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Purification and Quantification of T and B Lymphocytes by an Affinity Method

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Summary. Affinity surfaces were produced by coupling human immunoglobulin (HGG) to the surface of tissue culture grade plastic-ware with a water-soluble carbodiimide followed by treatment with anti-HGG antisera. Surface immunoglobulin (SIg) bearing human B lymphocytes attach to these surfaces when centrifuged on to them and unattached cells could be recovered by inverting the trays or dishes. Optimal cell attachment conditions could be rapidly evaluated by counting cells attached to representative areas of multi-well trays and percentage of SIg-bearing cells quantified. Evidence was obtained for cell attachment through Fc receptors as well as SIg using unrelated antigen–antibody-coated trays. This could be prevented by using the $F(ab')_2$ fragments of the antisera. Under these conditions specific attachment through κ and λ light chains could be achieved with normal and chronic lymphocytic leukaemic lymphocytes. Using tissue culture plastic Petri dishes and relatively small quantities of antiserum, larger numbers of lymphocytes could be processed to produce T lymphocytes containing less than 1 per cent of contaminating SIg-positive cells.

INTRODUCTION

The understanding of many aspects of immunological function requires the use of purified preparations of B and T lymphocytes. Several methods for B- and T-cell separation have been described based on differential adherence to glass beads (Shortman, 1966; Hunt, 1973), or nylon fibres (Greaves and Brown, 1974) and by free-flow electrophoresis which depends on variations of surface charge between the two cell types (Seiler, Johannsen, Sedlacek and Zeiller, 1974). Affinity chromatographic methods have also been devised utilizing the known surface markers of B lymphocytes such as the receptor for the Fc region of IgG (Basten, Sprent and Miller, 1972) and presence of immunoglobulin on the surface (SIg) of B cells (Schlossman and Hudson, 1973). While the affinity methods offer the advantages of separating cells via well established and extensively investigated surface phenomena, in their present form, whereby lymphocytes are presented to affinity columns of coated beads, several disadvantages exist. Thus extensive investigations of the optimal parameters required for cell separation are laborious to perform and relatively large quantities of specific antibody are necessary. Furthermore precise quantification of the non-specific removal of potentially important minor cell population is difficult to achieve.

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This communication describes an alternative method for the selective removal of B lymphocytes based on the attachment of the SIg (Unanue, Grey, Rabellino, Campbell and Schmidtke, 1971) to antibody-coated plastic tissue culture trays and dishes as an affinity surface. The method offers advantages in that the quantification of cell attachment can be easily achieved so that the parameters for specific and non-specific attachment can be rapidly investigated. Furthermore relatively small quantities of whole antisera can be used without the need to isolate specific antibody.

MATERIALS AND METHODS

Immunoglobulins

Commercially available Cohn fraction II (Sigma Chemical Company, London) was used as the source of human gamma-globulin (HGG). IgM was precipitated from Waldenström's macroglobulinaemic serum by dilution with nine volumes of water and partially purified by two further cycles of redissolving in saline and dilution with water. IgA was precipitated from myeloma sera with ammonium sulphate to 40 per cent saturation and purified by gradient elution from DEAE–Sephadex A-50. Bence–Jones proteins of type κ and λ were isolated by gel filtration on Sephadex G-100 of the total urinary proteins of myeloma patients isolated by ultrafiltration and lyophilization. They were further purified by gradient elution from DEAE–Sephadex A-50. Rat immunoglobulin (RaIg) was isolated by Pevikon block electrophoresis and used without further purification.

Antisera

Rabbit antiserum to human gamma-globulin (anti-HGG) was produced by immunization with Cohn fraction II, and anti-IgM and anti-IgA by immunizing with the corresponding immunoglobulin isolated as above. Rabbit antiserum was also raised by immunization with rat gamma-globulin (anti-RaIg). These antisera were used without attempts to render them monospecific.

Rabbit anti-κ and anti-λ light chain antisera were produced by immunization with pooled samples of the corresponding Bence–Jones protein and the resulting antisera were adsorbed with the appropriate light chain coupled to Sepharose with cyanogen bromide (Porath, Axen and Ernback, 1967) to ensure specificity.

$F(ab')_2$ fragments of certain of the anti-globulin antisera were prepared by pepsin hydrolysis (Nisonoff, Markus and Wissler, 1961).

Peripheral blood lymphocytes

Peripheral blood from normal donors was collected into heparinized bottles and erythrocytes sedimented by the addition of 0·6 per cent dextran. The leucocyte-rich supernatant was centrifuged over a Ficoll–Triosil (Nyegard, Oslo) layer (Thorsby and Bratlie, 1970) to collect lymphocytes at the interface. The lymphocytes were washed three times with 0·2 per cent bovine serum albumin (BSA) in Hepes-buffered Hanks's balanced salt solution (HBSS), finally resuspending in the same medium for use.

Quantification of lymphocyte attachment

Preparation of trays. For quantitative investigations of the attachment of lymphocytes to surfaces treated under varying conditions, the flat-bottomed wells of multi-well tissue

culture grade trays were used (Linbro FB-48-TC; Biocult, Paisley). Varying dilutions of immunoglobulins in saline (50 μl per well) were added to the wells and coupling to the plastic achieved by the addition of freshly prepared solutions (50 μl) of a water-soluble carbodi-imide (1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)-carbodi-imide metho-*p*-toluene-sulphonate) (Aldrich Chemical Company, Milwaukee, Wisconsin) (CMC) in saline. After mixing, the plates were left to stand at room temperature for 30 minutes. A ratio of 5:1 CMC:protein on a weight:weight basis was used for coupling. After coupling the wells were washed thoroughly with saline. Coupled trays were either stored overnight at 4° under saline and rewashed briefly before use or used immediately. The wells were then treated with heat-inactivated normal rabbit serum (NRS) (100 μl) for 30 minutes at room temperature which was subsequently thoroughly washed out with phosphate-buffered saline, pH 7·3 (PBS). This step was found to be essential in order to prevent subsequent non-specific sticking to the surface of antibodies from the antiserum and also non-specific attachment of lymphocytes to the plastic. Varying dilutions of rabbit antisera to immunoglobulins in 2 per cent NRS in PBS (100 μl) were then added to each well and left for 30 minutes at room temperature. The wells were thoroughly washed out with jets of saline and finally filled with 0·2 per cent BSA in HBSS.

Attachment of cells and scoring. The treated trays were cut in half to fit centrifuge carriers (Microtiter), the medium drained out of the wells and replaced by 100 μl of the lymphocyte suspension containing 1×10^5 cells. The plates were immediately centrifuged at 280 ***g*** for 3 minutes at 4°. Using an inverted microscope fitted with an eyepiece graticule the number of cells in six standard areas was counted at regular intervals across a diameter of a random selection of wells. The mean total number of cells in the six standard areas of a well was used as a measure of the total number of lymphocytes available for attachment.

The wells were then filled with ice cold 0·2 per cent BSA in HBSS, covered with Saran wrap, exluding all air bubbles, inverted, and left at 4° for 30 minutes for unattached cells to fall off the bottom of the well. The wells were then gently rinsed by filling with medium and inverting twice and attached cells recounted under the standard conditions as before. The number of cells attached to this standard area of a well was expressed as a percentage of the total number of cells available for attachment as counted before inverting the trays.

Preparative scale removal of B cells

Preparation of dishes. For experiments where recovery of the unattached cells was required 35 x 10 mm tissue culture grade Petri dishes (Falcon Plastics, Oxnard, California) were used. Optimal conditions for immunoglobulin coupling and antiserum treatment were selected from preliminary titrations using the multi-well trays. Typically, 1 ml of HGG (100 μg/ml) in saline was added to each dish followed by 1 ml of CDI (500 μg/ml) and after mixing allowed to stand for 30 minutes at room temperature. After thorough washing with saline the dishes were treated with NRS (0·3 ml) for 30 minutes at room temperature, rewashed and then treated with 2 ml of rabbit anti-HGG diluted in 2 per cent NRS in PBS (typically 1:50 dilution) for 30 minutes. The dishes were then rewashed with saline and filled with 0·2 per cent BSA in HBSS before use.

Cell separation. The dishes were emptied and 2 ml of a lymphocyte suspension containing 1×10^7 cells in the same medium added. The dishes were then centrifuged at 280 ***g*** for 3 minutes at 4°, filled with cold medium and the lid applied excluding air bubbles. They were then inverted and left at 4° for 30 minutes by which time unattached cells had fallen away

from the Petri dish bottom. The unattached cells were recovered by removing the dish from the lid leaving them in suspension on the lid.

Surface immunoglobulin staining

Test cells were incubated with a polyvalent rabbit antiserum to human immunoglobulin for 20 minutes at room temperature and washed three times with 0·2 per cent BSA in HBSS. They were then incubated with fluorescein-conjugated goat anti-rabbit immunoglobulin (Nordic Pharmaceuticals, Tilburg, Holland) for a further 20 minutes, rewashed and examined by dark ground illumination in a Zeiss microscope fitted with an Osram HBO 200 mercury vapour lamp.

E-binding lymphocytes

E rosettes were formed by mixing the lymphocytes with sheep erythrocytes in minimum essential medium (MEM) incubating at 37°, centrifugation and overnight incubation at 4° as described previously (Smith, Barker, Clein and Collins, 1973). At least 200 cells were scored in a haemocytometer counting cells binding three or more erythrocytes as positive.

RESULTS

OPITMAL ATTACHMENT CONDITIONS

Direct and indirect coating of the surface with anti-immunoglobulin

Preliminary experiments were carried out in the multi-well trays to determine the conditions under which the highest percentage of lymphocytes could be made to specifically attach to treated surfaces. Direct coupling of anti-HGG to the surface with CMC was compared with the indirect method whereby HGG was first coupled with CMC and then treated with anti-HGG. Summaries of these experiments are shown in Table 1. Serial dilutions of anti-HGG, or the immunoglobulin fraction prepared from it by ammonium sulphate precipitation, were coupled with CMC to the wells. Alternatively HGG dilutions were coupled to the wells and then treated with dilutions of anti-HGG in a checker board system. The wells were treated with normal rabbit serum, lymphocytes added, centrifuged

TABLE 1

ATTACHMENT OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TO PLASTIC SURFACES FOLLOWING VARIOUS TREATMENTS WITH HUMAN IMMUNOGLOBULIN (HGG) AND ANTI-HGG ANTISERUM

Expt no.	Coupled to surface with CMC	Subsequent treatment	Percentage cell attachment at optimal dilutions	Percentage cells SIg-positive by fluorescent staining
1	Anti-HGG	—	12	35
	Polyvalent anti-Ig†	—	18	
	NRS*	—	0·8	
	HGG	Anti-HGG	37	
	HGG	NRS	0·8	
2	Anti-HGG	—	10	22
	Ig fraction of anti-HGG	—	18	
	HGG	Anti-HGG	23	
	HGG	NRS	1·6	

* Normal rabbit serum.

† Selectively pooled antisera shown to contain anti-γ, -μ, -α, -κ and -λ activity.

in the cold, scored, filled with cold medium, inverted and rescored as described in the Materials and Methods section. For simplicity only the optimal percentage of cell attachment is shown for each treatment. It was found that direct coupling of anti-HGG whole antiserum direct to the wells could produce some lymphocyte attachment. Using a polyvalent anti-immunoglobulin, formed by combining individual antisera to Cohn Fraction II, Waldenström IgM, myeloma IgA and Bence–Jones κ and λ light chains, slightly higher binding would be achieved. However, the highest level of attachment was achieved by indirect coating with anti-HGG, i.e. by coupling HGG to the well followed by a treatment with anti-HGG which will leave a free valency of the anti-HGG available for B-cell attachment. Under these conditions the percentage of cells attached to the surface agreed well with the percentage of B cells detected in the original preparation by fluorescent antibody staining for SIg. An improvement on the percentage of attached cells in the direct coupling of antibody to the plastic could be achieved if the Ig fraction of the rabbit antiserum was isolated before coupling but the percentage was still lower than in the indirect method.

Cell presentation

The physical conditions essential for optimal cell attachment were also investigated. Wells were coupled with dilutions of HGG and CMC followed by anti-HGG which have previously been shown to give maximum attachment of lymphocytes. Control wells were treated with RaIg and CMC followed by anti-RaIg or with CMC alone followed by NRS. Lymphocyte suspensions were added to the wells and allowed to settle to the bottom of the wells at: (a) 4°; (b) 37° for 90 minutes; alternatively the trays were centrifuged at 4° and either (c) filled with cold medium and inverted immediately or (d) left to incubate at 37° for a further 90 minutes before inverting. The results shown in Table 2

TABLE 2

EFFECT OF PHYSICAL CONDITIONS OF PRESENTATION OF CELLS TO AFFINITY SURFACES ON THE ATTACHMENT OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES

Cell presentation	Coupled to surface with CMC†		
	HGG	RaIg*	—
	Antiserum treatment†		
	Anti-HGG	Anti-RaIg	NRS
	(Percentage lymphocyte attachment)		
Settling 4°, 90 minutes	5·3	0·2	0·3
Settling 37°, 90 minutes	4·8	2·1	1·2
Centrifuge at 4°	16·5	2·0	0·2
Centrifuge and incubate 90 minutes at 37°	12·9	3·6	1·4

* Rat immunoglobulin.

† Wells were treated with immunoglobulin (1 mg/ml) and CMC (5 mg/ml) followed by the antisera at 1 : 100 dilution.

indicated that centrifugation of the lymphocytes onto the bottom of the well produced satisfactory specific attachment while allowing cells to settle at 37° or in the cold were unsatisfactory. Subsequent incubation at 37° after centrifugation apparently led to some cells being lost from the surface. Where cells were left in contact with control dishes at 37° (methods (b) and (d)) a small proportion of lymphocytes (1–2 per cent) attached non-

specifically. In control wells coated with RaIg and anti-RaIg a larger number of cells attached than with the uncoated wells. This attachment was thought to occur through the antigen–antibody complex on the surface and the Fc receptor known to exist on the B cell (Paraskevas, Lee, Orr and Israels, 1972). It appeared to be enhanced by centrifugation and incubation at 37°. In view of these findings in all subsequent experiments the cells were centrifuged on to the bottom of the well in the cold, and the wells filled with cold medium and inverted without further incubation.

Immunoglobulin and antiserum titration.

As mentioned previously, immunoglobulin coupling of the surface and antiserum treatment was optimalized by a checker board titration of the HGG coupled to the well and the anti-HGG used in the second treatment. A typical titration is shown in Table 3. A

TABLE 3

EFFECT OF LEVEL OF HGG COUPLING TO THE PLASTIC SURFACE AND ANTI-HGG TREATMENT OF LYMPHOCYTE ATTACHMENT

HGG coupling*		Percentage lymphocyte attachment						
		Rabbit anti-HGG treatment† dilution						NRS‡
HGG/ml	CMC/ml	1 : 10	1 : 50	1 : 250	1 : 1250	1 : 6250	1 : 31250	1 : 50
10 mg	50 mg	21·3	19·4	23·6	16·9	20·7	12·9	5·7
1 mg	5 mg	21·7	22·9	18·7	12·1	7·6	4·0	5·1
100 μg	1 mg	19·8	21·5	25·0	17·3	8·2	5·5	3·0
10 μg	1 mg	24·3	25·4	21·1	13·4	5·5	2·0	0·9
1 μg	1 mg	25·9	27·3	23·0	15·3	6·0	1·2	1·4
0·1 μg	1 mg	17·1	9·5	6·1	3·4	3·0	0·9	1·5
0·01 μg	1 mg	3·1	3·0	0·5	1·0	0·5	0·3	0·1
Saline	1 mg	1·2	1·2	1·1	1·0	0·7	0·5	0·7

* Coupling effected by adding 50 μl of HGG in saline at the concentrations indicated followed by 50 μl of the appropriate dilution of carbodi-imide (CMC) to each well.

† Wells treated with 100 μl of the appropriate dilution of rabbit anti-HGG in 2 per cent NRS.

‡ Normal rabbit serum.

plateau of attachment can be observed down to an HGG coupling level of 1 μg/ml and to 1:250 dilution of the particular anti-HGG antiserum used. Higher levels of HGG coupling (10 mg–100 μg/ml) caused increased 'non-specific' attachment of lymphocytes detected in the NRS controls.

To ensure that maximum attachment was being achieved a parallel tray with wells coupled with dilutions of a mixture of HGG (Cohn Fraction II), IgA and IgM was treated with dilutions of a polyvalent anti-immunoglobulin antiserum with known reactivity to γ, α, μ, κ and λ chains. The mean percentage attachment in the plateau area of this titration was not increased over that obtained in parallel titration of HGG and anti-HGG.

Inhibition and use of $F(ab')_2$ fragment of antibodies

If attachment of lymphocytes was occurring solely by reaction of the spare valence of the anti-HGG antibody on the well surface with the SIg of the B lymphocyte, attachment should be inhibitable by exposure of the prepared wells to HCG before cell additions. Wells coated with HGG at (100 μg/ml) and treated with anti-HGG (1:100 dilution) were

TABLE 4

INHIBITION OF ATTACHMENT BY HGG

HGG coupling:*	100 μg/ml								Saline
Antiserum treatment:†	Anti-HGG (1:100 dilution)								NRS (1:50)
Inhibition by HGG/ml:‡	10 mg	1 mg	100 μg	10 μg	1 μg	0·1 μg	NRaS¶	—	—
Percentage attachment§	7·6±1·4¶	6·5±0·9	6·8±0·8	5·5±0·6	8·7±1·0	8·1±0·9	20·0±1·2	20·1±2·3	1·5±0·3

* Wells were treated with HGG (50 μl, 100 μg/ml) and CMC (50 μl, 1 mg/ml) for 30 minutes.
† Wells were treated with antiserum (100 μl) for 30 minutes.
‡ Wells were treated with dilutions of HGG, NRaS or saline (100 μl) after HGG and anti-HGG treatment for further 30 minutes.
§ Expressed as mean ± s.d.
¶ Normal rat serum.

incubated with serial dilutions of HGG for 30 minutes in an attempt to inhibit attachment. Control wells were left untreated or treated with a source of non-related immunoglobulin (normal rat serum). In the test illustrated in Table 4, 20·1 per cent of lymphocytes were attached to HGG–anti-HGG-treated wells and this attachment was not inhibited by treatment with normal rat serum. HGG, however, was capable of partially inhibiting at as little as 0·1 μg/ml although as much as 10 mg/ml did not reduce attachment to the levels found in completely untreated wells. A similar result was obtained when the HGG was replaced by normal human serum (NHS) as an inhibitor. The possibility was considered that the uninhibitable attachment was occurring through interaction of the Fc receptor, known to exist on B cells, and HGG–anti-HGG complex built up on the wells. Further evidence for this mode of attachment was obtained when wells were coated with RaIg and treated with anti-RaIg. Under optimal conditions, determined by a checker board titration, 8·2±1·8 per cent of a preparation of human lymphocytes attached to the RaIg–anti-RaIg-treated wells compared to 17·1±2·7 per cent to HGG–anti-HGG control wells.

Perhaps the most conclusive evidence that B lymphocyte attachment could occur both via SIg and the Fc receptor to the HGG–anti-HGG-treated surface was obtained in a direct comparison of the inhibitability of attachment achieved using a whole anti-HGG antiserum and the $F(ab')_2$ fragment of the immunoglobulin of that serum. Wells were coated with dilution of HGG and treated with either whole anti-HGG antiserum or a $F(ab')_2$ fragment of the immunoglobulin from the same serum and related wells were then treated with NHS to block the spare anti-HGG valence. From the results in Table 5 it can

TABLE 5

INHIBITION OF LYMPHOCYTE ATTACHMENT TO HGG-COATED WELLS TREATED WITH EITHER WHOLE ANTI-HGG ANTISERUM OR THE $F(ab')_2$ FRAGMENT THEREOF BY HUMAN SERUM

Dilution of antiserum	Inhibitor dilution	Percentage lymphocyte attachment*	
		Whole anti-HGG	$F(ab')_2$ fragment
1 : 10	—	24·9±2·3	25·0±5·0
1 : 50	—	22·0±2·2	24·4±5·0
1 : 250	—	20·2±3·1	19·1±2·6
1 : 1250	—	14·4±2·1	11·3±2·2
1 : 61250	—	10·8±4·2	3·5±0·5
1 : 10	NHS† 1 : 10	12·7±1·2	1·7±0·8
1 : 50	NHS 1 : 10	10·0±1·1	2·0±0·3
Saline		2·5±0·3	2·2±0·3

Wells were coated with HGG (10 μg/ml) and CMC (1 mg/ml) prior to treatment with either dilutions of whole anti-HGG antiserum or an equivalent dilution of the $F(ab')_2$ fragment of the immunoglobulin of this antiserum. Inhibition was attempted by the addition of diluted serum to the wells which was washed out before the addition of lymphocytes.

* Expressed as mean ±s.d.

† Normal human serum.

be seen that use of the $F(ab')_2$ fragment of the antiserum did not impair the percentage of lymphocytes attaching at optimal dilutions. However, whereas only approximately 50 per cent of the attachment achieved with whole antiserum could be inhibited by human serum, the attachment obtained with the $F(ab')_2$ fragment could be inhibited to within control levels by the same treatment.

ATTACHMENT BY MEANS OF κ AND λ LIGHT CHAINS OF SIg

Using specific antisera to κ and λ light chains it should be possible to obtain attachment of only those populations of lymphocytes bearing the corresponding class of SIg. Wells were coupled with predetermined optimal Bence–Jones proteins type κ and λ and HGG and then treated with the $F(ab')_2$ fragments of anti-κ, -λ and -HGG. The $F(ab')_2$ fragments of the antisera were used in order to avoid the attachment of B lymphocytes through the Fc receptor as described previously which would obscure the specificity of attachment through the individual light chains. Table 6 illustrates the results obtained under these

TABLE 6

SPECIFIC ATTACHMENT OF LYMPHOCYTES TO ANTIBODY-COATED PLASTIC SURFACES THROUGH κ AND λ LIGHT CHAINS OF THE SURFACE IMMUNOGLOBULIN

Treatment of wells		Lymphocyte source		
		Normal	CLL1†	CLL2‡
		WBC/mm³		
Coupled*	$F(ab')_2$ fragment of antisera†		9200	14,000
BJ-κ	Anti-κ	14·1 ± 0·6*	52·1 ± 6·8	1·4 ± 0·1
BJ-κ	Anti-λ	1·1 ± 0·1	1·3 ± 0·3	1·2 ± 0·2
BJ-λ	Anti-λ	15·7 ± 1·4	1·6 ± 0·5	6·4 ± 0·3
BJ-λ	Anti-κ	1·4 ± 0·2	0·3	0·2
HGG	Anti-HGG	28·3 ± 1·0	90·8 ± 2·8	9·0 ± 2·5
Saline	NRS	0·7 ± 0·2	0·2	0·2

* Wells were treated with protein (50 μl, 100 μg/ml) and CMC (50 μl, 1 mg/ml) for 30 minutes.

† Wells subsequently treated with 1 : 50 dilution of $F(ab')_2$ fragments of antisera (100 μl) for 30 minutes.

‡ Chronic lymphocytic leukaemia.

§ Expressed as mean ± s.d. of the percentage lymphocyte attachment.

conditions with lymphocytes obtained from an example of normal peripheral blood and two cases of chronic lymphocytic leukaemia (CLL). In the normal lymphocytic preparation the percentages of cells attached through the κ and λ chains to wells coupled with BJ-κ and BJ-λ and treated with the $F(ab')_2$ fragment of the corresponding antisera (14·1 and 15·7 per cent respectively) are within statistical agreement with the total SIg-bearing lymphocyte percentage attached to the HGG–anti-HGG-treated wells (28·3 per cent). The controls with the antisera crossed over show little attachment above the untreated control wells. One example of chronic lymphocytic leukaemic lymphocytes (CLL1) showed 90 per cent attachment to HGG–anti-HGG-treated wells which is to be expected in view of the known B-cell nature of the majority of cases of this disease. The monotypy of the lymphocytes was also clearly demonstrated in that only 1·6 per cent attached to wells of λ specificity, whilst 52 per cent attached to those with κ. The lower percentage attachment to the λ wells than the HGG wells is not at present understood but may reflect lower levels of SIg on CLL cells than on the normal B cell. Alternatively this may reflect unequal expression of heavy and light chains on the surface of CLL cells, as would be indicated by other group findings using class-specific immunofluorescent-staining (Grey, Rabellino

TABLE 7

PREPARATIVE SCALE REMOVAL OF B LYMPHOCYTES ON HGG- AND ANTI-HGG-TREATED PETRI DISHES

Expt no.	Analysis of unattached cells from dishes treated with:								Percentage of cells attaching to small wells under optimal conditions‡
	Unfractionated cells		Saline+NRS (control)		HGG+anti-HGG*		HCG+anti-HGG† repeated 2×		
	SIg (%)	E-ros (%)	SIg (%)	E-ros (%)	SIg (%)	E-ros (%)	SIg (%)	E-ros (%)	
1	22	68	21	66	4	87	n.d.	n.d.	n.d.
2	44	39	36	32	7	58	1	67	45·3±4·1
3	18	54	18	56	5	72	0	77	18·6±2·4
4	22	55	18	56	1	67	<1	72	23·0±2·2
5	25	53	23	51	6	66	3	71	28·3±1·0

* 35-mm tissue culture Petri dishes were treated with HGG (1 ml, 100 μg/ml) and CMC (1 ml, 1 mg/ml) and after washing with rabbit anti-HGG (2 ml, 1 : 50 dilution).

† Cells left unattached to first dish were presented to fresh HGG–anti-HGG-treated dish and cells not attached to this dish were taken for analysis.

‡ Expressed as mean±s.d.

and Pirofsky, 1971). More extensive studies of optimal attachment conditions for CLL cells will have to be made to resolve this aspect. The second case of CLL (CLL2) examined produced surprisingly little attachment under any of the treatment conditions. Indirect immunofluorescent staining and sheep erythrocyte rosetting of these cells revealed that they lacked detectable quantities of SIg and failed to form E rosettes.

LARGE-SCALE REMOVAL OF B CELLS USING PETRI DISHES

Proof that the attachment observed under optimal coating conditions of the multi-well plates represented complete attachment of B cells required a modification of the technique which allowed recovery of the unattached cells for subsequent analysis. For this purpose and also to provide preparative quantities of relatively pure T cells the method was scaled up to use the bottoms of 35-mm tissue culture grade Petri dishes. Petri dishes were coated with HGG and treated with anti-HGG at dilutions previously determined to be within the plateau region of a preliminary 'checker board' type titration in the multi-well plates. Typically, coating with 1 ml of 100 μg of HGG per millilitre and 1 ml of 1 mg/ml of CMC followed by treatment with 2 ml of 1:100 dilution of rabbit anti-HGG (1·5 mg of antibody per millilitre) was found to be adequate. Control dishes were treated with saline and CMC followed by a dilution of NRS. Both test and control dishes were treated with NRS as described in the methods to block non-specific sticking of lymphocytes to the plastic. After addition of a lymphocyte suspension, centrifugation and inversion the unattached lymphocytes could be recovered from the lid of the Petri dish. Preliminary tests revealed that up to 10^7 cells could be processed in a 35-mm Petri dish by this method.

The unattached cells were examined by indirect immunofluorescent anti-HGG staining for the SIg of B cells and by sheep erythrocyte rosetting (E rosette) for T cells. The results of a series of preparations are reported in Table 7. The fluorescent staining for SIg and E rosettes revealed that exposure to control Petri dishes had little effect on the B- and T-cell proportions. The percentage of cells attaching to the wells of trays under optimal coating conditions was in agreement with the percentage of cells found positive for SIg by immunofluorescence. From early experiments it was found that a single treatment on HGG–anti-HGG dishes left significant contamination (1–7 per cent) with SIg-positive cells. However, a higher degree of purity could be obtained if the unattached cells from a Petri dish were reapplied and the process repeated with a second HGG–anti-HGG treated dish. Under these conditions lymphocyte preparations containing 1 per cent or less SIg-positive B cells were obtained in three out of four experiments.

DISCUSSION

Using anti-immunoglobulin bound to the wells of multi-well tissue culture trays it was relatively simple to obtain specific attachment of SIg-positive lymphocytes, to quantify the percentage attachment and to investigate the parameters involved in optimalizing the specific attachment. It was found that covalent coupling of HGG to the surface followed by treatment with anti-HGG led to better lymphocyte attachment than direct coupling of the immunoglobulin fraction of anti-HGG. This was possibly due to dilution of anti-immunoglobulin molecules by non-antibody immunoglobulin in the direct coupling method together with loss of effective antibody molecules through coupling of

the antigen-combining sites to the plastic, resulting in a lower density of available antibody molecules on the plastic surface. A brief centrifugation to bring the lymphocytes in contact with the base of the well was all that was necessary to establish specific lymphocyte attachment. However, it was found essential to treat the wells with a serum and to apply the cell suspension in a serum-containing medium to prevent non-specific attachment of the cells to the plastic.

Using these conditions, in wells coupled with optimal concentrations of HGG and treated with optimal dilutions of anti-HGG the percentage of attached lymphocytes was in good agreement with percentage expressing SIg determined by immunofluorescent staining. Surprisingly however, the attachment could not be completely inhibited by blocking the anti-HGG on the wells with an excess of HGG. This uninhibitable attachment was attributed to attachment of lymphocytes to the plastic through the Fc receptor known to exist on a subpopulation of B lymphocytes (Paraskevas *et al.*, 1972) and the Fc region of the antibody coating on the plastic surface. This was confirmed by the demonstration that when the $F(ab')_2$ fragment was used in place of whole anti-HGG the attachment could be reduced to control levels by blocking with HGG. For total removal of B lymphocytes the attachment of cells via the Fc receptor as well as via the SIg was not considered a disadvantage. However, $F(ab')_2$ fragments of antisera should also obviously be employed in experiments where the small subpopulation of SIg-negative cells with Fc receptors are to be investigated (Parish and Hayward, 1974). Moreover, if specific removal by light or heavy chain type of the SIg was required then the $F(ab')_2$ fragment of specific antisera had to be used. Using this system with plates coated with Bence–Jones protein and $F(ab')_2$ fragments of specific anti-κ or -λ antisera, it was shown that the proportion of cells with κ or λ light chain SIg could be quantitated. Furthermore, the total of $\kappa+\lambda$-positive cells agreed with the total Sig-positive cells. Preliminary investigations of CLL lymphocytes successfully detected SIg on 90 per cent of the cells of one case and also demonstrated the κ monotypy of the light chain. Attachment was not achieved in a second case which was shown by immunofluorescent staining to be SIg-negative.

Using larger dishes (35 mm diameter) the method was successful in yielding lymphocyte preparations with very low levels of SIg-positive cells. Two sequential treatments in anti-HGG dishes were generally required to obtain high purity preparations possibly as a result of uneven cell sedimentation preventing some cells making contact with the affinity surface in the first treatment. Thus 10^7 cells, a number calculated to form a single layer of cells over the bottom of the dish assuming even distribution, could be fractionated on a plate treated with as little as 2 ml of 1 : 100 dilution of antiserum (equivalent to 3 μg of antibody). It is felt that this represents a considerable economy of antibody over some previously published cell separation methods (Schlossman and Hudson, 1973).

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