cannulated prior to renal homograft in an attempt to deplete the T lymphocytes. The TDL were sedimented in an International Centrifuge PR-2 at 3000 *g* for 15 min. Single cell suspensions were made from surgically removed tonsils, from patients from 3 to 11 years of age, and from portions of normal thymus glands removed at the time of thoracic surgery, from patients from 1 to 15 years of age, by gently pushing minced tissue through a nylon tea strainer.

All lymphocyte preparations were washed at 4°C a total of eight times, each with 25 vol. (twice with Hanks-0·1% bovine serum albumin, once with 0·83% NH_4Cl with 0·2% BSA to lyse any red cells, and five times with veronal saline buffer-0·1% BSA). Viability, by eosin exclusion, was usually greater than 95% and never less than 90%.

Antisera

Anti-gamma, -mu and -kappa antisera were raised in New Zealand white rabbits by immunization with: gamma chains of normal human IgG (prepared in Professor R. R. Porter's laboratory); mu chains prepared from a purified human cold agglutinin (Cooper, 1968); or with normal light chains made from the human IgG. The resulting antisera were rendered chain-specific by suitable absorption with purified human normal, myeloma and Waldenström immunoglobulins. These antisera were decomplemented by heating at 56°C for 30 min, were exhaustively absorbed with human red cell ghosts and were passed through a 0·45 mμ Millipore® filter. The anti-gamma, -mu and -kappa sera were diluted to 1/50, 1/200 and 1/100, respectively, with 0·05 M phosphate-buffered saline pH 7·1 with 0·5% BSA (PBS-BSA) prior to use in the lymphocyte absorption step.

Absorption of antisera by lymphocytes

Six aliquots of 5×10^6 to 2×10^7 purified, washed and viable lymphocytes from each preparation were pelleted by centrifugation at 4°C in conical tubes and the supernatants thoroughly removed. Three of the pellets were lethally frozen at −80°C for 10 min and then thawed. Two hundred microlitres of diluted anti-gamma, anti-mu or anti-kappa were each added to one viable and to one frozen–thawed lymphocyte pellet and all tubes were mixed at 4°C overnight. A control tube for each lymphocyte preparation consisted of 500 μl of the PBS-BSA added to a pellet of 5×10^7 viable lymphocytes and mixed overnight at 4°C. The next morning all seven tubes for each lymphocyte preparation were centrifuged and a portion of the supernatant removed for testing of residual anti-immunoglobulin activity in the Technicon AutoAnalyzer. The cell pellets were again tested, and those which had been frozen were 100% dead, and the others had no significant loss of viability.

Quantitation of immunoglobulins with the AutoAnalyzer

The methodology involved in quantitating gamma, mu and kappa chains with the Technicon AutoAnalyzer will be published in detail elsewhere. Briefly, two basic quantitative haemagglutination methods were employed. In the first method, used for quantitation of mu and kappa chains, a purified human IgM-kappa cold agglutinin (100 ng/ml) was used to agglutinate normal PBS washed human red cells in a continuous flow system. Agglutination could be prevented by the addition of the anti-mu (final dilution 1/2000) or anti-kappa (final dilution 1/1000). The inhibition by the anti-mu could be reversed and agglutination restored in a step-wise quantitative manner by the prior addition of increasing amounts (5–100 ng) of a standard purified 19S IgM or 8S IgM subunits. The inhibition by the anti-kappa could likewise be reversed by type kappa IgG or IgM standards (5–100ng).

In the second method, used for the quantitation of gamma chains, washed group 0 positive red cells were lightly coated with an incomplete IgG hyperimmune anti-D sera, at 37°C, and the cells were then washed eight times with PBS. These IgG-coated cells could then be agglutinated by aliquots of the anti-gamma (final dilution 1/500) in the continuous flow system, and this agglutination could be inhibited in a quantitative step-wise manner by the prior addition of increasing amounts of a standard IgG (1–100 ng).

Using these automated systems, the amounts of lymphocyte-associated gamma, mu and kappa chains could be quantitated from the diminution in the activity of the respective specific antisera following lymphocyte absorption. The quantity of specific antisera absorbed by intact viable lymphocytes indicated the quantities of each surface-membrane immunoglobulin chain. It is our experience that absorptions must be carried out at 4°C and that lymphocyte viability must be high both before and after absorption for meaningful quantitation of cell-surface immunoglobulins. Aliquots of the PBS supernatants of the control lymphocyte pellets were added to aliquots of each antiserum in order to measure any immunoglobulin not bound to the lymphocytes and any such immunoglobulin found was subtracted from the membrane-bound values. The quantity of antisera absorbed by the frozen–thawed lymphocytes indicated the quantities of total (membrane plus internal) lymphocyte immunoglobulin of each chain type. The quantity of each internal immunoglobulin chain was obtained then by subtracting the membrane content from the total immunoglobulin chain value. All immunoglobulin data were expressed as nanograms of the specific chain per 10^7 cells.

The quantitation of human gamma, mu and kappa chains by the haemagglutination and

haemagglutination-inhibition methods proved extremely sensitive and reproducible. Gamma chain could be measured in amounts as low as 1 ng, mu chains as low as 4 ng and kappa chains as low as 2 ng with the reproducibility in each system of about $\pm 10\%$ for soluble free immunoglobulin and about $\pm 20\%$ for lymphocyte-associated immunoglobulin. The lymphocyte absorbed antisera and the control supernatants could be kept for up to several weeks and a large number tested at one time along with aliquots of antisera absorbed overnight with purified immunoglobulin standards. Specificity in each system was doubly insured by the specificity of each anti-immunoglobulin and by the chain restriction of each anti-red cell antibody. The three systems allowed multiple cross-checks for specificity. As further confirmation of the validity of the techniques, the surface immunoglobulins of several different numbers of cells were measured and approximately the same value was obtained when expressed as ng per 10^7 cells.

RESULTS

Normal peripheral blood lymphocytes (PBL)

All of the lymphocyte preparations tested (Table 1) had measurable amounts of surface-membrane gamma chains with moderate variability amongst lymphocytes from different donors (4–23 ng gamma chains per 10^7 lymphocytes). Kappa chains were measurable on the surface of those normal lymphocytes having the higher gamma values. Mu chains could not be detected and, from the known sensitivity and number of cells tested, it could be calculated that the maximum average amount of mu chains which could have gone

TABLE 1. Quantitation of surface-membrane and internal immunoglobulin chains*

Lymphocyte specimen	Surface-membrane			Internal		
	Gamma	Mu	Kappa	Gamma	Mu	Kappa
Normal Peripheral Blood						
1	23	<5	6	33	<5	22
2	4	<7	<3	31	<7	20
3	19	<8	4	93	33	28
4	10	<8	<3	52	<8	18
5	4	<6	<3	58	<6	22
6	13	<8	8	>50	<8	36
7	6	N.D.+	N.D.	34	N.D.	N.D.
Mean	11	<7	<5	50	<11	24
Tonsil						
1	18	20	13	120	20	27
2	30	25	30	150	0	40
3‡	30	5	25	150	0	50
4‡	25	5	25	150	0	50
5	50	<7	25	300	0	75
6	38	12	15	230	0	>35
7	26	19	13	295	10	87
8	18	10	14	190	0	22
9	20	23	21	310	7	180
Mean	28	13	20	210	4	63

Thoracic duct						
1	<1	<4	<3	7	<4	4
2	<1	<3	<3	5	<3	5
3	<1	<7	3	20	<7	8
Mean	<1	<5	<3	11	<5	6
Thymus						
1	7	<4	2	33	<4	7
2	1	<4	<2	8	<4	5
3	<1	<4	<2	22	<4	9
4	8	<4	5	25	<3	5
5	2	<3	<2	5	<3	0
6	4	<2	<2	9	<2	0
7	5	<2	<2	30	<2	10
Mean	4	<3	<3	19	<3	5
Chronic lymphatic leukaemia						
1	7	<7	<3	N.D.	N.D	N.D.
2	<1	4	6	N.D.	N.D.	N.D.
3	8	<7	3	N.D.	N.D.	N.D.
4	4	<7	<3	7	63	<6
5	1	<7	2	6	135	50
6	<1	<7	<3	3	75	<3
7	2	<7	10	30	110	50
7, 6 weeks later	4	7	7	10	185	43
8	5	<7	3	41	<14	25
9	<1	<7	<3	<1	<14	<6
9, 3 months later	4	<7	<3	9	<14	<6
Lymphosarcoma						
1	3	60	<3	7	100	<3
2	8	90	40	12	60	40
3	<1	51	<3	<1	18	<3
4	3	19	<3	5	5	<3
5	8	30	25	27	<7	<3
6	>15	<7	5	>15	<7	10

* Values are for ng/10^7 cells.
† N.D. Not done.
‡ Identical twins.

undetected on the surface was 7 ng/10^7 cells. There was a much larger amount of intracellular gamma and kappa chain, an average of 50 and 24 ng/10^7 cells, respectively. Only one of the normals had appreciable amounts of internal mu chain; this finding was confirmed on repeat measurement.

Tonsil lymphocytes

The tonsil cells consistently had large amounts of surface-membrane immunoglobulin chains with an average of 28 ng of gamma, 13 ng of mu and 20 ng of kappa/10^7 cells. Very large amounts of internal gamma and kappa chains were consistently found with an average of 210 and 63 ng/10^7 cells, respectively. In contrast, internal IgM was either small or non-detectable in most cases.

Thoracic duct lymphocytes (TDL)

The three preparations showed a consistent pattern of little or no detectable surface-membrane immunoglobulin and a small amount of internal gamma and kappa chains (average of 11 ng and 5 ng/10^7 cells, respectively).

Thymocytes

Small amounts of gamma chain were demonstrated on the surface of all but one of the thymus lymphocyte preparations and kappa chain was present on the two preparations having the most gamma. Larger amounts of internal IgG were always found, but in no instance could mu chains be detected on the surface or internally, even when a very high ratio of cells to anti-mu was used.

Additional confirmatory studies were performed in which 10^9 washed, viable human thymocytes were suspended in Tris buffer (pH 8·2) frozen and thawed four times, spun at 20,000 *g* for 15 min and the supernatant passed through a 0·45 mμ Millipore® filter. This supernatant was put on a 110 × 2·5 cm Sephadex G-200 column which earlier had been calibrated with purified 19S IgM, IgG and albumin. Ten millilitre fractions were taken and a 200-μl aliquot of each tube was tested using the AutoAnalyzer for gamma and mu chains. A sharp peak of gamma chains was found in the anticipated amount at the known point of elution of IgG. None of the tubes had mu activity.

Chronic lymphatic leukaemia (CLL)

All of the lymphocyte preparations had either small amounts or undetectable surface-membrane immunoglobulin. However, all but one of the preparations examined for internal immunoglobulin had moderate to large amounts of immunoglobulin which was predominantly either mu (four patients) or gamma (one patient). Approximately equivalent amounts of kappa to heavy chains were found in three of these, while no kappa was found in two cases; these immunoglobulins later were presumed to be lambda type. Patient 9 had no detectable surface or internal gamma, mu or kappa chains on the first examination and only a small amount of gamma on repeat examination 3 months later. We are currently examining the possibility that this patient could have an IgA-lambda intracellular immunoglobulin.

Lymphosarcoma

In contrast to the CLL lymphocytes, the lymphocytes from patients with lymphosarcoma and leukemia had moderate to large amounts of surface-membrane immunoglobulin which was predominantly mu in five patients and gamma in one patient. Approximately equivalent amounts of kappa to heavy chains were found on the surface membranes of three of these patients and were absent from the surface of three, these presumably having lambda chains. Moderate to large amounts of internal immunoglobulin heavy chain were found and usually corresponded to the class found on the surface. An interesting exception was patient 5 who had mainly mu chain on the surface and gamma chain internally. A large lymphosarcoma-infiltrated spleen had been surgically removed 2 weeks previously, and the patient had developed the leukaemia for the first time post-operatively. Her lymphocyte count was 50,000/mm^3 at the time this specimen was obtained.

DISCUSSION

We have measured gamma, mu and kappa chains on the surface-membrane and inside various populations of human lymphocytes. These values have been expressed as nanograms (ng) of each chain per 10^7 cells. For purposes of discussion, we can assume that the gamma and mu chains are bound to the equivalent amount of kappa chains or lambda chains and can convert the values for ng of gamma or mu heavy chains to ng of IgG or IgM per 10^7 lymphocytes. Our values for kappa chains relative to heavy chains were consistent with this assumption.

We found that each type of lymphocyte examined had a consistent pattern with considerable differences amongst the different populations. Normal PBL had an average of about 15 ng of surface-membrane IgG and about 75 ng of intracellular IgG per 10^7 lymphocytes. We were unable to detect mu chains on the surface, but could determine that there was <10 ng of surface-membrane IgM/10^7 cells. One of the normal preparations, from the oldest normal donor, had an internal mu chain equivalent of about 45 ng of IgM. The other PBL preparations had less than 10 ng IgM/10^7 cells. Tonsil cells generally had larger amounts of surface IgG (average 40 ng/10^7 cells), had measurable amounts of surface IgM (average about 17 ng IgM/10^7 cells) and very large amounts of internal IgG (about 300 ng/10^7 cells). Thoracic duct lymphocytes had little or no detectable surface-membrane immunoglobulin, but had an average of 17 ng of intracellular IgG. In contrast, thymic lymphocyte preparations had an average of 6 ng/10^7 cells of surface-membrane IgG and 25 ng/10^7 cells of intracellular IgG. No IgM was detectable on or within TDL or thymocytes even when these were tested at a high cell to anti-mu ratio.

Fluorescent studies on normal PBL (Pernis *et al.*, 1971; Cooper *et al.*, 1971; Papamichail *et al.*, 1971; Siegal, Pernis and Kunkel, 1971; Froland, Natvig and Berdal, 1971; Grey, Rabellino and Pirofsky, 1971; Preud'homme *et al.*, 1971) have varied considerably in the total percentage of surface-immunoglobulin-positive cells, and the immunoglobulin class distribution (IgG predominant-versus-IgM predominant). It is likely that there are subpopulations of B cells, some having a higher density and some with absent or low density of each surface immunoglobulins. If the assumption is made, based on the various fluorescent studies, that the high-density IgG or IgM surface-bearing populations each consists of about 10% of the total PBL, one can calculate from the ng/10^7 data and from the molecular weight for IgG and 8S IgM (Parkhouse & Askonas, 1969; Vitetta, Baur & Uhr, 1971) that there would be an average of 60,000 molecules of surface IgG and fewer than 35,000 molecules of surface 8S IgM per cell on the high density subpopulations. It cannot be said whether the internal IgG is similarly distributed.

Our finding of larger amounts of surface IgG and measurable amounts of surface IgM on the tonsil cells, as compared with PBL, indicates either a higher density of surface immunoglobulins is present on the positive cells or that surface immunoglobulin is present on a larger subpopulation of the B cells. A recent study (Zucker-Franklin & Berney, 1972) using an EM hybrid rosette technique, showed that the surface-membrane immunoglobulin-positive cells were also restricted to a small subpopulation of the total tonsil lymphocytes. This study also showed that tonsil cell suspensions contained only 2–10% plasma cells, half of which had surface immunoglobulins by the rosette method. Our finding of surface IgM, but low or absent intracellular IgM, might indicate that some of the IgM-bearing cells have internal IgG (Pernis *et al.*, 1971).

Our finding of significant amounts of surface-membrane and internal IgG in thymocytes has bearing on the present controversy concerning the nature of the antigen receptor on T cells. Although there have been functional demonstrations that the receptors are immunoglobulin (Basten *et al.*, 1971; Mason & Warner, 1970; Greaves, Torrigiani & Roitt, 1971; Lesley, Kettman & Dutton, 1971) most morphologic studies fail to demonstrate these or find only a small fraction of positive cells (Perkins, Karnovsky & Unanue, 1972; Hammerling & Rajewsky, 1971; Gonatas *et al.*, 1972). Using the similar methods for surface radioiodinating thymocytes and attempting to identify heavy and light immunoglobulin chains from solubilized cells, Vitetta *et al.* (1972) fail to identify these molecules, whereas Marchalonas, Atwell & Cone (1972) have found light chains and heavy chains on human and murine thymocytes. Using a sandwich autoradiographic method (Nossal *et al.*, 1972) small numbers of immunoglobulin molecules have been demonstrated on a high percentage of murine thymocytes.

Although we cannot say whether the IgG we find on and within thymocytes is present on a small subpopulation or present at a low density on a large percentage of cells, our findings provide an independent demonstration that there are appreciable amounts of thymocyte-associated immunoglobulins which we can measure and isolate. The amount of IgG found on and within thymocytes (about one-third the level of PBL) would appear to rule out their origin from a B cell population resident within the thymus unless this population of B cells had a much larger amount of IgG per cell than PBL. On the other hand, our failure to detect surface-membrane immunoglobulin on TDL would indicate that if the IgG was present in small amounts on large numbers of thymocytes, it was subsequently lost or greatly diminished upon peripheralization, analogous to some other thymic antigens (Raff, 1971).

Turning to the leukaemic lymphocytes, there was a consistent difference between chronic lymphatic leukaemic cells and lymphosarcoma cells, both of which have been shown to be B cells (Wortis *et al.*, in preparation). Despite the fact that immunofluorescent (Pernis *et al.*, 1971; Papamichail *et al.*, 1971; Grey *et al.*, 1971; Preud'homme *et al.*, 1971) and radioautographic (Wilson & Nossal, 1971) studies on CLL cells have shown that most preparations have a high percentage of surface-immunoglobulin-positive cells, our finding of small amounts of surface-immunoglobulin per 10^7 cells indicates that each cell would have no more than a few thousand immunoglobulin molecules. In contrast, the CLL cells had large amounts of intracellular immunoglobulin which was predominantly one class of heavy chains (usually mu) and an equivalent amount of kappa chains or else absent kappa chains (presumably lambda present). A recent report of two atypical cases of CLL (Hurez, 1972) describes large amounts of intracellular IgM. Our findings indicate that unreleased IgM is present in most CLL preparations even though it is not detectable by routine fluorescent staining (Preud'homme *et al.*, 1971). The LS cells had much larger amounts of surface-membrane immunoglobulin which was likewise restricted in chain composition and usually IgM. Our findings of the difference in surface-membrane immunoglobulin content of the CLL and LS lymphocytes are especially interesting in view of the morphologic differences in the two cell types (Schrek, 1972). Our findings on the LS cells are analogous to findings in some cases of Burkitt lymphoma (Sherr *et al.*, 1972).

We have recently studied a group of patients with acute lymphatic leukaemia. Despite the fact that these lymphocytes can be shown to usually be B cells (to be published), they have very small amounts of surface and moderate amounts of internal IgG. Thus, there appear

to be different types of B cell leukaemia which differ morphologically and with regard to their surface-membrane and intracellular immunoglobulins. It is possible (Pernis *et al.*, 1971) that each type is a neoplastic analogue of B cells in different stages of differentiation. The ALL cells could represent a precursor B cell with a low density of surface receptors; the lymphosarcoma cells would be analogous to stimulated B cells with a higher density of IgG or IgM receptors such as are found in the tonsil; and the CLL cells could represent a pre-secretory B cell with lower surface, but higher internal immunoglobulin. The myeloma and Waldenström cells would, of course, be the counterpart of the final antibody secreting B cells. It is possible that agents may be found which act selectively on B cells or on different stages of differentiation of B cells, and that these might be of therapeutic usefulness in the treatment of the various neoplasms of human lymphocytes.

ACKNOWLEDGMENTS

We thank Miss Virginia MacCarthy, Dr D. Billig, Dr W. Maloney, Dr R. Handin, Dr J. Mitus, Dr N. Jaffe, Dr J. Gozzo, Dr W. Chasin, Dr R. Schwartz and Dr P. Levine for their help in providing specimens for this study. This study was made possible through the generous loan of AutoAnalyzer equipment by the Technicon Instrument Corporation.

We thank Miss Julia Johnson for her excellent technical assistance, and Dr M. H. Flax, and Dr S. Leskowitz for helpful discussion and support.

This study was supported by U.S. Public Health Grants AI 10071 and AM 15369 and by a grant from the Massachusetts Chapter of the American Cancer Society.

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