CHARACTERIZATION OF IMMUNOGLOBULIN STRUCTURES FROM THE SURFACE OF CHRONIC LYMPHOCYTIC LEUKEMIA CELLS*

By TROND ESKELAND,‡ M.D., EVA KLEIN,§ M.D., MASAHARU INOUE,‖ M.D., AND BO JOHANSSON,¶ M.D.

(*From the Anatomical Institute, University of Oslo, Oslo 1, Norway*)

There is evidence that normal lymphoid cells synthesize and accumulate immunoglobulin structures on their surface (1, 2). Much of the evidence is provided by experiments in which anti-immunoglobulin antibodies were found to react with the cell surface. By these studies antigenic structures of the various parts of serum immunoglobulin molecules have been detected on lymphoid cells. There is, however, scanty additional information on the relationship between the surface structures and serum immunoglobulin and how the former are incorporated and kept in the surface membrane.

Recently, it has been found that cells from some Burkitt tumors (3–5) and chronic lymphocytic leukemias (1, 6) have IgM[1] heavy chain (mu chain) and kappa light chain structures on the surface in sufficient amounts to be demonstrated by fluorescein-labeled antibodies in fluorescence microscopy. The intracellular amounts of immunoglobulins were apparently very small. Mu and kappa structures have also been demonstrated on normal cells by the same technique (1, 7, 8).

The major fraction of IgM in human serum has a sedimentation coefficient of about 19S and the molecules consist of five subunits (9) bound together by disulfide bonds. The subunits have a sedimentation coefficient of about 7S and are composed of two mu and two light chains linked together by disulfide as well as noncovalent bonds. A minor fraction of IgM in normal serum is similar in size to 7S IgM (10, 11). In cells engaged in 19S IgM secretion, 7S IgM and only small amounts of 19S IgM were found (12).

In order to learn more about surface immunoglobulin structures, the leu-

kemia cells from one of the patients mentioned above have been studied. Mu and kappa structures have been released and their size determined. A preliminary report has been presented elsewhere (13).

Material and Methods

Human 19S IgM.—IgM with kappa chains was purified in large scale from the sera of patients with Waldenström's macroglobulinemia by zone electrophoresis using Pevikon (Kema Nord, Stockholm, Sweden) as supporting medium (14), followed by gel filtration on Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden).

Human 7S IgM.—By reduction of purified 19S IgM with 0.2 M 2-mercaptoethanol, followed by alkylation with iodoacetamide, 7S IgM was obtained (15). The material was finally passed through Sephadex G-200 to ascertain that only 7S IgM was present.

Human Kappa Chains.—Kappa chains were isolated by chromatography of the 7S IgM on a Sephadex G-100 column equilibrated with 1 N propionic acid (16).

Human IgG.—A solution of Kabi gamma globulin 16.5% (Kabi, Stockholm, Sweden) was used.

By double diffusion in gel and immunoelectrophoresis antibodies against transferrin, IgM, IgG, kappa chains, lambda chains, α_2-macroglobulin, and human serum proteins revealed no impurities in the IgM (4 mg/ml) and kappa chain (0.5 mg/ml) preparations.

Rabbit 7S Gamma Globulin.—Rabbit gamma globulins, more than 98% pure on electrophoresis (Mann Research Laboratories Inc., New York) were used. The protein was passed through a Sephadex G-200 column. Two smaller protein peaks emerged first, then a large peak. The latter peak had the position in the elution diagram characteristic for human 7S gamma globulin. Fractions from this peak were pooled, concentrated to 7 mg/ml, and labeled with ^{125}I using an electrolytic method (17).

After determination of the labeling efficiency (15), the solution was dialyzed in order to remove free iodine. As a final purification step the protein solution was run in the ultracentrifuge on a sucrose density gradient. In experiments to be described, only material from the 7S peak was used.

The labeling efficiency was 84%, mean iodine content 0.8 moles/mole of protein.

Antisera.—Rabbit immunoglobulin preparations from Dakopatts A/S, delivered by Brostex A/S, Copenhagen, Denmark, against human serum proteins, IgG, IgM, kappa chains, and lambda chains, were used. Rabbit antisera against transferrin and α_2-macroglobulin were supplied by Behringwerke AG, Marburg-Lahn, Germany.

The reagents were tested against the protein preparations described above and normal human serum by immunoelectrophoresis and double diffusion in gel. As the anti-IgM reagent reacted with IgM, but not with IgG or free kappa chains, this reagent will be designated anti-mu reagent or anti-mu antibodies.

For fluorescence microscopy, fluorescein-labeled goat globulins from Hyland Laboratories, Los Angeles, Calif., against human kappa chains and IgM were used. The latter reagent will also be designated anti-mu reagent or anti-mu antibodies. The reactivity could not be blocked by free kappa chains. For the detection of human HL-A transplantation antigens, fluorescein-conjugated globulins from the serum of a patient (Berlin) who had obtained multiple transfusions was used. The serum was received from Dr. Dausset.

Protein Determinations.—Protein determinations were made by a modified Folin technique (18) or by optical density at 280 nm. The standard curves were obtained with samples from Kabi's 16.5% gamma globulin solution. The protein content was assumed to be 165 mg/ml.

Gel Diffusion Techniques.—The Ouchterlony technique was used for double diffusion, employing 1% agarose in 0.11 M Na-barbital-HCl buffer, pH 8.6. The wells were 3 mm in

diameter, with a center-to-center distance of 7 mm. Immunoelectrophoresis was performed by the technique of Wadsworth and Hanson (19).

Leukemia Cells.—Leukemia cells with mu and kappa structures on the cell surface were obtained from the blood of T. P., an 82 yr old male with chronic lymphocytic leukemia. His case report (6) and a study of the immunoglobulin structures on the cell surface (1) have recently been published. In the electron microscope, the cells were similar to lymphoblasts (20). At the time of this study the leukocyte count in the peripheral blood was about 400,000 per mm^3, most of the cells being small lymphocytes.

For the purification of white blood cells, fresh blood (with citrate added) was used. 3 volumes of blood were mixed with 1 volume of 3% dextran T-250 (Pharmacia Fine Chemicals) in 0.15 M NaCl. The suspension was incubated at 37°C for half an hour. After incubation, the upper layer with mostly leukemia cells was removed and the cells were washed one to two times in Hanks' balanced salt solution (BSS)^2 containing 1% gelatin (BSSg). About 1 ml of packed cells with a concentration of about 2.5 × 10^9 leukocytes/ml was usually obtained from 10 ml of blood. In order to lyse the remaining erythrocytes, a solution containing 0.14 M NH$_4$Cl and 0.01 M ethylenediaminetetraacetate (EDTA) was added (21). The suspension was placed at 4°C for 10 min; the leukocytes were then sedimented by centrifugation at low speed. The treatment was repeated once if some erythroctyes were still intact. The purified leukocytes were washed three times in BSSg and finally once in BSS. Most of the leukocytes were still viable after this procedure, as judged from their ability to exclude trypan blue.

In the later stages of this study the erythrocytes were removed by NH$_4$Cl-lysis only. The yield of leukocytes increased somewhat wiht this treatment.

Homogenization and Subcellular Fractionation.—The procedures were performed at 0–5°C. Usually 9 ml of a solution containing 0.033 M tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 8.0, and 0.005 M MgCl$_2$ was added to 1 ml of viable or frozen and thawed cells. The suspension was homogenized in a Potter-Elvehjem homogenizer run at 1800 rpm. The pestle diameter was 2.4 cm. The homogenization was finished when most of the nuclei seemed to be free of cytoplasm as examined by phase contrast microscopy, usually after 50 strokes. The homogenate was then centrifuged at a speed sufficient to sediment nuclei and unbroken cells, that was about 2000 g^3 for 10 min. The precipitate was designated Prec. 1a, the supernatant Sup. 1a. Tris buffer was added to Prec. 1a in the original volue, and the homogenization and centrifugation was repeated. Sup. 1b and Prec. 1b were then obtained (Fig. 3).

For the sedimentation of all cellular particles (22), Sup. 1a and Sup. 1b were subjected to ultracentrifugation in a Spinco type 65 fixed angle rotor for 2 hr at 35,000 rpm giving about 105,000 g. The supernatants, called Sup. 2a and Sup. 2b, were then removed, while Prec. 2a was suspended in Tris buffer in the original volume and subjected to 50 strokes in the Potter-Elvehjem homogenizer. After homogenization, the solution was subjected to ultracentrifugation at 105,000 g for 2 hr. Prec. 2a was thus divided in Sup. 2c and Prec. 2c (Fig. 3). Finally, Sup. 2a, Sup. 2b, and Sup. 2c were concentrated about 10 times by vacuum dialysis using a Collodion filter No. 13200 (Sartorius-Membranfilter GmbH, Göttingen, West Germany).

Initially, the sedimentation of material in Sup. 1a was performed by running at 5000 g for 15 min, followed by runs for 30 min each at 5000, 10,000, 20,000, 40,000, and 80,000 g. After each run, the precipitate was dissolved in a small volume of Tris buffer, while the supernatant was subjected to further centrifugation.

For quantitation of mu and kappa structures (see below), small samples were taken from

---

^{2} *Abbreviations used in this paper:* BSS, Hanks' balanced salt solution; BSSg, BSS containing 1% gelatin; FS, fluorescence staining; PBS, phosphate-buffered saline, PBSg, PBS containing 0.1% gelatin; PHA, passive hemagglutination.

^{3} The g values given in this article are the values obtained at the bottom of the tubes (g_{max}).

the solution of frozen and thawed cells, homogenate, supernatants, and precipitates. With the exception mentioned above, the samples from the precipitates were taken after the precipitates had been resuspended to the original volume and homogenized.

Extraction by Washing of Frozen and Thawed Cells.—Cells frozen and thawed once were suspended in BSSg to a 20% solution and kept on ice for 15 min. The cells were then sedimented at 2000 g for 10 min. The precipitate was resuspended in the original volume and the treatment repeated. After centrifugation, the precipitate was dissolved in Tris buffer in the original volume and homogenized in a Potter-Elvehjem homogenizer as described. The solution was then run at 2000 g for 10 min. Finally, the three supernatants were run at 105,000 g for 2 hr and the mu and kappa structures quantitated.

Mercaptoethanol Extraction.—To 1 ml of packed viable cells was added 1.5 ml 0.1 M 2-mercaptoethanol in 0.1 M Tris-HCl buffer, pH 7.2. After 1 hr at 37°C with agitation, the supernatant obtained after sedimentation at 800 g for 10 min was removed and the treatment repeated once. The two supernatants were pooled and dialyzed against 120 ml 0.4 M iodoacetamide in 0.1 M Tris-HCl buffer, pH 7.2. The supernatant was then fractionated by adding solid $(NH_4)_2SO_4$. The precipitate formed between 15 and 40% saturation was sedimented, dissolved in 0.4 ml 0.1 M Tris-HCl buffer, pH 7.2, and dialyzed against BSS. Mu and kappa structures were assayed in the final solution.

Density Gradient Ultracentrifugation.—The centrifugation was performed by using 4.4 ml of a 40-10% sucrose gradient in 0.07 M Tris-HCl buffer, pH 8.0, and a Spinco swinging bucket-type SW 65 rotor at 5°C for 17 hr at 36,000 rpm giving about 140,000 g.

Gel Filtration.—After concentration Sup. 2a was filtrated on a 1 × 100 cm Sephadex G-200 column, equilibrated with a solution containing 0.3 M NaCl, 0.01 M boric acid–NaOH, pH 8.0, and 0.02% NaN_3. The flow rate was approximately 2 ml/hr.

Inhibition of Passive Hemagglutination.—For detection and quantitation of mu and kappa structures in the various cellular fractions inhibition of passive hemagglutination was performed.

IgM-coated cells were prepared as follows: to a 3% solution of formalinized sheep red blood cells in 0.135 M NaCl and 0.01 M phosphate buffer, pH 7.0 (PBS), was added an equal volume of a 0.005% solution of tannic acid (E. Merck AG, Darmstadt, West Germany) and the suspension was incubated at 37°C for 10 min. After washing three times with PBS and resuspending the cells in the original volume, an equal volume of PBS containing 0.5 mg 19S IgM/ml was added, and the suspension was incubated for 15 min at 37°C. The cells were then washed three times with PBS containing 0.1% gelatin (PBSg), and were finally resuspended in PBSg in the double of the original volume.

The inhibition tests were performed in disposable plastic trays (Linbro Chemical Co., New Haven, Connecticut, model MVC-96 with V-shaped 0.3 ml volume cups). 1 drop of anti-mu reagent diluted $\frac{1}{1000}$ to $\frac{1}{2000}$ with PBSg, or 1 drop of anti-kappa reagent diluted $\frac{1}{200}$ to $\frac{1}{500}$, and 1 drop of PBSg was added to 1 drop of undiluted or doubling dilutions of test material. After incubation for 45 min at 37°C, 1 drop of the solution of IgM-coated cells was added, and the incubation continued for 45 min. The trays were then placed at room temperature and read the next morning. In the cases where the test material contained particulate material, equal volumes of the test material, PBSg, and antibody reagent were added to small centrifuge tubes. After incubation, the tubes were run at 2200 g for 15 min, and three drops of the supernatants from each tube transferred to the trays. The procedure was then continued as described above.

By comparison of the inhibition efficiency of the test material in serial dilutions and known amounts of IgM or kappa chains, the amounts of mu and kappa structures were quantitated. In these quantitations only cups with complete inhibition were counted.

The dilution of anti-mu and anti-kappa reagents were the highest dilutions giving complete agglutination of the test cells when no inhibitory material was present.

Inhibition of Fluorescence Staining.—Inhibition of the reaction of viable leukemia cells with

fluorescein-labeled anti–HL-A, anti-mu, or anti-kappa reagents was also assayed in some instances. The antibody reagents were incubated with test material before the leukemia cells were added (for details see reference 1). At least two antibody dilutions were used and selected as the highest dilutions giving bright staining of the cells in the absence of inhibitory material. Comparable results were usually obtained with this method and with inhibition of passive hemagglutination.

RESULTS

Liberation of Mu and Kappa Structures from the Cells by Homogenization.—After homogenization of viable or frozen and thawed cells, the homogenate was exposed to 2000 g for 10 min. The supernatant (Sup. 1a) which was free of nuclei and unbroken cells was found to contain mu and kappa structures by using inhibition of passive hemagglutination or fluorescence staining. The supernatant also inhibited the staining of viable leukemia cells with fluorescein-conjugated antibodies against HL-A antigens, showing that mu and kappa structures were not the only membrane structures liberated.

The size of the immunoglobulin structures in Sup. 1a was investigated by differential centrifugation as shown in Table I. Although the efficiency of the various fractions in inhibiting anti-mu antibodies in passive hemagglutination and fluorescence staining did not correspond exactly, there seems to be an increasing sedimentation of mu and HL-A antigens as the g value increased. But even 80,000 g for 30 min left mu structures in the supernatant.

Since the membrane fragments carrying mu structures were sedimented at a broad range of g values, the centrifugation of the homogenate was simplified to include a run at 2000 g for 10 min, followed by an ultracentrifugation at 105,000 g for 2 hr. The ultracentrifugation is supposed to sediment all membranes, but the supernatant, Sup. 2a, still contained mu and kappa structures as judged from its ability to inhibit the corresponding antibodies in passive hemagglutination and fluorescence staining.

Determination of the Size of the Free Immunoglobulin Structures.—For analysis of the size of the immunoglobulin structures in Sup. 2a, it was concentrated and a small amount of rabbit 7S gamma globulin labeled with ^{125}I was added as marker substance. Sephadex G-200 filtration and density gradient ultracentrifugation were then performed. For comparison, 5 μl of normal human serum to which was added ^{125}I-labeled rabbit gamma globulin was run in a parallel tube in the ultracentrifuge. The fractions obtained were tested for mu and kappa structures by inhibition of passive hemagglutination. It was found that the mu and some of the kappa structures sedimented in a similar manner to 7S rabbit gamma globulin (Fig. 1) and were eluted from the gel in the same fractions (Fig. 2), while some of the kappa structures were apparently of smaller size. The most likely interpretation seems to be that the mu structures and some of the kappa structures are chains forming 7S IgM molecules, while some of the kappa structures occur as free kappa chains.

Quantitations of Immunoglobulin Structures in Various Fractions.—The quan-

tities of mu and kappa structures in the various fractions shown by Fig. 3 were compared by their ability to inhibit passive hemagglutination with anti-mu and anti-kappa antibodies.

The inhibitory efficiency of the fractions was compared to that of known amounts of IgM and kappa chains. 19S IgM and 7S IgM were found to have

TABLE I

		Anti–HL-A FS	Anti–mu FS	Anti–mu PHA
2000 g	Prec. 1a			
10 min	Sup. 1a	—	—	— (1/4)
5000 g	Prec.	—	—	+?
15 min	Sup.	—	—	— (½)
5000 g	Prec.	—	—	+?
30 min	Sup.	+	+—	— (½)
10,000 g	Prec.	—	—	+?
30 min	Sup.	+	+—	— (½)
20,000 g				
30 min	Prec.	+	+—	—
40,000 g	Sup.	+	+—	— (½)
30 min				
80,000 g	Prec.	+	+—	—
30 min	Sup.	+—	+	— (½)

Inhibition of the reactivity of anti-mu antibodies in passive hemagglutination (anti-mu, PHA) and in fluorescence staining (anti-mu, FS), and of anti–HL-A antibodies in fluorescence staining (anti–HL-A, FS) by various cellular fractions is shown. Before testing a small volume of Tris buffer was added to the precipitates.

— Inhibition. The numbers in parentheses show the highest dilution giving inhibition.

+— Very weak, if any inhibition.

+ No inhibition.

+? No inhibition, probably due to large particles in the precipitates.

similar inhibiting power on a weight basis of anti-mu and anti-kappa antibodies, and for convenience 19S IgM was used as reference. The inhibition of anti-kappa antibodies by known amounts of kappa chains and IgM is shown in Table II, right. By assuming a molecular weight of 22,500 for kappa chains and 67,500 for mu chains (9), it was calculated that free kappa chains have less inhibiting power than kappa chains in IgM.

The results of one quantitation experiment are presented in Table II. Similar results were obtained in several experiments. The left part of Table II shows that the same amounts of mu structures were found in whole cells (frozen and thawed) as in the homogenate. Sup. 1a, which was devoid of nuclei and unbroken cells,

contained most of the mu structures found in whole cells. Rehomogenization of the precipitate (Prec. 1a) liberated only small amounts of mu structures. After ultracentrifugation of Sup. 1a, the precipitate (Prec. 2a) and the supernatant (Sup. 2a) contained approximately equal amounts of mu structures. Reho-
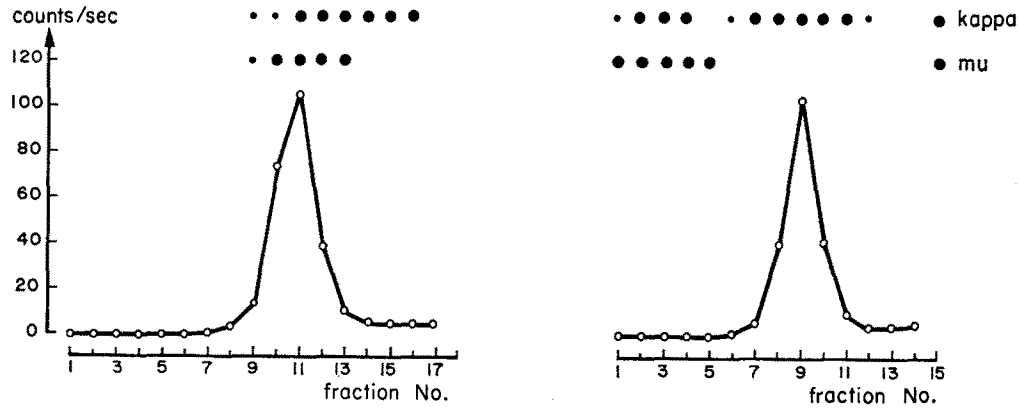

FIG. 1. Sucrose density gradient ultracentrifugation of Sup. 2a after concentration (left) and normal human serum (right). ^{125}I-labeled 7S rabbit gamma globulin was added to both samples. The bottom fraction is fraction No. 1. The open circles show the amount of 7S rabbit gamma globulin expressed as counts per second. Kappa and mu structures were localized by inhibition of passive hemagglutination. Small filled circles (•), weak inhibition; large filled circles (●), strong inhibition.
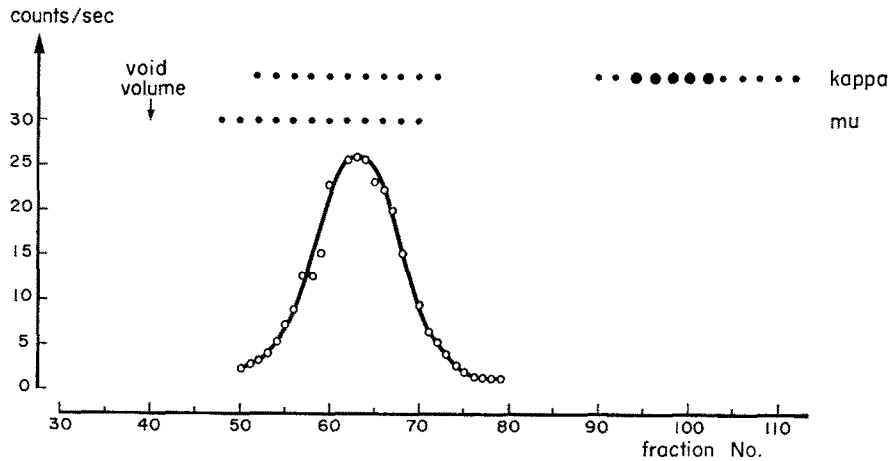

FIG. 2. Sephadex G-200 filtration of ^{125}I-labeled 7S rabbit gamma globulin and Sup. 2a after concentration. Each fraction contained approximately 0.5 ml. Void volume of the gel bed was determined by filtration of 19S IgM. The open circles show the amount of 7S rabbit gamma globulin expressed as counts per second. Every second fraction was tested for mu and kappa structures by inhibition of passive hemagglutination. Small filled circles (•), weak inhibition; large filled circles (●), strong inhibition.

mogenization of Prec. 2a liberated small amounts of mu structures which were only detectable in the supernatant (Sup. 2c) after concentration.

When the various fractions were tested for inhibition of anti-kappa antibodies (Table II, right), the inhibiting material was found to be distributed among the fractions similar to the mu structure. In order to estimate the inhibition of anti-kappa antibodies caused by IgM only, the smallest amount of
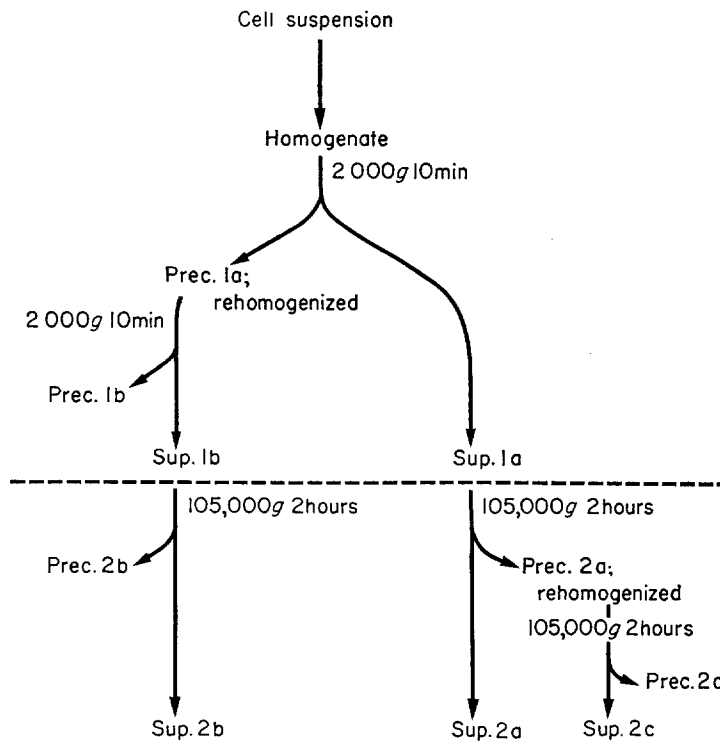

FIG. 3. Homogenization and fractionation procedure of frozen and thawed leukemia cells. The precipitates were homogenized with the original volume of Tris buffer.

19S IgM which inhibited the anti-kappa antibodies was divided by the calculated amount of IgM in the various fractions. However, the titers calculated in this way (Table II) were invariably lower than the observed titers, indicating that the various fractions also contained kappa structures not associated with IgM. This is in agreement with results presented above for Sup. 2a.

After concentration, Sup. 2a gave in gel diffusion tests a distinct precipitation line with anti-kappa antibodies, and a faint line with anti-mu antibodies (Fig. 4). In contrast, the anti-mu reagent gave more distinct precipitation lines

with small amounts of pure 7S IgM than the anti-kappa reagent. This also shows that there were more kappa than mu structures in Sup. 2a.

After concentration and density gradient ultracentrifugation of Sup. 2c, kappa and mu structures were found in the levels of the tube as already described for Sup. 2a.

TABLE II

The Amount of Mu and Kappa Structures in Various Cellular Fractions Determined by Inhibition of Passive Hemagglutination

	Anti–mu antibodies			Anti-kappa antibodies			
	Titer (observed)	Sensitivity of test in μg of IgM/ml	Amount in μg of IgM/ml	Sensitivity of test in μg of IgM/ml	Titer (calculated) if only IgM was present	Titer (observed)	Sensitivity of test in μg of kappa chains/ml
Cell suspension (10%)	1/8	0.38	3.0	1.5	1/2	1/4	1.5–0.75
Homogenate	1/8		3.0		1/2	1/4	
Prec. 1a	1/2		<0.75		<1/1	1/1	
Sup. 1a	1/8		3.0		1/2	1/4	
Prec. 1b	1/2		0.75	3.0	<1/1	1/1	
Sup. 1b	1/1		0.38		<1/1	1/1	
Prec. 2a	1/2	0.75	1.5	1.52	1/1	1/4	0.75
Sup. 2a	1/2		1.5		1/1	1/2–1/4	
Prec. 2c	1/2		1.5		1/1	1/2	
Sup. 2c	<1.1		<0.75		<1/1	<1/1	
Prec. 2b	≦1/1		≦0.75		<1/1	<1/1	
Sup. 2b	<1/1		<0.75		<1/1	<1/1	
Sup. 2c, conc.	1/4	0.75	3.0	3.0	1/1	1/2	1.5
Sup. 2b, conc.	1/1		0.75		<1/1	1/1	

Titer (observed) shows the highest dilution of the fractions which completely inhibited passive hemagglutination with anti-mu or anti-kappa antibodies. *Sensitivity of the test* is the smallest amount of 19S IgM or free kappa chains giving complete inhibition. *Amount of IgM* is calculated from the titer (observed) and sensitivity of the test. *Titer (calculated)* gives an estimation of the inhibition of anti-kappa antibodies caused by IgM only. These titers were obtained by dividing the smallest amount of 19S IgM which inhibited anti-kappa antibodies (sensitivity of the test) by the calculated amount of IgM in the various fractions.

Liberation of Mu and Kappa Structures by Freezing and Thawing.—Frozen and thawed cells were washed twice with BSSg and finally with Tris buffer. The first two washings were performed very gently, while the third washing was done as a homogenization. The supernatants obtained after the washings (Sup. A, Sup. B, and Sup. C) were then run in the ultracentrifuge in order to sediment all membranes. By inhibition of passive hemagglutination it was found that a large proportion of the mu and kappa structures were liberated as molecules by the first two washings (Table III).

Liberation of Mu and Kappa Structures by Mercaptoethanol Treatment.—Before it became evident that homogenization released mu and kappa structures, viable cells were exposed to 2-mercaptoethaol. This treatment killed the cells. Material precipitated from the supernatant with ammonium sulphate was found to contain both mu and kappa structures. By density gradient ultracentrifugation of this material, the mu structures were found to sediment somewhat slower than mu structures liberated by homogenization. The position of kappa structures was not investigated.
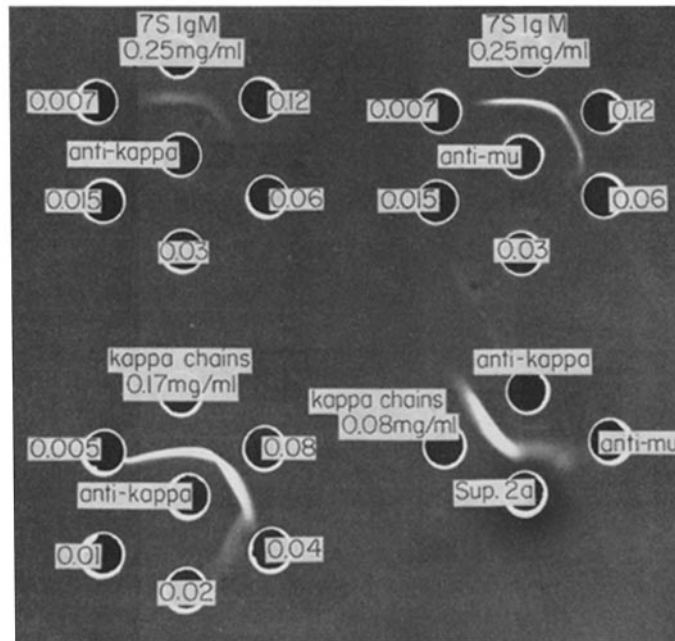

FIG. 4. Double diffusion in agarose gel. Sup. 2a after concentration and various dilutions of 7S IgM and kappa chains were tested against anti-mu and anti-kappa antibodies.

DISCUSSION

The Origin of the Isolated Immunoglobulin Structures.—As we intended to study mu and kappa structures made by small lymphocytes and found on the surface of the cells, it is, of course, of the utmost importance to ascertain that the isolated immunoglobulin structures were not directly derived from an intracellular pool or any exterior source.

Three different approaches were used to exclude that significant amounts of immunoglobulins were directly derived from the interior of the cells.

The first set of experiments included comparison of the staining of fixed and viable cells using fluorescein-labeled anti-mu and anti-kappa antibodies. The

fluorescence seemed to be localized solely on the cell membrane even in the case when fixed cells were stained (1). Moreover, the inhibition of the fluorescence staining observed when viable cells were exposed first to unconjugated antibodies, and later to a fluorescein-labeled reagent, was equally effective as when viable cells were exposed to unconjugated antibodies, followed by fixation and treatment with conjugated antibodies (1).

Secondly, comparison of the absorbing efficiency of viable and frozen and thawed cells showed that equal amounts of anti-kappa and anti-mu antibodies were removed by fixed numbers of cells (1).

TABLE III

Inhibition of Passive Hemagglutination Used to Compare the Amount of Mu and Kappa Structures Liberated as Molecules by Two Washings (A and B) Followed by Homogenization (C) of Frozen and Thawed Cells

Dilution of the test material →	Anti-mu antibodies						Anti-kappa antibodies					
	1/1	1/2	1/4	1/8	1/16	1/32	1/1	1/2	1/4	1/8	1/16	1/32
Cell suspension			−	−	+−	+	−	−	−		+(+)	+++
Sup. A	+	−	−	+−	++	++(+)	−	−	−	−	++(+)	+++
Sup. B	−	+	++	++(+)	+++	+++	−	+	+++	+++	+++	+++
Sup. C	+	++	++(+)	+++	+++	+++	−	++(+)	+++	+++	+++	+++

Before testing, the supernatants were run at 105,000 *g* for 2 hr.
−, +− Complete inhibition.
+, +(+), ++ Some inhibition.
++(+) Weak, if any inhibition.
+++ No inhibition.

The third approach to exclude the presence of intracellular immunoglobulin is described in this paper. The distribution of mu and kappa structures among various fractions was investigated, and no increase of immunoglobulin structures during the fractionation procedure was found. About half the amount of immunoglobulin structures on the cells was liberated as isolated molecules.

Blood cells absorb small quantities of plasma proteins, but it is not likely that we were dealing with proteins absorbed from the exterior. Only mu and kappa structures were found on the cells (6), and before homogenization the cells were extensively washed with BSS containing gelatin.

Unfortunately the leukemia cells have not been established in culture. But Daudi cells, derived from a Burkitt lymphoma and kept in culture for more than 2 yr, carry mu and kappa structures on the surface in similar amounts as the leukemia cells (1, 3, 4). This supports the assumption that the leukemia cells synthesized their surface immunoglobulin structures.

7S IgM and kappa chains constitute only a very minor fraction of the immunoglobulins in serum except in some patients with Waldenström's macro-

globulinemia (23) or multiple myeloma. In the serum from patient T.P. the immunoglobulin level was slightly subnormal (6), and agarose electrophoresis revealed a normal immunoglobulin pattern (unpublished observation). It seems also pertinent to mention that anti-mu and anti-kappa antibodies were cytotoxic to these cells in the presence of complement (1).

In conclusion, no support has been found for the assumption that a significant number of immunoglobulin structures liberated from the leukemia cells came from sources other than the cell surface.

The Size of the Isolated Molecules.—The analysis of molecular size included sucrose density gradient ultracentrifugation and gel filtration. As the mu and some of the kappa structures were found in the same fractions as the 7S rabbit gamma globulin, we suggest that 7S IgM is present and has a structure similar to that of 7S IgM from other sources.

As mentioned above, 7S IgM molecules constitute the subunit of 19S IgM molecules (9). 7S IgM also exists in cells secreting 19S IgM (12), and has been found in normal serum (10, 11) and in sera from patients with various diseases (23–28). 7S IgM serum seems to be secreted as 7S IgM molecules (29). 7S IgM molecules probably consist of two mu and two light chains, although the presence of a third light chain has been suggested (30).

In order to explain that 19S IgM antibodies invariably are secreted earlier than IgG antibodies during an immune response, it has been proposed (31) that the IgM and IgG receptors on the immunocompetent cells are similar to the antibodies found in serum. 19S IgM receptors should be more effective than IgG receptors in binding antigens due to the higher number of combining sites, and 19S IgM-producing cells would be more quickly stimulated to make an immune response. The finding that 7S IgM was liberated from the surface of lymphoid cells indicates that the proposal just mentioned may be revised.

The mu structures extracted from the cells by mercaptoethanol sedimented somewhat more slowly than the IgM extracted by homogenization. The reduction in size was probably due to cleavage of chains or disulfide bonds (32–34).

The free kappa structures found by ultracentrifugation and gel filtration of various supernatants are compatible with the assumption that free kappa chains were also present. Free kappa chains have previously been found in cells secreting immunoglobulins and in their secretion product (35–37).

The Binding Forces Keeping the IgM Molecules and Kappa Chains in the Surface Membrane.—The liberations of 7S IgM and kappa chains were done under conditions not favorable for disruption of covalent bonds. On one occasion an extract was prepared by homogenization from living cells in one sequence at a temperature near 0°C until a density gradient ultracentrifugation had been performed. Examination of the fractions revealed mu and kappa structures in the same levels of the tube as in the other experiments.

Approximately half of the mu and kappa structures present on the cell surface could be isolated by homogenization of viable or frozen and thawed cells. Even

careful washing of cells which had been frozen and thawed once was sufficient to liberate a large proportion of the molecules. In contrast, these cells had been washed about six times in the viable state during their purification. This suggests that the binding of these molecules required an intact membrane.

Some mu and kappa structures remained in the membrane in spite of extensive homogenization. The forces keeping these structures in the membrane are presently under investigation.

The Number of IgM Molecules and Kappa Chains on the Cell Surface.—In the following calculations it will be assumed that all the immunoglobulin structures on the cell surface are similar to those liberated as isolated molecules by homogenization.

It has previously been shown (1) that 10^9 viable leukemia cells carry mu structures on their surface equivalent to 25 μg IgM. This value corresponds well with the number obtained in the experiments reported here. A 10% cell solution, containing about 2.5 $\times$ 10^8 cells/ml, was shown to have 3 μg IgM/ml. It should be pointed out that the latter value was obtained with an inexact titration method only. For quantitations of this kind, the ideal situation is that the antigen used for reference is physically identical with the antigen to be quantitated. This was obviously not possible to achieve. Since 7S and 19S IgM had similar inhibiting power on a weight basis, we assumed that 19S IgM could be used as a reference in our experiments.

Assuming 25 μg IgM/10^9 cells and a molecular weight of 180,000, 80,000 7S IgM molecules have to be present on each cell. The local density of IgM molecules on the cell surface is difficult to estimate, since the fluorescence staining showed an uneven distribution of mu structures (1, 6).

Inhibition of passive hemagglutination revealed that the cell suspension had a large excess of kappa structures as compared to mu structures (Table II). This was also found in the precipitates (Table II), and in Sup. 2a and Sup. 2c. isolated kappa chains were demonstrated.

The amount on the cell surface of kappa chains not forming a part of IgM can only be roughly calculated. This is due to the fact that anti-kappa antibodies also were inhibited by IgM. Moreover, the finding that the inhibitory efficiency of kappa chains derived from 19S IgM by reduction and alkylation was less than that of kappa chains in IgM suggests that kappa chains on the membranes and liberated chains behave differently in inhibition experiments.

An estimation based on the numbers in Table II gives the results that the amount of kappa chains not associated with IgM was at least equal to the amount of kappa chains in IgM.

SUMMARY

Chronic lymphocytic leukemia cells with relatively large amounts of mu and kappa immunoglobulin structures on the surface, and apparently very small amounts intracellularly, were subjected to homogenization or washing after

freezing and thawing. After a light centrifugation, which sedimented the nuclei and unbroken cells, most of the immunoglobulin structures were found in the supernatant. Ultracentrifugation, which was performed to remove the membranes from the supernatant, sedimented only half the amount of the immunoglobulin structures.

By sucrose density gradient ultracentrifugation and Sephadex G-200 filtration, the unsedimented immunoglobulin structures were shown to consist of 7S IgM and free kappa chains. About 80,000 7S IgM molecules were calculated to be present on each cell. The amount of kappa chains not associated with IgM was estimated to be equal to the amount of kappa chains in IgM. Inhibition of passive hemagglutination was used to detect and quantitate the immunoglobulin structures.

BIBLIOGRAPHY

1. Klein, E., T. Eskeland, M. Inoue, R. Strom, and B. Johansson. 1970. Surface immunoglobulin-moieties on lymphoid cells. *Exp. Cell Res.* **62**:133.
2. Singhal, S. K., and H. Wigzell. 1971. Cognition and recognition of antigen by cell associated receptors. *Progr. Allergy.* In press.
3. Klein, E., G. Klein, J. S. Nadkarni, J. J. Nadkarni, H. Wigzell, and P. Clifford. 1968. Surface IgM-kappa specificity on Burkitt lymphoma cells in vivo and derived culture lines. *Cancer Res.* **28**:1300.
4. Nadkarni, J. S., J. J. Nadkarni, P. Clifford, G. Manolov, E. M. Fenyö, and E. Klein. 1969. Characteristics of new cell lines derived from Burkitt lymphomas. *Cancer.* **23**:64.
5. Klein, E. 1971. Immunoglobulin production of lymphoid cells. *In* Symposium on Human Tumor Virology and Immunology, Tokyo. In press.
6. Johansson, B., and E. Klein. 1970. Cell surface localized IgM-kappa immunoglobulin reactivity in a case of chronic lymphocytic leukemia. *Clin. Exp. Immunol.* **6**:421.
7. Pernis, B., L. Forni, and L. Amanti. 1970. Immunoglobulin spots on the surface of rabbit lymphocytes. *J. Exp. Med.* **132**:1001.
8. Rabellino, E., S. Colon, H. M. Gray, and E. R. Unanue. 1971. Immunoglobulins on the surface of lymphocytes. I. Distribution and quantiation. *J. Exp. Med.* **133**:156.
9. Miller, F., and H. Metzger. 1965. Characterization of a human macroglobulin. I. Molecular weight and its subunits. *J. Biol. Chem.* **240**:3325.
10. Killander, J. 1963. Separation of human immunoglobulins by gel filtration and zone electrophoresis. *Acta Soc. Med. Upsal.* **68**:230.
11. Solomon, A. 1969. Molecular heterogenity of immunoglobulin-M (γM-globulin). *J. Immunol.* **102**:496.

12. Parkhouse, R. M. E., and B. A. Asconas. 1969. Immunoglobulin M biosynthesis. Intracellular accumulation of 7s subunits. *Biochem. J.* **115**:163.

13. Klein, E., and T. Eskeland. 1971. Surface IgM on lymphoid cells. *In* Cell Interactions and Receptor Antibodies in Immune Responses. Academic Press, Inc., New York. 91.

14. Müller-Eberhard, H. J. 1960. A new supporting medium for preparative electrophoresis. *Scand. J. Clin. Lab. Invest.* **12**:33.

15. Harboe, M., B. G. Solheim, and J. Deverill. 1969. Individual antigenic specificity of human macroglobulins. Localization of antigenic determinants and their use to demonstrate formation of hybrid molecules after reduction and reassociation. *J. Exp. Med.* **129**:1217.

16. Fleischman, J. B., R. H. Pain, and R. R. Porter. 1962. Reduction of γ-globulins. *Arch. Biochem. Biophys. Suppl.* **1**:174.

17. Rosa, U., G. A. Scassellati, F. Pennisi, N. Riccioni, A. Giagnoni, and R. Giordani. 1964. Labelling of human fibrinogen with ^{131}I by electrolytic iodination. *Biochim. Biophys. Acta.* **86**:519.

18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265.

19. Wadsworth, C., and L. Å. Hanson. 1960. Comparative analysis of immune electrophoretic precipitates employing a modified immune electrophoretic technique. *Int. Arch. Allergy Appl. Immunol.* **17**:165.

20. Hammond, E. 1970. Ultrastructural characteristcs of surface IgM reactive malignant lymphoid cells. *Exp. Cell Res.* **59**:359.

21. Boyle, W. 1968. An extension of the ^{51}Cr-release assay for the estimation of mouse cytotoxins. *Transplantation.* **6**:761.

22. Siekevitz, P. 1962. Preparation of microsomes and submicrosomal fractions: mammalian. *Methods Enzymol.* **5**:61.

23. Bush, S. T., H. A. Swedlund, and G. J. Gleich. 1969. Low molecular weight IgM in human sera. *J. Lab. Clin. Med.* **73**:194.

24. Rothfield, N. F., B. Frangione, and E. C. Franklin. 1965. Slowly sedimenting mercaptoethanol-resistant antinuclear factors related antigenically to M-immunoglobulins (γ_1M-globulin) in patients with systemic lupus erythematosus. *J. Clin. Invest.* **44**:62.

25. Gleich, G. J., J. W. Uhr, J. H. Vaughan, and H. A. Swedlund. 1966. Antibody formation in dysgammaglobulinemia. *J. Clin. Invest.* **45**:1334.

26. Klein, F., P. Mattern, H. Radema, and L. van Zwet. 1967. Slowly sedimenting serum components reacting with anti-IgM sera. *Immunology.* **13**:641.

27. Solomon, A., and H. G. Kunkel. 1967. A "monoclonal" type, low molecular weight protein related to γM-macroglobulins. *Amer. J. Med.* **42**:958.

28. Stobo, J. D., and T. B. Tomasi. 1967. A low molecular weight immunoglobulin antigenically related to 19S IgM. *J. Clin. Invest.* **46**:1329.

29. Solomon, A., and C. L. McLaughlin. 1970. Biosynthesis of low molecular weight IgM (7S) and high molecular weight (19S) immunoglobulin M. *J. Clin. Invest.* **49**:150.

30. Suzuki, T., and H. F. Deutsch. 1967. Dissociation, reaggregation and subunit structure studies of some human γM-globulins. *J. Biol. Chem.* **242**:2725.

31. Mäkelä, O., E. Kostiainen, T. Koponen, and E. Ruoslahti. 1967. The timing and

quality of IgA, IgG and IgM responses in rabbits immunized with hapten. *Nobel Symp. Gamma Globulins, 3rd.* 505.

32. Frank, M. M., and J. H. Humphrey. 1968. The subunits in rabbit anti-Forssman IgM antibody. *J. Exp. Med.* **127**:967.

33. Albritton, W. L., R. E. Schrohenloher, and J. C. Bennett. 1970. Release of Fab- and Fc-like fragments from human IgM by reductive cleavage. *J. Immunol.* **104**:518.

34. Morris, J. E., and F. P. Inman. 1970. Dissociation of a small proportion of a human IgM population into Fc μ, Fab μ, and free light chains following reduction. *J. Immunol.* **105**:451.

35. Shapiro, A. L., M. D. Scharff, J. V. Maizel, and J. W. Uhr. 1966. Synthesis of excess light chains of gamma globulins by rabbit lymph node cells. *Nature (London).* **211**:243.

36. Asconas, B. A., and A. R. Williamson. 1967. Balanced heavy and light chain synthesis in immune tissue and disulphide bond formation in IgG-assembly. *Nobel Symp. Gamma Globulins, 3rd.* 369.

37. Zolla, S., J. Buxbaum, E. C. Franklin, and M. D. Scharff. 1970. Synthesis and assembly of immunoglobulins by malignant human plasmacytes. I. Myelomas producing γ-chains and light chains. *J. Exp. Med.* **132**:148.